Denise de Araujo Alves

Estratégias reprodutivas em *Melipona*, com
ênfase em pequenas populações de
Melipona scutellaris (Apidae, Meliponini)

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em pequenas populações de *Melipona scutellaris*
(Apidae, Meliponini)

Reproductive strategies in *Melipona*, with emphasis
in small populations of *Melipona scutellaris*
(Apidae, Meliponini)

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*Dedico esse trabalho aos meus pais
Eutália e Graciliano e às minhas
"fadas-madrinhas" Vera e Inês,
pelo apoio constante e amor incondicional em
todos os momentos.*

*"A abelha por Deus foi amestrada
Sem haver um processo bioquímico
Até hoje não houve nenhum químico
Pra fazer a ciência dizer nada
O buraco pequeno da entrada
Facilita a passagem com franqueza*

*Uma é sentinela de defesa
E as outras se espalham no vergel
Sem turbina e sem tacho fazem mel
Como é grande o poder da natureza"*

V. Paraíba, P. Norte, B. Tavares

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Resumo

As abelhas sem ferrão exercem importante papel ecológico como polinizadores de muitas espécies vegetais das regiões tropicais e tem significativo potencial para uso na polinização de culturas agrícolas. Contudo, com a contínua degradação de habitats, as populações de inúmeras espécies tem se tornado cada vez menores e separadas umas das outras por grandes distâncias. A criação de espécies de abelhas é um componente essencial para a conservação da biodiversidade, além de uma alternativa de fonte de renda. Para tanto, esforços de conservação e programas de criação em escala comercial requerem uma combinação de fatores, como o conhecimento biológico mais amplo, principalmente os relacionados à produção de sexuais e à diversidade genética necessária para manter a viabilidade de pequenas populações destas abelhas. Nesse contexto, os principais objetivos desta tese foram avaliar a variabilidade genética em populações manejadas, sob condições de isolamento genético ou não, e a produção de machos e rainhas nessas populações e o papel na reprodução. Para isso estudamos duas populações de *Melipona scutellaris* mantidas em regiões geográficas distintas, uma no município de Igarassu (PE; 7°50'S 34°54'W), onde a espécie ocorre naturalmente e outra no município de São Simão (SP; 21°26' 47°34'W), onde a população foi iniciada com duas colônias e criada por mais de 10 anos, quando chegou, a partir de sucessivas multiplicações, a 55 ninhos. Assim, embora a população de S. Simão tivesse maior redução na diversidade alélica e maior frequência de machos diplóides, quando comparada à mantida em Igarassu, ela foi criada com sucesso por um extenso período (ca. 10 anos). Provavelmente o baixo número de alelos sexuais em S. Simão, e a conseqüente produção de machos diplóides, foi a principal explicação para a frequência significativamente maior de sexuais criados nessa população. Como contraponto à alta produção de machos diplóides, as substituições das rainhas-mãe foram mais frequentes e as colônias produziram mais rainhas. Além disso, a alta produção de machos e rainhas também pode ser entendida em termos de benefícios reprodutivos individuais. Tanto as rainhas fisogástricas como as operárias poedeiras foram responsáveis pela maternidade de machos haplóides. Contudo, 80% dos machos filhos de operárias foram produzidos por operárias filhas da rainha-mãe substituída, indicando que essas operárias especiais tem sobrevida maior que as demais e parasitam reprodutivamente a força de trabalho da geração seguinte, que são menos relacionadas a elas. Quanto à super-produção de rainhas, detectou-se que 25% das colônias órfãs, após a perda da rainha-mãe, eram invadidas por rainhas que foram produzidas e vieram de colônias próximas. Nessas colônias não-natais, elas iniciaram suas atividades de postura. Este importante resultado muda as bases para melhoramento genético destas abelhas estabelecidas até o momento. Outro estudo relacionado à alta produção de rainhas foi realizado em colônia poligínica de *M. quadrifasciata*, em que oito rainhas fisogástricas co-existiam. Ao contrário da hipótese de que alguma das rainhas poderia ter vindo de outro ninho, todas eram irmãs completas. Isto sugere novas estratégias reprodutivas ainda desconhecidas para as abelhas do gênero *Melipona*.

Abstract

Stingless bees play an important ecological role as pollinators of many wild plant species in the tropics and have significant potential for the pollination of agricultural crops. However, as a consequence of habitat degradation, populations of a number of bee species have become increasingly small and separated from one another by large distances. Thus, stingless beekeeping is an essential component of biodiversity conservation, as well as a profitable business. Therefore, conservation efforts and breeding programme on a large scale require a combination of factors, including a broader biological knowledge, especially those related to production of sexuals and to the genetic diversity needed to maintain the viability of small population. In this context, the main goals were to evaluate the genetic variability in managed populations under or not genetic isolation and the production of males and queens. Two *Melipona scutellaris* populations were studied and they were kept in different geographic regions, one in Igarassu (PE; 7°50'S 34°54'W), in the species' natural area of occurrence, and the other in São Simão (SP; 21°26' 47°34'W), where the population was started from only two foundress colonies and which after a breeding programme of ten years increased to about 55. Despite a great reduction in the number of alleles and an increased frequency of diploid males in the S. Simão population, it could be successfully bred and maintained for a prolonged period (ca. 10 years). Probably the low number of sex alleles in S. Simão population, leading to production of diploid males, was the main reason for the highest level of sexual production. To counter-balance the high production of diploid males, the replacement of mother queen was more frequent leading to higher levels of queen production by the colonies. Furthermore, the high production of males and queens can also be explained by the individual fitness benefits. Queen and reproductive workers were haploid males' mothers. However, 80% of the workers' sons had genotypes that were compatible with them being the sons of workers that were the offspring of a superseded queen, indicating that these workers greatly outlive all other workers and reproductively parasitize the next-generation workforce, that are less related individuals. Related to queen overproduction, 25% of all queenless colonies were invaded by unrelated queens that fly in from unrelated hives nearby. In these non-natal colonies, the alien queens started their egg laying activities. Another study related to the high queen production was conducted in a polygyne colony of *M. quadrifasciata*, where eight physogastric queens coexisted. Contrary to the hypothesis that some of these queens could be an alien queen, it was confirmed that they were full-sisters. This suggests new reproductive strategies that are unknown for *Melipona* bees.

INTRODUÇÃO GERAL

M. Hrnčir

IMPORTÂNCIA DAS ABELHAS SEM FERRÃO, EM ESPECIAL AS DO GÊNERO *Melipona*

As abelhas sem ferrão, Meliponini, compreendem o grupo mais diverso de abelhas eusociais (Michener 1974; Sakagami 1982), com distribuição nas regiões tropicais e subtropicais do globo (Michener 1974). Face à extrema diversidade de espécies, os Meliponini apresentam uma gama de variações quanto a aspectos comportamentais, sistemas de comunicação, estratégias de forrageamento, densidades populacionais, arquiteturas de ninhos, entre outros (Michener 1974; Sakagami 1982).

Nesse grupo diverso, destaca-se o gênero *Melipona*, que compreende cerca de 65 espécies distribuídas pela região Neotropical (Camargo & Pedro 2007). Muitas delas são conhecidas por polinizarem eficiente e efetivamente uma ampla variedade de espécies vegetais em ambientes naturais (Wilms *et al.* 1996; Ramalho 2004). Como a polinização é um dos serviços ecossistêmicos essenciais para manter e melhorar o bem-estar humano (Costanza *et al.* 1997), as abelhas *Melipona*, assim como outras espécies de Meliponini, são agentes de importância crucial para a conservação da biodiversidade natural nos trópicos (Roubik 1989).

Além do valor ecológico, as abelhas *Melipona* tem importância tanto em aspectos culturais, associados a rituais religiosos, quanto econômicos (Quezada-Euán *et al.* 2001). Por armazenarem considerável quantidade de mel e pólen em seus ninhos, e produzirem bastante cerume, essas abelhas representam importante complemento de renda para muitas famílias (Cortopassi-Laurino *et al.* 2006). Também, muitas espécies são polinizadoras-chave de culturas agrícolas economicamente importantes, tais como tomate, urucum, pimentão e berinjela (Heard 1999; Slaa *et al.* 2006). A necessidade de colônias para uso em áreas agrícolas abertas ou em casas de vegetação, atualmente, é uma demanda crescente, e a venda de ninhos para polinização dessas culturas tem se tornado mais uma potencial fonte de renda (Cortopassi-Laurino *et al.* 2006).

CONSERVAÇÃO DAS PEQUENAS POPULAÇÕES DE *Melipona*

As espécies de *Melipona* são sensíveis a distúrbios antropogênicos, especialmente ao desmatamento (Brown & Albrecht 2001), pois utilizam cavidades arbóreas como substrato de nidificação (Michener 1974). Assim como em outras abelhas sem ferrão, nos eventos naturais

de reprodução, por exameagem, o vínculo entre colônia-filha e colônia-mãe permanece por certo período, já que a primeira depende de recursos (alimento e materiais de construção) armazenados na colônia-mãe (Nogueira-Neto 1954). Essa estreita relação no início do ciclo das colônias-filhas impede que elas se dispersem para grandes distâncias e uma vez que a colônia é fundada e se estabelece no oco, ela permanece ali até o fim do seu extenso ciclo de vida, pois a rainha perde a capacidade de voar (Michener 1974). Devido a essas características, as colônias são altamente dependentes da existência de árvores que abrigam seus ninhos e as protegem de potenciais predadores (Brown & Albrecht 2001; Eltz *et al.* 2003), bem como de recursos florais que as provem alimento, e a perda de habitats se torna uma das maiores ameaças à sua contínua existência (Goulson *et al.* 2008).

Como consequência da degradação de habitats (Fig. 1), as populações de inúmeras espécies de *Melipona* tem se tornado cada vez menores, fragmentadas e separadas umas das outras por grandes distâncias (Brown & Albrecht 2001). Com a diminuição do número de ninhos em uma área, e a consequente redução na diversidade genética, as populações enfrentam uma ameaça adicional à sua sobrevivência, o endocruzamento (Goulson *et al.* 2008; Zayed 2009).

Na tentativa de minimizar os efeitos negativos do endocruzamento em pequenas populações, o manejo ou criação de espécies de abelhas se torna um componente essencial para a conservação da biodiversidade (Jaffé *et al.* 2010). Além das razões econômicas (e.g, venda do mel, pólen e ninhos), a criação de abelhas sem ferrão (conhecida como meliponicultura), no cenário atual, surge como uma atividade de desenvolvimento sustentável indicada para preservação e uso dos recursos naturais (Nogueira-Neto 1997; Cortopassi-Laurino *et al.* 2006). O crescente interesse do público em geral, comunidade científica e órgãos públicos, pela meliponicultura, pode ajudar a compensar, em moderada extensão, a contínua perda de habitat e garantir a polinização adequada de flores de espécies em ambientes naturais e de culturas agrícolas.

Embora no Brasil a meliponicultura tenha crescido rapidamente e haja técnicas de manejo para criação de diferentes abelhas sem ferrão que são constantemente implementadas (Nogueira-Neto 1997), a maioria dos criadores ainda mantem um pequeno número de ninhos de mesma espécie (Cortopassi-Laurino *et al.* 2006). Para atender às atuais e às futuras demandas, há a necessidade da criação de colônias em ampla escala e, para isso, a multiplicação de ninhos se torna necessária. Mas para que isso ocorra, num futuro próximo,

assim como acontece com algumas espécies de *Bombus* no hemisfério norte (Velthuis & van Doorn 2006), uma combinação de fatores se faz necessária, entre eles o conhecimento biológico mais amplo, principalmente os relacionados à produção de sexuais (rainhas e machos).

PRODUÇÃO DE RAINHAS E MACHOS EM *Melipona*

Em geral, a produção de sexuais em abelhas sem ferrão é influenciada por condições externas às colônias, como fatores climáticos que agem diretamente na disponibilidade de recursos tróficos, e condições internas, tais como o número de operárias no ninho (Roubik 1982; Bego 1990). Contudo, possivelmente, fatores intrínsecos às colônias sejam mais pronunciados na produção de rainhas e machos, já que cada uma possui sua própria dinâmica (Velthuis *et al.* 2005).

Ao contrário do que ocorre em outros gêneros de Meliponini, todos os indivíduos (rainhas, machos e operárias) em colônias de *Melipona* são produzidos em células de cria de mesmas dimensões e, portanto, eles devem ingerir quantidades similares de alimento (Michener 1974; Hartfelder *et al.* 2006). Além disso, as rainhas de *Melipona* são produzidas em alto número (Kerr 1969; Sommeijer *et al.* 2003; Wenseleers & Ratnieks 2004; Santos-Filho *et al.* 2006). Na tentativa de compreender essa super-produção de rainhas, algumas teorias foram propostas, baseadas em níveis diferentes de explicação (em termos proximais (*como?*) e em termos finais (*por quê?*)). Kerr foi o primeiro a propor uma explicação proximal de determinação de castas baseada em um modelo de dois-loci-dois-alelos, em que indivíduos duplo-heterozigotos podem se tornar rainhas (Kerr 1948, 1950). Posteriormente, a teoria foi implementada e condições alimentares foram consideradas importantes para direcionar o destino de casta das larvas femininas determinadas geneticamente a se tornarem rainhas (Kerr 1969; Velthuis & Sommeijer 1991; Jarau *et al.* 2010). A explicação evolutiva, ou final, para essa alta produção é dada por considerações teóricas acerca do conflito de castas e estrutura de parentesco (Bourke & Ratnieks 1999; Ratnieks 2001). De acordo com esse modelo, uma larva fêmea tem certo controle sobre seu destino de casta e obtém mais benefícios, em termos de aptidão direta, em se tornar uma rainha devido à reprodução direta (Bourke & Ratnieks 1999; Ratnieks 2001; Wenseleers *et al.* 2003; Wenseleers & Ratnieks 2004; Ratnieks & Helanterä 2009).

Outro aspecto importante na biologia do gênero se refere à frequente presença de

operárias reprodutivas não apenas em colônias órfãs, mas também na presença da rainha-mãe (Koedam *et al.* 1999; Sommeijer *et al.* 1999; Tóth *et al.* 2002; Velthuis *et al.* 2002; Alves *et al.* 2009). Essas operárias tem ovários desenvolvidos e podem botar óvulos não-fertilizados, que darão origem a machos. Isso acarreta um conflito reprodutivo entre rainha-operária, bem como entre operária-operária, na produção de machos, já que cada uma delas é mais relacionada aos seus próprios filhos, do que aos seus irmãos ou sobrinhos (ver Tóth *et al.* 2004; Wenseleers & Ratnieks 2006).

OBJETIVOS E ORGANIZAÇÃO DA TESE

Nesse contexto, o objetivo geral dessa tese foi investigar a produção de machos e rainhas em pequenas populações de *Melipona*. Nosso principal modelo de estudo foi a espécie *M. scutellaris* (capítulos 1 a 4), por diferentes motivos. O primeiro foi a possibilidade de estudar uma população iniciada com apenas duas colônias e que estava geneticamente isolada por, aproximadamente, 10 anos (Nogueira-Neto 2002). O segundo motivo diz respeito à extensa distribuição geográfica de *M. scutellaris* em áreas florestadas da Mata Atlântica da região Nordeste do Brasil (Camargo & Pedro 2007), onde ela é amplamente criada por meliponicultores (Cortopassi-Laurino *et al.* 2006; Carvalho-Zilse *et al.* 2009). O último se refere a questões práticas, tais como ninhos populosos e resistentes à manipulação humana.

De forma geral e resumida, estávamos interessados nas seguintes questões:

- (a) as consequências da criação prolongada, em relação à diversidade genética e à produção de machos diplóides, de uma população fundada com um baixo número de colônias (capítulo 2);
- (b) o conflito reprodutivo entre rainha-operária e operária-operária na produção de machos haplóides (capítulo 3);
- (c) as estratégias reprodutivas vinculadas à super-produção de rainhas (capítulos 4 e 5).

Assim, a tese está dividida em cinco capítulos no formato de manuscritos para publicação. Devido a essa razão, repetições de alguns assuntos poderão ser encontradas entre os capítulos. A seguir, os objetivos de cada capítulo são apresentados.

Capítulo 1. Produção de sexuais em pequenas populações de *Melipona scutellaris*

O objetivo principal foi apresentar uma análise descritiva e comparativa quanto à produção de rainhas e machos em duas pequenas populações mantidas em diferentes regiões geográficas, uma onde *M. scutellaris* está isolada geneticamente (São Simão, 21°26'

47°34'W) e a outra onde a espécie ocorre naturalmente (Igarassu, 7°50'S 34°54'W) (Fig. 1).

Capítulo 2. Successful maintenance of a stingless bee population despite a severe genetic bottleneck

O principal objetivo foi estudar o efeito do gargalo genético imposto artificialmente através da fundação de uma população a partir de apenas duas colônias em uma região fora da área de ocorrência natural da espécie. Em especial, avaliar as consequências da criação prolongada dessa população e comparar a diversidade genética e a presença de machos diplóides com a população de origem.

Capítulo 3. The queen is dead – long live the workers: intraspecific parasitism by workers in the stingless bee *Melipona scutellaris*

Dada a ocorrência de operárias reprodutivas em colônias de abelhas sem ferrão, nosso objetivo geral foi investigar o conflito reprodutivo entre rainha e operárias na produção de machos. Como em estudos prévios foi detectada a presença de operárias menos relacionadas (i.e., não eram irmãs das operárias presentes num dado momento) ou mesmo operárias não-natais), em determinadas colônias, os objetivos específicos desse estudo foram testar se essas operárias produzem machos e em qual extensão.

Capítulo 4. Intraspecific queen parasitism in the stingless bee *Melipona scutellaris* (Hymenoptera, Apidae)

Estudo recente mostrou que rainhas virgens de *Melipona* escapam de serem mortas pelas operárias e especulou sobre a possibilidade dessas rainhas tentarem penetrar ninhos órfãos e lá iniciarem suas atividades reprodutivas. Nosso objetivo foi testar essa hipótese e verificar se as rainhas que escapam vivas de suas colônias são bem-sucedidas ao entrarem em ninhos onde não foram produzidas e, assim, criarem oportunisticamente sua própria prole.

Capítulo 5. First discovery of a rare polygyne colony in the stingless bee *Melipona quadrifasciata* (Apidae, Meliponini)

Dado que rainhas penetram em ninhos não-natais e lá são bem-sucedidas em suas atividades de postura, o principal objetivo desse estudo foi verificar se múltiplas rainhas fisogástricas, aceitas simultaneamente, em colônia poligínica eram aparentadas ou não. Em particular, nós avaliamos como o grau de relacionamento entre essas rainhas e como elas compartilhavam as posturas de ovos, ou seja, se todas as rainhas participavam das atividades de postura ou apenas algumas. Embora esse estudo tenha sido realizado com *M. quadrifasciata*,

evento similar também ocorreu em *M. scutellaris*, em que cinco rainhas co-existiram em uma colônia por alguns meses (Carvalho com. pes.).

Fig. 1. Remanescentes florestais (11,73%) da Mata Atlântica (cobertura original 150 milhões de ha) e ocorrências de *M. scutellaris* (pontos negros). Destaque para população isolada geneticamente no município de São Simão, SP (modificado de Ribeiro *et al.* 2009).

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CAPÍTULO 1.

Produção de sexuais em pequenas
populações de *Melipona scutellaris*

Produção de sexuais em pequenas populações de *Melipona scutellaris*

RESUMO

A produção de sexuais em insetos sociais é um evento desencadeado por uma complexa interação entre fatores intra- e extra-coloniais. Embora muitos fatores possam influenciar na produção de sexuais em abelhas sem ferrão, esse é primeiro que compara duas pequenas populações mantidas em diferentes regiões geográficas, uma em área onde *Melipona scutellaris* está isolada geneticamente (São Simão, 21°26' 47°34'W) e a outra em área onde a espécie ocorre naturalmente (Igarassu, 7°50'S 34°54'W). Em 38 colônias amostradas em Igarassu, 7,19% corresponderam a machos (n= 16.557 pupas) e 5,60% eram rainhas (n= 14.902 fêmeas). Surpreendentemente, em S. Simão as frequências de sexuais foram significativamente maiores em relação ao encontrado em Igarassu, pois 21,76% eram machos (n= 18.532 pupas) e 14,76% (n= 14.413 fêmeas). Embora houvesse diferenças inter-coloniais em cada população, a produção de sexuais foi constante ao longo do tempo, sem variações sazonais. Além disso, os tamanhos das abelhas também não diferiram entre as populações, o que possivelmente, significa que colônias das duas localidades alocam quantidades similares de alimento para a produção de cria. Diante desse contexto, discutimos relação entre a alta produção de sexuais na população mantida em S. Simão e o baixo número de alelos sexuais.

INTRODUÇÃO

Embora as abelhas sem ferrão (Meliponini) possuam similaridades biológicas com as abelhas melíferas (Apini), como a reprodução colonial por enxameagem (Michener 1974), algumas diferenças são marcantes, principalmente as relacionadas aos aspectos reprodutivos. Uma delas é o número de machos que se acasalam com a rainha-mãe. Em *Apis*, as rainhas se acasalam com muitos machos (Palmer & Oldroyd 2000), mas nos Meliponini, geralmente, a cópula única é a regra (Peters *et al.* 1999; Strassmann 2001). Outra diferença acentuada se refere à forma como as células de cria são aprovisionadas. Nas abelhas sem ferrão, o aprovisionamento é massal, ou seja, cada célula é preenchida com alimento larval líquido regurgitado pelas operárias, seguido pela oviposição da rainha e pelo fechamento das células pelas operárias (Sakagami & Zucchi 1963; Zucchi *et al.* 1999). Dessa forma, as larvas possuem todo o alimento necessário para o seu desenvolvimento, sem a necessidade de nenhum contato com as operárias adultas (Michener 1974; Sakagami 1982). Em contraste, as larvas de *Apis* tem contato com

operárias adultas durante seu desenvolvimento, pois ficam em células de cria abertas e são alimentadas progressivamente por elas (Michener 1974). Em comum nos dois grupos de abelhas, Apini e Meliponini, bem como em outros insetos haplo-diplóides, a rainha controla o sexo dos ovos que ela põe, através do controle na liberação dos espermatozóides armazenados em sua espermateca, dando origem a fêmeas, quando os óvulos são fertilizados, ou a machos, quando não o são (Hamilton 1964). Dessa forma, a produção de rainhas e machos constitui o investimento em reprodução, e a de operárias representa o investimento em crescimento e manutenção da colônia (Oster & Wilson 1978).

Na grande maioria das abelhas eusociais, as rainhas são criadas em células reais, maiores em altura e largura em relação às demais. Nessas espécies, a determinação de castas é regulada troficamente pela quantidade de alimento larval (Michener 1974; Hartfelder *et al.* 2006). Contudo, as espécies pertencentes ao gênero *Melipona* criam todos os indivíduos em células de cria de mesmas dimensões e, portanto, eles devem ingerir quantidades similares de alimento. Além disso, diferentemente do que ocorre em outros gêneros, as rainhas de *Melipona* são produzidas em alto número (Kerr 1969; Sommeijer *et al.* 2003 ; Wenseleers & Ratnieks 2004; Santos-Filho *et al.* 2006). Na tentativa de compreender essa alta taxa de produção de rainhas, algumas teorias foram propostas, baseadas em níveis diferentes de explicação (em termos proximais (*como?*) e em termos finais (*por quê?*)).

Ao contrário das rainhas, as operárias não podem se acasalar, mas em muitas espécies elas tem a capacidade de botar óvulos não-fertilizados, que darão origem a machos. Isso acarreta um conflito reprodutivo entre a rainha-mãe e operárias na produção de machos, já que cada uma delas é mais relacionada aos seus próprios filhos, do que aos seus irmãos ou sobrinhos (para revisão ver Tóth *et al.* 2004; Wenseleers & Ratnieks 2006a). Em abelhas melíferas, operárias reprodutivas são mais raras (Ratnieks 1993; Wenseleers & Ratnieks 2006a, 2006b), porém em abelhas sem ferrão, elas são mais frequentes, não apenas em colônias órfãs, mas também na presença da rainha-mãe (Beig 1972; Bego 1982; Koedam *et al.* 1999; Tóth *et al.* 2004; Wenseleers & Ratnieks 2006a; Alves *et al.* 2009a).

Em muitas espécies de abelhas sem ferrão, em nível populacional, os sexuais são produzidos ao longo de todo o ano (Sommeijer *et al.* 2003; Velthuis *et al.* 2005; Alves *et al.* 2009b), enquanto que, em nível colonial, eles são produzidos em períodos distintos (Chinh *et al.* 2003; Chinh & Sommeijer 2005; Velthuis *et al.* 2005). Estudos avaliaram fatores extra- e/ou intra-colônias que possivelmente exercem influência em tal produção, tais como a disponibilidade de

recursos tróficos no ambiente (Roubik 1982), a quantidade de alimento estocado (Bego 1982; Moo-Valle *et al.* 2001; Morais *et al.* 2006), a população do ninho (Bego 1982; van Veen *et al.* 1992), a produção de cria (Koedam 1999; Santos-Filho *et al.* 2006; Alves *et al.* 2009b) e a presença de operárias reprodutivas (Bego 1990; Koedam *et al.* 1999; Paxton *et al.* 2003; Tóth *et al.* 2002; Sommeijer *et al.* 2003; Koedam *et al.* 2005).

Embora diferentes fatores, como mencionado acima, influenciem na produção de sexuais, nenhum avaliou os efeitos do isolamento genético nas pequenas populações de abelhas sem ferrão. Dado que as pequenas populações isoladas geneticamente possuem menor número de alelos sexuais do que as populações que se encontram em sua região de ocorrência natural (Cook & Crozier 1995), esperamos que elas produzam mais machos. Isso porque o menor número de alelos sexuais em insetos com sistema de determinação de sexo por CSD (“*complementary sex determination*”, Whiting 1943) acarreta a produção de machos diplóides, o que implica desvantagens e alto custo para as colônias, além dos machos haplóides naturalmente produzidos (para revisão Cook & Crozier 1995; Heimpel & de Boer 2008; Zayed 2009).

Nesse contexto, nosso objetivo foi avaliar a frequência com que machos e rainhas são produzidos por pequenas populações tanto em condições de isolamento genético quanto na ausência de isolamento. Para isso, utilizamos colônias de *Melipona scutellaris*, espécie comum no nordeste brasileiro, mantidas em uma região onde a espécie ocorre naturalmente (Igarassu, PE) e em outra região onde a espécie encontra-se isolada há 10 anos (São Simão, SP). Além disso, a fim de avaliarmos a alocação de recursos na produção de cria nas duas populações, mensuramos o tamanho de machos, rainhas e operárias produzidos.

MATERIAL E MÉTODOS

Locais de estudo, período e amostragem

No período de janeiro de 2006 a maio e 2007, nós usamos 38 e 18 ninhos mantidos na Granja São Saruê (Igarassu, Pernambuco, 7°50'S 34°54'W) e na Fazenda Aretuzina (São Simão, S. Paulo; 21°26'S 47°34'W), respectivamente (Tabela 1). Em Igarassu, local de ocorrência natural de *M. scutellaris*, aproximadamente, 750 ninhos eram mantidos no meliponário (Cortopassi-Laurino *et al.* 2006), cercado por área florestada (Pierrot & Schlindwein 2003), onde ninhos naturais poderiam ser encontrados. Já em S. Simão, onde a espécie é mantida

fora de sua área de ocorrência natural, a população foi iniciada com duas colônias provenientes de Igarassu em 1996 (Nogueira-Neto 2002) e, após 10 anos de sucessivas multiplicações, o número chegou a 55 ninhos.

Nós amostramos favos de cria contendo pupas com olhos pigmentados, para avaliar o número de machos, rainhas e operárias. Caso a coleta do favo requeresse intensa manipulação ou o favo contivesse poucas células de cria (< 40 células), optávamos por não amostrá-lo. Devido a essas razões, utilizamos diferentes ninhos a cada amostragem. Para cada favo, desoperculamos todas as células de cria a fim de distinguirmos o sexo dos indivíduos e, no caso de fêmeas, a casta (rainha ou operária), de acordo com as características morfológicas de suas cabeças. Para análises genéticas e morfométricas, coletamos 10 operárias, 10 rainhas (quando possível) e todos os machos produzidos, que foram preservados em álcool absoluto a 20°C. O restante do favo era devolvido à colônia de origem.

Medidas morfométricas

Para medirmos as abelhas, colocávamos cada uma delas em um aparato de metal preenchido por material esponjoso com cavidades de diferentes tamanhos. Uma vez dentro dessa cavidade, dispúnhamos uma placa de vidro sobre o indivíduo, a fim de que sua cabeça e tórax fossem mantidos em posição adequada para as medições (Ribeiro & Alves 2001). Depois desse procedimento, com uma câmera fotográfica acoplada a um estereomicroscópio (Leica MZ16), capturávamos a imagem de cada abelha, com auxílio de câmera digital (Leica DFC500) e realizávamos as medidas nas fotografias, usando o software Programa Leica Soft IM50.

Escolhemos, de acordo com Hartfelder & Engels (1992) e Camargo (com. pes.), cinco caracteres da cabeça e do tórax: largura máxima da cabeça; distância interorbital, medida acima da sutura alveolar; comprimento da cabeça, entre o término do ocelo médio e o início do labro; distância intertegular, e; comprimento do mesoscuto. Contudo, para as análises realizadas no presente trabalho, apenas consideramos a distância intertegular, medida-padrão utilizada em outras espécies de Meliponini (e.g. Araújo *et al.* 2004) e em outros grupos de abelhas, como *Bombus* (Goulson *et al.* 2002; Couvillon & Dornhaus 2010) e nas espécies solitárias (Cane 1987).

Em média, medimos cerca de 10 operárias, 9 rainhas e 9 machos para cada favo de cria

(Tabela 1). Com algumas exceções, não medimos as abelhas de favos em que os machos estavam ausentes.

Análises genéticas

A fim de avaliarmos quais colônias produziam machos diplóides (Tabela 1), utilizamos marcadores de microssatélites (Alves *et al.* submetido, Cap. 2), seguindo o protocolo descrito em Alves *et al.* (2009a).

RESULTADOS

Produção de sexuais

Em Igarassu, um total de 16.557 indivíduos foi amostrado em 38 colônias em três meses de coleta (janeiro e setembro de 2006 e maio de 2007). Desses, 1.655 eram machos, 861 corresponderam a rainhas e 14.041 a operárias. Nesse período, a produção média de rainhas e machos, como porcentagem de fêmeas e da produção total, respectivamente, foi de 5,60% (95% intervalo de confiança: [4,75 – 6,45%], variação: 0 – 14,13%) e 7,19% (95% intervalo de confiança: [4,24 – 10,14%], variação: 0 – 47,33%) (Tabela 1). Embora para ambos os sexos, a maior produção tenha sido no mês de maio (Fig. 1A), não houve diferenças mensais (machos: $H = 0,3059$, $p = 0,8582$; rainhas: $H = 4,8727$, $p = 0,0875$).

Durante um ano de estudo em S. Simão (março de 2006 a fevereiro de 2007), em 18 colônias, amostramos 18.532 abelhas, das quais 4.119 eram machos, 2.169 eram rainhas e 12.244 corresponderam a operárias. A produção média de rainhas, considerado a prole feminina, foi de 14,76% (95% intervalo de confiança: [13,34 – 16,19%], variação: 3,36 – 27,23%) (Tabela 1). Já a produção média de machos, entre os indivíduos, foi de 21,76% (95% intervalo de confiança: [16,49 – 27,03%], variação: 0 – 69,69%). Embora haja variação na taxa de produção tanto de rainha quanto de machos entre os meses (Fig. 1B), a diferença mensal não foi significativa (machos: $H = 9,8497$; $p = 0,5440$; rainhas: $H = 14,3803$, $p = 0,2127$).

Quando comparamos a produção de machos e rainhas nas duas populações, as colônias mantidas em S. Simão produziram significativamente mais sexuais em relação às colônias de Igarassu (machos: $H = 29,5500$; $p = 0,0000$; rainhas: $H = 66,10420$, $p = 0,0000$). Em Igarassu apenas em 23,19% dos favos (16 em 69) continham mais de 10% de machos e em 11,59% dos favos (8 em 69) tinham mais de 10% de rainhas entre as pupas femininas. Em contraste, as frequências de

machos e rainhas foram superiores em favos coletados em S. Simão, com 61,11% (33 em 54) e 83,33% (45 em 54), respectivamente (Fig. 2).

Tamanho dos sexuais

As rainhas produzidas em Igarassu ($n = 273$ rainhas; média $\pm$ d.p. = $2,71 \pm 0,07$ mm; 95% intervalo de confiança: [2,69 – 2,74]) foram ligeiramente menores que as produzidas em S. Simão ($n = 454$, média $\pm$ d.p. = $2,75 \pm 0,05$ mm, 95% intervalo de confiança: [2,73 – 2,77]), porém a diferença não foi significativa entre as duas localidades ($H = 10,510$; $p = 0,2310$). Contudo, as colônias de Igarassu produziram tanto machos ($n = 220$, média $\pm$ d.p. = $2,87 \pm 0,53$ mm; 95% intervalo de confiança: [2,68 – 3,06]) quanto operárias ($n = 302$; média $\pm$ d.p. = $3,01 \pm 0,08$ mm; 95% intervalo de confiança: [2,98 – 3,04]) maiores que as mantidas em S. Simão (machos: $n = 404$, média $\pm$ d.p. = $2,55 \pm 1,06$ mm, 95% intervalo de confiança: [2,25 – 2,86]; operárias: $n = 469$, média $\pm$ d.p. = $2,99 \pm 0,07$, 95% intervalo de confiança: [2,97–3,01]). Embora haja diferenças no tamanho dos indivíduos produzidos em colônias das duas localidades, elas não são significativas (machos: $H = 0,3737E-3$, $p = 0,9846$; operárias: $H = 1,9354$, $p = 0,1642$).

Da mesma forma que a produção de sexuais não variou significativamente ao longo dos meses em Igarassu e em S. Simão, o tamanho dos indivíduos também não, com exceção dos machos em Igarassu ($H = 13,1338$, $p = 0,0014$). Ou seja, os machos (S. Simão: $H = 7,8534$, $p = 0,7264$), rainhas (Igarassu: $H = 0,5208$, $p = 0,7708$; S. Simão: $H = 9,5956$; $p = 0,5671$) e operárias (Igarassu: $H = 0,5216$; $p = 0,7704$; S. Simão: $H = 18,5232$, $p = 0,0702$).

Tabela 1. Colônias de *Melipona scutellaris* mantidas em Igarassu (IG) e São Simão (SS), com informações sobre o número total de pupas, a porcentagem de machos e rainhas (entre as fêmeas) nos favos de cria amostrados, número (n) de machos, rainhas e operárias medidos, e os respectivos tamanhos, dados pelas distâncias intertegulares, (média $\pm$ desvio-padrão).

colônia e rainha	data de coleta	favo de cria			machos		rainhas		operárias	
		número de pupas	machos (%)	rainhas entre as fêmeas (%)	n	distância intertegular (mm)	n	distância intertegular (mm)	n	distância intertegular (mm)
IG 01	01/2006	267	0,00	5,37						
IG 01-a	09/2006	441	36,05	4,96	10	3,01 $\pm$ 0,07	9	2,71 $\pm$ 0,11	10	3,05 $\pm$ 0,05
IG 01	05/2007	370	0,00	2,71						
IG 02-a	01/2006	288	0,00	10,07			10	2,74 $\pm$ 0,13	11	3,09 $\pm$ 0,06
IG 02	09/2006	357	0,00	10,64						
IG 02	05/2007	289	2,05	8,48	2	3,12 $\pm$ 0,04	10	2,81 $\pm$ 0,08	10	3,07 $\pm$ 0,04
IG 03-a	01/2006	386	16,06	10,19	10	2,97 $\pm$ 0,09	10	2,79 $\pm$ 0,10	10	3,11 $\pm$ 0,06
IG 04-a	01/2006	199	0,00	1,01						
IG 05	01/2006	160	0,00	9,38						
IG 05-a	09/2006	341	18,48	6,47	10	2,92 $\pm$ 0,06	10	2,66 $\pm$ 0,08	10	2,98 $\pm$ 0,08
IG 06	01/2006	333	1,50	7,01	4	2,62 $\pm$ 0,10	10	2,73 $\pm$ 0,08	9	3,11 $\pm$ 0,09
IG 06-a	09/2006	426	24,41	6,21	10	2,97 $\pm$ 0,06	8	2,80 $\pm$ 0,05	10	3,08 $\pm$ 0,06
IG 06	05/2007	349	5,44	8,79	10	2,96 $\pm$ 0,05	10	2,78 $\pm$ 0,05	10	3,08 $\pm$ 0,07
IG 07-a	01/2006	281	0,00	0,00						
IG 07	09/2006	165	0,00	0,00						
IG 07	05/2007	88	0,00	12,92						
IG 08-a	01/2006	323	8,36	6,08	10	2,99 $\pm$ 0,07	10	2,59 $\pm$ 0,13	10	2,99 $\pm$ 0,04
IG 09-a	01/2006	100	0,00	6,00						
IG 10-a	01/2006	102	0,00	0,00						
IG 11	01/2006	243	0,00	7,82						
IG 11-a	05/2007	488	45,08	12,69	10	2,95 $\pm$ 0,12	9	2,59 $\pm$ 0,08	10	2,77 $\pm$ 0,17
IG 12	01/2006	190	0,00	2,63						
IG 12-a	09/2006	380	28,42	7,72	10	2,99 $\pm$ 0,06	10	2,73 $\pm$ 0,09	10	2,96 $\pm$ 0,22
IG 13-a	01/2006	365	28,77	3,85	10	2,92 $\pm$ 0,10	10	2,74 $\pm$ 0,06	10	3,06 $\pm$ 0,11
IG13	09/2006	227	0,00	7,49						
IG13	05/2007	379	21,64	4,71	10	3,10 $\pm$ 0,05	10	2,85 $\pm$ 0,08	9	3,08 $\pm$ 0,12
IG 14-a	01/2006	164	2,44	2,50	4	2,87 $\pm$ 0,04	4	2,70 $\pm$ 0,03	10	2,97 $\pm$ 0,09
IG 14	05/2007	75	0,00	2,67						
IG 15-a	01/2006	525	0,19	9,73	1	2,96	10	2,75 $\pm$ 0,07	10	3,09 $\pm$ 0,13
IG 16-a	01/2006	282	33,69	3,74	10	2,92 $\pm$ 0,09	7	2,72 $\pm$ 0,13	10	3,03 $\pm$ 0,08
IG 16	09/2006	497	0,53	4,32	2	2,92 $\pm$ 0,06	10	2,67 $\pm$ 0,08	10	2,94 $\pm$ 0,12
IG 17-a	01/2006	188	0,00	1,06						
IG 18-a	01/2006	241	19,09	3,08	10	2,70 $\pm$ 0,15	5	2,70 $\pm$ 0,19	10	2,85 $\pm$ 0,12
IG 18	09/2006	294	0,00	4,76						
IG 18	05/2007	445	0,59	16,48	2	2,96 $\pm$ 0,03	10	2,74 $\pm$ 0,04	10	2,97 $\pm$ 0,11
IG 19-a	01/2006	455	21,10	9,47	10	2,87 $\pm$ 0,13	10	2,60 $\pm$ 0,09	10	2,87 $\pm$ 0,06
IG 20	01/2006	405	1,23	3,50	4	2,94 $\pm$ 0,07	10	2,65 $\pm$ 0,11	10	2,97 $\pm$ 0,21
IG 20-a	05/2007	368	17,93	7,28	10	3,04 $\pm$ 0,06	10	2,71 $\pm$ 0,11	10	3,02 $\pm$ 0,09
IG 21	09/2006	242	0,00	4,76						
IG 21-a	05/2007	66	1,52	9,23	1	3,02	5	2,77 $\pm$ 0,09	5	3,05 $\pm$ 0,10
IG 22-a	09/2006	168	0,00	13,15						
IG 23-a	09/2006	269	1,86	1,14	4	2,90 $\pm$ 0,05	3	2,58 $\pm$ 0,04	10	2,91 $\pm$ 0,10
IG 24-a	09/2006	162	0,00	7,41						
IG 24	05/2007	356	0,00	20,19

colônia e rainha	data de coleta	favo de cria			machos		rainhas		operárias	
		número de pupas	machos (%)	rainhas entre as fêmeas (%)	n	distância intertegular (mm)	n	distância intertegular (mm)	n	distância intertegular (mm)
IG 25-a	09/2006	123	0,00	1,63						
IG 25	05/2007	177	0,00	5,65						
IG 26-a	09/2006	282	21,99	7,27	10	3,05 ± 0,07	9	2,76 ± 0,07	10	3,04 ± 0,13
IG 27-a	09/2006	314	6,37	5,44	6	3,02 ± 0,07	10	2,82 ± 0,13	9	3,15 ± 0,08
IG 28-a	09/2006	191	0,00	4,71						
IG 29-a	09/2006	328	0,00	4,27						
IG 30-a	09/2006	326	19,63	2,29	9	3,03 ± 0,23	6	2,67 ± 0,18	9	2,97 ± 0,18
IG 31-a	09/2006	265	0,00	3,40						
IG 31	05/2007	134	0,00	11,19						
IG 32-a	05/2007	72	0,00	0,00						
IG 33-a	05/2007	366	0,00	3,28						
IG 34-a	05/2007	162	0,00	9,26						
IG 35-a *	05/2007	262	47,33	8,70	10	3,15 ± 0,09	10	2,64 ± 0,10	10	3,00 ± 0,08
IG 36-a	05/2007	217	29,03	5,19	10	3,03 ± 0,05	8	2,68 ± 0,05	10	2,96 ± 0,07
IG 38-a	05/2007	176	0,57	9,71	1	2,98	10	2,66 ± 0,09	10	3,02 ± 0,08
IG 39-a	05/2007	423	9,69	9,95	10	3,02 ± 0,06	10	2,78 ± 0,07	10	3,06 ± 0,08
<i>Média:</i>		240,20	7,19	5,60		2,87		2,71		3,01
SS 01-a *	03/2006	279	66,31	14,89	10	2,82 ± 0,39	5	2,70 ± 0,07	10	2,95 ± 0,12
SS 02-a *	03/2006	445	51,46	15,28	10	3,11 ± 0,09	10	2,75 ± 0,07	10	2,97 ± 0,11
SS 02	04/2006	137	43,80	16,88	10	3,09 ± 0,07	5	2,71 ± 0,06	5	2,92 ± 0,05
SS 02-b	02/2007	201	8,96	7,65	10	2,92 ± 0,03	10	2,64 ± 0,07	10	2,85 ± 0,18
SS 04-a	04/2006	247	13,77	27,23	10	2,93 ± 0,12	10	2,62 ± 0,15	10	3,02 ± 0,08
SS 04-a	06/2006	409	18,09	20,60	10	2,91 ± 0,09	10	2,69 ± 0,03	10	3,00 ± 0,08
SS 06-a	05/2006	351	24,22	15,04	10	2,90 ± 0,14	9	2,66 ± 0,08	10	2,94 ± 0,10
SS 06-a	07/2006	347	15,85	11,64	10	3,01 ± 0,05	10	2,77 ± 0,11	10	3,11 ± 0,12
SS 06-a	08/2006	223	36,77	22,70	10	2,95 ± 0,10	10	2,83 ± 0,10	10	3,06 ± 0,12
SS 06-b	12/2006	195	5,13	11,89	9	2,76 ± 0,08	10	2,66 ± 0,07	10	2,87 ± 0,12
SS 06	01/2007	233	0,43	12,93			10	2,66 ± 0,09	10	2,85 ± 0,07
SS 06	02/2007	460	0,00	11,96			10	2,74 ± 0,10	9	2,99 ± 0,08
SS 08-a *	03/2006	244	61,89	8,60	10	3,01 ± 0,11	6	2,74 ± 0,08	10	2,96 ± 0,11
SS 08	06/2006	195	3,00	11,58	10	2,76 ± 0,17	5	2,74 ± 0,06	10	2,82 ± 0,37
SS 08-b	10/2006	329	17,02	16,12	10	3,01 ± 0,15	10	2,77 ± 0,10	10	3,02 ± 0,06
SS 08	12/2006	208	3,85	10,50	7	2,93 ± 0,10	10	2,70 ± 0,08	10	2,87 ± 0,12
SS 10-a *	01/2007	295	52,54	24,29						
SS 15-a	03/2006	457	21,01	13,30	10	3,03 ± 0,05	10	2,71 ± 0,05	10	2,96 ± 0,04
SS 15-a	04/2006	361	23,82	19,64	10	3,06 ± 0,11	10	2,75 ± 0,06	10	3,07 ± 0,06
SS 15-a	07/2006	145	28,28	11,54	10	3,01 ± 0,06	10	2,75 ± 0,04	10	3,05 ± 0,05
SS 15	01/2007	321	35,51	16,91	10	2,96 ± 0,06	10	2,66 ± 0,09	10	2,90 ± 0,08
SS 25-a	09/2006	201	0,50	17,00			10	2,81 ± 0,04	10	2,97 ± 0,09
SS 25	01/2007	167	0,60	21,69						
SS 28-a	03/2006	420	10,00	15,87	10	3,01 ± 0,11	10	2,80 ± 0,08	9	3,06 ± 0,06
SS 28-a	06/2006	380	26,05	14,23	10	3,04 ± 0,09	10	2,84 ± 0,05	10	3,15 ± 0,17
SS 28-a	08/2006	511	42,47	18,03	10	3,09 ± 0,06	10	2,80 ± 0,04	10	3,07 ± 0,09
SS 28-a	10/2006	338	4,14	11,42	9	2,85 ± 0,13	10	2,76 ± 0,04	10	3,08 ± 0,11
SS 28-a	01/2007	376	27,39	20,15	11	3,04 ± 0,13	10	2,74 ± 0,10	10	3,04 ± 0,10
SS 34-a	03/2006	403	0,00	11,66			10	2,70 ± 0,08	10	3,00 ± 0,06
SS 36-a	03/2006	354	5,65	13,17	10	2,99 ± 0,09	10	2,75 ± 0,06	10	3,04 ± 0,09
SS 36-b *	09/2006	357	41,74	22,60	10	3,05 ± 0,09	10	2,78 ± 0,06	10	3,02 ± 0,08
SS 36-b *	11/2006	320	69,69	10,31	10	3,04 ± 0,09	5	2,75 ± 0,11	5	3,03 ± 0,12

colônia e rainha	data de coleta	favo de cria			machos		rainhas		operárias	
		número de pupas	machos (%)	rainhas entre as fêmeas (%)	n	distância intertegular (mm)	n	distância intertegular (mm)	n	distância intertegular (mm)
SS 43-a	03/2006	404	4,70	12,73	9	2,92 ± 0,06	10	2,75 ± 0,05	10	3,00 ± 0,06
SS 43-a	05/2006	501	0,00	21,16			10	2,74 ± 0,12	10	2,97 ± 0,12
SS 43-a	07/2006	479	19,42	18,39	10	3,09 ± 0,09	10	2,83 ± 0,05	10	3,07 ± 0,09
SS 55-a	09/2006	425	0,00	8,24			10	2,72 ± 0,07	10	2,81 ± 0,16
SS 55-a	01/2007	577	9,01	15,62						
SS 59-a	03/2006	338	0,00	8,28			10	2,81 ± 0,15	10	3,05 ± 0,16
SS 59-a	05/2006	389	26,99	8,80	10	3,00 ± 0,13	10	2,74 ± 0,12	10	3,01 ± 0,08
SS 59-a	06/2006	340	30,88	16,17	10	3,05 ± 0,05	10	2,75 ± 0,07	10	2,98 ± 0,24
SS 59-a	09/2006	724	24,72	16,51	10	3,04 ± 0,11	9	2,80 ± 0,09	12	2,96 ± 0,35
SS 59-a	11/2006	501	36,13	14,06	10	2,97 ± 0,12	10	2,73 ± 0,07	10	2,98 ± 0,10
SS 59	12/2006	520	23,65	14,61	10	2,99 ± 0,07	10	2,75 ± 0,06	10	2,95 ± 0,24
SS 59-b	01/2007	381	38,06	7,20	10	2,95 ± 0,07	10	2,82 ± 0,09	11	2,97 ± 0,27
SS 59-b	02/2007	482	13,28	16,99	10	3,03 ± 0,09	10	2,82 ± 0,08	10	3,02 ± 0,10
SS 63	03/2006	251	56,57	23,85	10	2,93 ± 0,05	10	2,74 ± 0,05	10	2,97 ± 0,05
SS 63-a *	04/2006	408	55,39	23,08	11	3,08 ± 0,18	10	2,84 ± 0,07	9	3,05 ± 0,06
SS 63-a *	05/2006	244	51,23	10,92	11	3,18 ± 0,15	10	2,84 ± 0,06	9	3,06 ± 0,05
SS 63	07/2006	277	1,81	17,65	5	2,93 ± 0,06	10	2,78 ± 0,08	10	3,05 ± 0,04
SS 63-b	11/2006	440	10,00	16,41	10	3,04 ± 0,10	10	2,75 ± 0,04	10	3,01 ± 0,11
SS 66-a	09/2006	281	14,95	9,21	10	2,99 ± 0,04	10	2,82 ± 0,10	10	3,01 ± 0,23
SS 67-a	01/2007	386	13,73	15,62						
SS 72-a	01/2007	275	1,45	15,87						
Média:		340,02	21,76	14,76		2,55		2,75		2,99

As letras nos códigos das colônias se referem à sucessão de diferentes rainhas-mãe.

* A rainha-mãe e seu parceiro sexual compartilhavam um mesmo alelo no locus sexual e, portanto, machos diplóides foram produzidos.

Fig. 1. Porcentagem média mensal de machos (% do total de indivíduos) e de rainhas (% das fêmeas) produzidos em colônias de *Melipona scutellaris* mantidas em Igarassu (A) e S. Simão (B). As barras representam os erros-padrão.

Fig. 2. Distriuição de frequências das proporções de machos, entre os indivíduos (A-B), e de rainhas, entre as fêmeas (C-D), em colônias de *Melipona scutellaris* mantidas em Igarassu (A, C) e em S. Simão (B, D).

Fig. 3. Tamanho médio de machos, rainhas e operárias produzidos mensalmente em colônias de *Melipona scutellaris* mantidas em Igarassu (A) e S. Simão (B). As barras representam os erros-padrão.

DISCUSSÃO

A produção de sexuais em insetos sociais é um evento desencadeado por uma complexa interação entre fatores intra- e extra-coloniais (Bego 1990; Crozier & Pamilo 1996). Embora muitos fatores (e.g. disponibilidade de alimento no ambiente, quantidade de alimento armazenado na colônia, população do ninho) possam influenciar na produção de sexuais em abelhas sem ferrão (Velthuis *et al.* 2005), esse é o primeiro estudo que compara duas pequenas populações mantidas em diferentes regiões geográficas, uma em área onde a espécie está isolada geneticamente e a outra em área onde a espécie ocorre naturalmente.

Nas colônias de *Melipona scutellaris* mantidas em Igarassu e em S. Simão, os sexuais foram produzidos constantemente, mas em frequências diferentes pelas várias colônias (Tabela 1). Em nível populacional, nossos resultados corroboram estudos anteriores sobre a produção contínua de rainhas em colônias de *Melipona* ao longo dos meses (Moo-Valle *et al.* 2001; Sommeijer *et al.* 2003; van Veen *et al.* 2004; Morais *et al.* 2006). Assim como em *M. beecheii* (van Veen *et al.* 2004) e em *M. favosa* (Sommeijer *et al.* 2003), a produção de rainhas de *M. scutellaris* foi constante ao longo do tempo, sem variações significativas entre os meses do ano. O mesmo foi observado para os machos nas duas populações de *M. scutellaris*, assim como em colônias de *M. beecheii*, em que a quantidade de alimento estocado nos ninhos não foi manipulada (Moo-Valle *et al.* 2001), e de *M. bicolor* (Alves *et al.* in prep.). Ou seja, em nível populacional, os sexuais de *M. scutellaris* sempre estão presentes, independente da época do ano, e a sua produção é menos afetada por fatores sazonais.

Em relação ao tamanho dos indivíduos produzidos, houve variação tanto em nível intra- como inter-colonial, seja numa população ou noutra (Tabela 1). A magnitude de variação de tamanho das operárias de um mesmo ninho, eventualmente, pode ser em decorrência de aloetismo, em que operárias de diferentes tamanhos se especializam em determinadas tarefas (Goulson *et al.* 2005). Outra possível explicação para tal diferença entre tamanhos, em termos adaptativos, é a maior longevidade de operárias pequenas quando a fonte de néctar é escassa, o que representaria um seguro para a colônia quando ela passa por momentos de escassez de néctar (Couvillon & Dornhaus 2010). Assim, em nível populacional, a variação média entre as colônias pode ser atribuída às suas condições alimentares. Portanto, colônias com menos alimento armazenado produziram a mesma quantidade de indivíduos que colônias em boas condições alimentares, porém menores em tamanho (Ramalho *et al.* 1998).

As colônias mantidas em S. Simão não produziram indivíduos com diferenças significativas em tamanho em relação às mantidas em Igarassu (Fig. 3). Assim, podemos considerar que o investimento de recursos na produtividade das colônias, ou seja, na produção de machos, rainhas e operárias, é similar nas duas populações. Mesmo em regiões geográficas distintas e com condições climáticas diferenciadas, as colônias mantidas em Igarassu e em S. Simão alocaram quantidade semelhante de alimento na produção de sexuais e de operárias. Assim, não consideramos que a disponibilidade de recursos alimentares em cada região seja o principal fator de influência na diferença significativa da produção de sexuais nas duas populações. Contudo, também não descartamos que as condições ambientais (e.g. fatores abióticos, disponibilidade de recursos), como estudos anteriores apontaram, sejam fatores importantes em tal produção.

Atribuímos à discrepante diferença do número de alelos sexuais entre as duas populações (Alves *et al.* submetido) como a principal força na significativa produção de sexuais em S. Simão. Enquanto em Igarassu o número estimado de alelos sexuais foi de 25,9, em S. Simão esse número foi, aproximadamente, seis vezes menor (3,8) (Alves *et al.* submetido). A menor diversidade alélica em S. Simão leva à maior produção de machos diplóides (Cook & Crozier 1995; Zayed 2009; Alves *et al.* submetido), além de machos haplóides que geralmente são produzidos (Alves *et al.* 2009a). Quanto maior for o empobrecimento genético da população, maior é a chance de rainhas de realizarem acasalamentos com machos haplóides que compartilham alelos sexuais idênticos (Whiting 1943; Cook & Crozier 1995), o que acarretará maior produção de machos diplóides (Heimpel & de Boer 2008; Zayed 2009). Caso esses machos sejam viáveis, como são em *M. scutellaris* (Carvalho 2001; Alves *et al.* submetido), eles usualmente são estéreis (Cook & Crozier 1995; Heimpel & de Boer 2008), representando um alto custo para as colônias em que são produzidos. Além do custo em termos reprodutivos e de uso de recursos em sua produção (e.g. construção de células de cria, aprovisionamento com alimento larval), eles também causam uma desaceleração no crescimento colonial, já que, cerca de 50% dos ovos diplóides destinados a se tornarem fêmeas (operárias) se tornam machos diplóides, que não realizam uma ampla variedade de tarefas indispensáveis para a manutenção e/ou sobrevivência da colônia (Chapman & Bourke 2001).

Dessa forma, o impacto negativo da produção de machos diplóides é contrabalanceado pela substituição da rainha-mãe (Camargo 1979; Alves *et al.* submetido). De fato, a expectativa de vida de rainhas que produzem machos diplóides corresponde à metade da expectativa observada em rainhas que não os produzem (Alves *et al.* submetido) e assim a taxa

de substituição de rainhas é maior. Embora o alto número de rainhas seja um custo para as colônias, no que se refere ao gasto de recursos – já que muitas são mortas pouco tempo após emergirem das células (Silva *et al.* 1972; Koedam *et al.* 1995) – e à diminuição na produção de operárias – para cada rainha, uma operária deixa de ser produzida (Wenseleers & Ratnieks 2004) –, o baixo número de alelos sexuais nas pequenas populações de abelhas sem ferrão pode ser uma forte pressão a fim de aumentar a produção de rainhas.

Outro fato que também ajuda a explicar a surpreendente alta taxa de produção de rainhas na população de S. Simão é o estabelecimento de rainhas que nasceram em outros ninhos (Wenseleers *et al.* submetido). Nesse estudo, os autores verificaram que, em 25% das colônias órfãs, rainhas vindas de outros ninhos e que, provavelmente, estavam recém-fecundadas, entravam em “colônias-hospedeiras” e lá iniciavam suas atividades de postura, normalmente. Corroborando com esses dados, verificamos que das cinco rainhas que produziram machos diplóides em S. Simão, uma foi substituída por rainha nascida em outra colônia (Wenseleers *et al.* submetido).

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CAPÍTULO 2.

Successful maintenance of a stingless bee population despite a severe genetic bottleneck

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Successful maintenance of a stingless bee population despite a severe genetic bottleneck

ABSTRACT

Stingless bees play an important ecological role as pollinators of many wild plant species in the tropics and have significant potential for the pollination of agricultural crops. Nevertheless, conservation efforts as well as commercial breeding programmes require better guidelines on the amount of genetic variation that is needed to maintain viable populations. In this context, we carried out a long-term genetic study on the stingless bee *Melipona scutellaris* to evaluate the population viability consequences of prolonged breeding from a small number of foundress colonies. In particular, it was artificially imposed a genetic bottleneck by setting up a population starting from only two foundress colonies, and continued breeding from it for a period of over 10 years in a location outside its natural area of occurrence. We show that despite a great reduction in the number of alleles present at both neutral microsatellite *loci* and the sex-determining locus relative to its natural source population, and an increased frequency in the production of sterile diploid males, the genetically impoverished population could be successfully bred and maintained for at least 10 years. This shows that in stingless bees, breeding from a small stock of colonies may have less severe consequences than previously suspected. In addition, we provide a simulation model to determine the number of colonies that are needed to maintain a certain number of sex alleles in a population, thereby providing useful guidelines for stingless bee breeding and conservation programmes.

INTRODUCTION

Pollination by animals is considered a key ecosystem service (Costanza *et al.* 1997), and bees play a crucial role in this as important vectors for the efficient and effective sexual reproduction of many wild plants and commercial crops (Kearns *et al.* 1998; Slaa *et al.* 2006). However, anthropogenic changes, such as deforestation, agricultural intensification and pollution, have contributed to the decline or even the extinction of many native and managed bee populations, with severe negative consequences for both natural ecosystems and agriculture (Biesmeijer *et al.* 2006; Goulson *et al.* 2008).

Bees have special needs with regard to suitable nesting sites and floral resources and

habitat loss is one of the major threats to their continued survival (Goulson *et al.*, 2008). In addition, population reduction and habitat fragmentation lead to genetic bottlenecks and increased genetic drift and hence to a much reduced genetic diversity, which can negatively affect population viability (Goulson *et al.* 2008; Zayed 2009). Indeed, due to the complementary sex determination system (CSD; Whiting 1943), bees and other haplodiploid insects are expected to be unusually vulnerable to such genetic impoverishment, since the loss of alleles at the sex locus will lead to an increased production of sterile diploid males (Cook & Crozier 1995; Packer & Owen 2001). Under a CSD sex determination system, diploid individuals that are heterozygous at the sex locus develop into females, whereas haploid hemizygous and diploid homozygous individuals develop into males (Whiting 1943). The loss of genetic diversity in bee populations therefore leads to an increased chance for queens to make matched matings, i.e. to mate with haploid males sharing identical sex alleles (Whiting 1943; Cook & Crozier 1995; Zayed 2009), and this will result in an increased production of diploid males (Zayed *et al.* 2004). The production of diploid males is highly disadvantageous and imposes a large cost (Zayed *et al.* 2004; Heimpel & de Boer 2008), since diploid males are usually either unviable or sterile (Cook & Crozier 1995; Heimpel & de Boer 2008), or only able to father sterile triploid offspring (Cook & Crozier 1995). In addition, for eusocial species, the production of diploid males will slow down colony growth and increase colony mortality (Ross & Fletcher 1986; Cook & Crozier 1995), since a large proportion of the female worker-destined progeny will end up developing into diploid males, which do not contribute any colony resources (Chapman & Bourke 2001).

Stingless bees are the most diverse group of all eusocial bees and can be found in most tropical or subtropical regions of the world (Michener 1974). They are characterised by having perennial colonies composed of a few hundreds to ca. 10,000 workers and typically, a single, once-mated queen (Peters *et al.* 1999). Stingless bees of the genus *Melipona* comprise approximately 65 species distributed throughout the Neotropical region (Camargo & Pedro 2007). They nest in tree cavities in which the bees store pollen and honey in large egg-shaped pots (Michener 1974), which are frequently harvested by local beekeepers (Cortopassi-Laurino *et al.* 2006). In addition, *Melipona* species are major pollinators of a wide variety of wild plants and agricultural crops that have flowers with poricidal anthers that release pollen through the bees' vibrations (Heard 1999), and are therefore of great importance for the conservation of natural biodiversity in the tropics. Unlike bumblebees (Slaa *et al.* 2006),

however, stingless bees have as yet not been commercially bred on a large scale for pollination purposes, although they have been shown to have great potential for the pollination of many crops, including avocado, coconut, coffee, guava, mango and rose apple grown in open culture, as well as for strawberry, sweet pepper and tomato kept under greenhouse conditions (Heard 1999; Slaa *et al.* 2006). One factor that has hampered the large-scale deployment of stingless bees for pollination purposes is that little information is available on how successful breeding programmes should be set up (Cortopassi-Laurino *et al.* 2006), for example with respect to the amount of genetic variation and the number of sex alleles that are needed to maintain viable populations. So far, only few estimates are available of the number of sex alleles present in managed *Melipona* populations (Kerr 1987; Aidar & Kerr 2001; Carvalho 2001), and none of these studies have considered the genetic consequences of prolonged breeding of stingless bee colonies from a limited stock.

In this context, we carried out a long-term genetic study on the stingless bee *Melipona scutellaris* in order to evaluate the population viability consequences of prolonged breeding from a small number of foundress colonies. In particular, it was artificially imposed a genetic bottleneck by setting up a population starting from only two foundress colonies, and continued breeding from it for a period of over 10 years in a location outside its natural area of occurrence. Subsequently, we compare the frequency of the production of diploid males and the number of sex alleles that are present in the small and isolated managed population and the large, managed source population that is located in the species' natural area of occurrence. In addition, we determine the number of colonies that are needed to maintain a certain number of sex alleles in a population, thereby providing useful guidelines for stingless bee breeding and conservation programmes.

MATERIALS AND METHODS

Study organism

The geographic distribution of *M. scutellaris* covers the Atlantic Rainforest areas in the Northeast of Brazil (Camargo & Pedro 2007), where it uses hollow trees as protected cavities for nesting. Their perennial nests are composed of egg-shaped pots in which they store honey and pollen and multilayered horizontal brood combs with same-sized cells, from which workers, queens and males are reared. All cells are constructed, mass-provisioned with liquid

larval food and are sealed by the workers immediately after egg deposition (Michener 1974). A colony consists of one functional single-mated queen, ca. 2,000 workers (Peters *et al.* 1999; Tóth *et al.* 2004), and some males and virgin queens. In the Northeast of Brazil, *M. scutellaris* is intensively reared, due to the amount and quality of its honey (Cortopassi-Laurino *et al.* 2006; Carvalho-Zilse *et al.* 2009). In addition, *M. scutellaris* is a key pollinator for a large number of commercial crops, such as avocado, guava and rose apple (Castro 2002).

Study sites

We carried out this study with managed colonies kept in stingless bees apiaries (known as meliponaries) inside and outside their natural area of occurrence (at Granja São Saruê, Igarassu, Pernambuco state, 7°S, 34°W, and the Aretuzina Farm, São Simão, São Paulo state, 21°S, 47°W, respectively). In Igarassu, the beekeeper increased the number of colonies from 200 to 750 in five years by splitting nests (Cortopassi-Laurino *et al.* 2006) and in addition to this high number of managed colonies that were kept, the meliponary was also surrounded by a forested area, where wild nests can be found housing in tree cavities. In S. Simão, where *M. scutellaris* is kept outside its natural range, the population was started from only two foundress colonies which were obtained from Igarassu in 1996 (Nogueira-Neto 2002) and which after a breeding programme of ten years increased to about 55.

Sampling

Between January 2006 and May 2007, we collected stingless bees from 38 and 18 colonies maintained in wooden free-foraging nest boxes in Igarassu and S. Simão (Table 1). We collected 10 worker pupae and any male pupae that we could find directly from the comb, after which we preserved them in absolute ethanol for genetic analysis; the remainder of the brood was reintroduced back into the natal nest. For the S. Simão population, some colonies were resampled when the mother queen (which was individually marked) was replaced by a new one, which resulted in the collection of brood derived from 24 distinct queens (Table 1).

Genetic analysis of diploid male production

To check for diploid male production we genotyped all the collected male brood from all the brood combs that contained more than 5% of males ($n = 38$ colonies; Table 1). Those

containing fewer males could be inferred not to produce diploid males, given that the production of 5% males was significantly lower than the expected 50% if a queen had made a matched mating (one-sided binomial test, $P < 10^{-15}$ given that the collected combs contained 65-524 cells with diploid brood (avg. 231)). We genotyped 10 worker pupae and an average of 11 male pupae (range 7-23, total 404; Table 1) from 38 colonies in Igarassu and 18 in S. Simão at three microsatellite *loci*, *Mbi-201*, *Mbi-254* (Peters *et al.* 1998), and *T4-171* (Paxton *et al.* 1999). DNA was extracted using the Chelex method and microsatellites were amplified using multiplex PCR reactions, followed by a touchdown programme, as described in Alves *et al.* (2009). After amplification, 1 μ L of the PCR product was mixed with 8.8 μ L formamide and 0.2 μ L Genescan 500 LIZ size standard (Applied Biosystems, Lennik, Belgium) and loaded onto an ABI-3130 Avant capillary sequencer. Alleles were called using the supplied Gene Mapper software and checked by eye.

We reconstructed the genotype of each mother queen and that of her mate from the genotypes of the diploid brood she produced. This was straightforward, given that in all cases, genotypes were consistent with the mother queens being singly mated and with just a single matriline being present. We categorized males as haploid if they were homozygous at all *loci* and as diploid if they were heterozygous at one or more *loci* and carried both paternal and maternal alleles. In order to guarantee the accuracy of all male genotypes, we re-amplified, rescored and rechecked all male genotypes that appeared to suggest that they were diploid. The percentage of diploid brood that consisted of diploid males was estimated as

$$p = \frac{M(D/(D+H))}{M(D/(D+H)) + F} \quad (1)$$

where M and F were the number of males and females (gyenes and workers) collected from each comb and D and H were the number of diploid and haploid males among the genotyped males. For queens that had made a matched mating, we checked for deviations from the expected percentage of 50% using the parametric bootstrap method (Efron & Tibshirani 1998) followed by a Bonferroni correction for multiple testing, assuming that M and D were binomially distributed and using 1,000,000 bootstrap replicates. Deviations from the expected 50:50 ratio could arise either as a result of occasional double mating (Carvalho 2001) or due to some of the diploid males being unviable.

Relative life expectancy of queens that had made a matched mating

In order to determine the longevity of queens that had or had not made a matched mating we transferred 10 *M. scutellaris* colonies headed by newly mated queens from Igarassu to free-foraging nestboxes in the bee laboratory at the University of São Paulo (São Paulo, São Paulo state, 23°S, 46°W). Subsequently, we monitored the colonies for queen replacement events, by marking all mother queens with a coloured plastic tag on their thorax, and regularly checked all queens over a total period of ca. 3 years. Subsequently, female and male pupae were sampled before and after each queen replacement event, and genotyped as described above. Since *M. scutellaris* does not naturally occur in São Paulo state and given the small population size, we expected matched matings to occur by chance.

Estimation of the number of sex alleles

The effective number of sex alleles present in each population was estimated using the equation of Laidlaw *et al.* (1956):

$$k_e = \frac{2(N+1)}{H+1} \quad (2)$$

where k_e is the effective number of sex alleles, N is the total number of colonies sampled and H is the expected number of colonies that produced diploid males out of a given total number of colonies. Given that the life expectancy of queens producing diploid males was only half as long as those producing haploid males (see Results), the power p to detect colonies producing diploid males using our sampling scheme was only 50%. Hence, H was estimated as the observed number of colonies producing diploid males H_{obs}/p . 95% confidence limits on n were calculated using the parametric bootstrap method using the percentile method and 1,000,000 bootstrap replicates (Efron & Tibshirani 1998), assuming that H was binomially distributed and taking p as a constant.

Effect of a population bottleneck on the sampled number of sex alleles

The effect of starting a population from a small number of initial colonies is expected to greatly reduce the number of sex alleles present in the population. To quantify this effect we used a simple stochastic simulation model in Wolfram *Mathematica* in which we determined the expected number of sampled sex alleles as a function of the number of colonies used to

set up a new isolated population and the number of sex alleles present in the source population, assuming that these would be present in the population in approximately equal frequencies. The source population was taken to comprise 1,000 colonies. 95% confidence limits on the expected number of sampled sex alleles were calculated as the 2.5% and 97.5% percentiles of the distribution of estimates obtained by repeating the simulation 1,000 times (Efron & Tibshirani 1986).

Decline in the number of sex alleles over multiple generations

Over multiple generations, the number of sex alleles in a small population is expected to further decline as a result of drift. Nevertheless, because of selection against common alleles (which will tend to be eliminated as a result of matched matings), this decline is expected to proceed more slowly than for neutral alleles (Kimura & Crow 1964; Yokoyama & Nei 1979). To determine the speed at which sex alleles would be lost by drift from the *S. Simão* population, we again used a stochastic *Mathematica* simulation model, which was set up as follows: (a) we started the simulation with an initial population of 2 colonies containing a total of 5 sex alleles (the most parsimonious estimate, see Results), (b) queens died and were superseded at a constant rate of 1/87 days and 1/175 days for queens that had or had not made a matched mating (these mortality rates correspond to the reciprocal of the mean life expectancies of queens, see Results), (c) new queens were always assumed to be daughters of the superseded queen (occasionally queens in this species are superseded by unrelated queens that fly in from other colonies, but this is rare enough that it could be ignored in the present context) and were allowed to mate with a random male in the population, (d) 23% of the males were assumed to be the workers' sons and 77% the queen's sons (Alves *et al.* 2009) and (e) the population was allowed to grow via colony splitting at an observed rate of ca. 5 colonies per year. This simulation was then run for a period corresponding to 10 years, and at each point in time the effective number of alleles (Kimura & Crow 1964) that were present in the population was calculated. The same simulation was then repeated for unlinked neutral *loci*, as well as for the case where the population would have been started from a larger number of 5 foundress colonies containing 12 alleles. Subsequently, 95% confidence intervals on the means were calculated as the 2.5% and 97.5% percentiles of the estimates obtained over 1,000 simulation runs.

Estimating the number of breeding colonies from the number of sex alleles

After many generations, the number of sex alleles maintained in a population will be set by a balance between drift and mutation (Kimura & Crow 1964; Yokoyama & Nei 1979; Cornuet 1980). Yokoyama & Nei (1979) showed that the effective number of sex alleles that can be maintained in a finite population k_e is given by

$$k_e = \frac{1}{\sqrt{-\frac{3}{4N_e} \ln(\mu \sqrt{12\pi N_e})}} \quad (3)$$

where μ is the rate of mutation to new sex alleles and N_e is the effective size of the population. By solving Equation (3) for N_e , the effective population size given the presence of k_e sex alleles can therefore be estimated as

$$N_e = \frac{3}{8} k_e^2 \text{ProductLog}\left(\frac{2}{9\pi k_e^2 \mu^2}\right) \quad (4)$$

Following Kerr (1986), we assume in our calculations that $\mu \cong 1.6 \times 10^{-6}$, which is also in line with more recent estimates based on molecular evidence in *Apis* honeybees (Hasselmann *et al.* 2008).

The effective population size N_e for social Hymenoptera has been calculated to be

$$N_e = \frac{(3-p)^2 N_f N_m}{2N_f + (2-p)^2 N_m} \quad (5)$$

where p is the proportion of males that are workers' sons (which in this equation are all assumed to be produced by different egg-laying workers) and N_f and N_m are the effective number of breeding queens and males in the population (Owen & Owen 1989). For stingless bees, where queens mate only once (Peters *et al.* 1999) $N_f = N_m = C$, the effective number of breeding colonies, whereas for honeybees, where queens are multiple mated (Estoup *et al.* 1994), $N_f = C$ and $N_m = M_e C$, where M_e is the effective queen mating frequency. From this, the effective number of breeding colonies in the population C corresponding to a certain effective population size can be calculated as

$$C = \frac{N_e (2 + (2-p)^2 M_e)}{(3-p)^2 M_e} \quad (6)$$

where for stingless bees $M_e = 1$ and p can range between 0 to 1, depending on the species (Tóth *et al.* 2004), whereas for *A. mellifera* $M_e \cong 12.4$ (Estoup *et al.* 1994) and $p \cong 0$ (Visscher 1989)

RESULTS

Neutral genetic variation

The three microsatellite *loci* used were reasonably polymorphic with a total of 8, 5 and 7 alleles present at *loci* *Mbi-201*, *Mbi-254* and *T4-171*. Nevertheless, as expected, the genetic variation was much reduced in the bottlenecked S. Simão population (allelic richness: 3, 3, 4; expected heterozygosity: 61%, 64%, 51%; effective number of alleles: 2.6, 2.8, 2.0) compared to the Igarassu source population (allelic richness: 8, 5, 7; expected heterozygosity: 77%, 64%, 74%; effective number of alleles: 4.3, 2.8, 3.8). Hence, the average allelic richness and the mean effective number of alleles present were reduced by ca. 50% and 32% in S. Simão compared to the Igarassu population.

Diploid male production

Out of 404 males genotyped, 48 (11.88%) were heterozygous at one or more *loci*, and were therefore definitely diploid males (Table 1). The observed proportion of colonies producing diploid males (H_{obs}) in the bottlenecked S. Simão population ($6/24 = 25.0\%$) was significantly higher than in the Igarassu source population ($1/38=2.6\%$) (GLZ with binomial error structure and logit link function, log-likelihood = -18.1204, one-sided $P = 0.003$). Given that in S. Paulo two mother queens that produced diploid males survived only half as long (for 59 and 115 days, i.e. for an average of 87 days) as 18 queens who did not produce diploid males (median life expectancy 175 days, Kaplan-Meier analysis), the estimated true proportion of newly established queens that would have produced diploid males was ca. twice as high, i.e. $12/24=50.0\%$ in S. Simão and $2/38=5.4\%$ in Igarassu.

As expected, colonies producing diploid males had higher mean proportions of males in their brood combs (mean $\pm$ s.d. = $57.80 \pm 8.30\%$; range 47.33-69.69%) than colonies that just produced haploid males (mean $\pm$ s.d. = $21.17\% \pm 10.65$; range 5.13-45.08%) (Table 1). In addition, in none of the 7 colonies where queens had made a matched mating did the estimated percentage of diploid brood that was male deviate from the expected 50% (Table

1, parametric bootstrap method, $P > 0.05$). This provides additional independent evidence for single mating and also shows that diploid males did not have a lower viability than haploid ones.

Estimated number of sex alleles and effective population size

From Equation (2) (Laidlaw *et al.* 1956) and the observed frequency of colonies producing diploid males, we estimate that the Igarassu source population contained an effective number of 25.9 sex alleles (95% CL [11.1-78.0], Table 2). The bottlenecked S. Simão population, by contrast, was estimated to contain an effective number of only 3.8 sex alleles (95% CL [2.4-6.0], Table 2). Given an estimated effective number of 25.9 sex alleles being present in the Igarassu population, an estimated mutation rate μ of 1.6×10^{-6} (Kerr 1986) and the fact that in *Melipona scutellaris* 23% of the males are workers' sons, we can also estimate that the source population must have had an effective size of ca. 3,732 (Equation (4)), which corresponds to ca. 2,497 effectively breeding colonies (Equation (6), Owen & Owen 1989), or a colony density of ca. 8.3 colonies per hectare (given a male flight range of ca. 1 km and a circular mating area of ca. 3 km², Carvalho-Zilse & Kerr 2004) (Table 2).

Simulation results

Our simulation results demonstrate that the low number of sex alleles present in S. Simão was mainly the result of the severe population bottleneck that was imposed by starting a population from only two source colonies. In fact, sampling two colonies from a source population containing an effective number of 25 sex alleles results in the sampling of an average number of 5.4 sex alleles (95% CL [4.0-6.0]), which is close to our estimate obtained after 10 years of continued breeding (3.8 sex alleles). This means that only very few – if any – sex alleles were lost by drift in the S. Simão population, an idea that is further reinforced by our simulations which show that alleles at the sex locus are lost by drift at a very low rate, and at a much lower rate than for neutral *loci* (Figure 1). This is due to the strong balancing selection at the sex locus, which favours rare alleles and tends to keep the different sex alleles in approximately equal frequencies, thereby reducing the likelihood of drift (Figure 1; Kimura & Crow 1964; Yokoyama & Nei 1979; Zayed *et al.* 2007).

Table 1. *Melipona scutellaris* colonies kept in Igarassu (IG) and São Simão (SS) with information on the total number of pupae and percentage of males present in the sampled brood combs, the number of workers and males genotyped and the estimated percentage of diploid brood that was male.

Colony and queen ¹	Collection date	Sampled brood combs		Number of individuals genotyped ²			Estimated % of diploid brood male ³
		Number of pupae	Percentage of males	Workers	Haploid males	Diploid males	
IG 01-a	Sep. 2006	441	36.05%	10	14	0	0.00%
IG 02-a	Jan. 2006	288	0.00%				0.00%
IG 03-a	Jan. 2006	386	16.06%	10	8	0	0.00%
IG 04-a	Jan. 2006	199	0.00%				0.00%
IG 05-a	Sep. 2006	341	18.48%	10	9	0	0.00%
IG 06-a	Sep. 2006	426	24.41%	10	10	0	0.00%
IG 07-a	Jan. 2006	281	0.00%				0.00%
IG 08-a	Jan. 2006	323	8.36%	10	9	0	0.00%
IG 09-a	Jan. 2006	100	0.00%				0.00%
IG 10-a	May 2007	102	0.00%				0.00%
IG 11-a	Sep. 2006	488	45.08%	10	9	0	0.00%
IG 12-a	Jan. 2006	380	28.42%	10	9	0	0.00%
IG 13-a	Jan. 2006	365	28.77%	10	9	0	0.00%
IG 14-a	Jan. 2006	164	2.44%				0.00%
IG 15-a	Jan. 2006	525	0.19%				0.00%
IG 16-a	Jan. 2006	282	33.69%	10	8	0	0.00%
IG 17-a	Jan. 2006	188	0.00%				0.00%
IG 18-a	Jan. 2006	241	19.09%	10	10	0	0.00%
IG 19-a	May 2007	455	21.10%	10	8	0	0.00%
IG 20-a	May 2007	368	17.93%	10	10	0	0.00%
IG 21-a	May 2007	66	1.52%				0.00%
IG 22-a	Sep. 2006	87	0.00%				0.00%
IG 23-a	Sep. 2006	269	1.86%				0.00%
IG 24-a	Sep. 2006	162	0.00%				0.00%
IG 25-a	Sep. 2006	123	0.00%				0.00%
IG 26-a	Sep. 2006	282	21.99%	10	10	0	0.00%
IG 27-a	Sep. 2006	314	6.37%	10	10	0	0.00%
IG 28-a	Sep. 2006	191	0.00%				0.00%
IG 29-a	Sep. 2006	328	0.00%				0.00%
IG 30-a	Sep. 2006	326	19.63%	10	10	0	0.00%
IG 31-a	Sep. 2006	265	0.00%				0.00%
IG 32-a	May 2007	72	0.00%				0.00%
IG 33-a	May 2007	366	0.00%				0.00%
IG 34-a	May 2007	162	0.00%				0.00%
IG 35-a	May 2007	262	47.33%	10	1	9	44.71%
IG 36-a	May 2007	217	29.03%	10	10	0	0.00%
IG 38-a	May 2007	176	0.57%				0.00%
IG 39-a	May 2007	423	9.69%	10	12	0	0.00%
Proportion of colonies that produced diploid males					2.63%		
SS 01-a	Mar. 2006	279	66.31%	10	7	3	37.12%
SS 02-a	Mar. 2006	445	51.46%	10	0	10	51.46%
SS 02-b	Feb. 2007	201	8.96%	10	10	0	0.00%
SS 04-a	Apr. 2006	247	13.77%	10	10	0	0.00%

Colony and queen ¹	Collection date	Sampled brood combs		Number of individuals genotyped ²			Estimated % of diploid brood male ³
		Number of pupae	Percentage of males	Workers	Haploid males	Diploid males	
SS 06-a	Jul. 2006	347	15.85%	10	8	0	0.00%
SS 06-b	Dec. 2006	195	5.13%	10	9	0	0.00%
SS 08-a	Mar. 2006	244	61.89%	10	5	4	41.92%
SS 08-b	Oct. 2006	329	17.02%	10	10	0	0.00%
SS 10-a	Jan. 2007	295	52.54%	10	0	10	52.54%
SS 15-a	Mar. 2006	457	21.01%	10	11	0	0.00%
SS 25-a	Sep. 2006	201	0.50%				0.00%
SS 28-a	Aug. 2006	511	42.47%	10	16	0	0.00%
SS 34-a	Mar. 2006	403	0.00%				0.00%
SS 36-a	Mar. 2006	354	5.65%	10	7	0	0.00%
SS 36-b	Nov. 2006	320	69.69%	10	8	5	46.93%
SS 43-a	Jul. 2006	479	19.42%	10	10	0	0.00%
SS 55-a	Sep. 2006	425	0.00%				0.00%
SS 59-a	Sep. 2006	724	24.72%	10	23	0	0.00%
SS 59-b	Jan. 2007	381	38.06%	10	20	0	0.00%
SS 63-a	Apr. 2006	408	55.39%	10	7	7	38.31%
SS 63-b	Nov. 2006	440	10.00%	10	9	0	0.00%
SS 66-a	Sep. 2006	281	14.95%	10	10	0	0.00%
SS 67-a	Jan. 2007	386	13.73%	10	10	0	0.00%
SS 72-a	Jan. 2007	275	1.45%				
<i>Proportion of colonies that produced diploid males</i>				<i>25.00%</i>			

¹The letters in the colony codes refer to the succession of different mother queens.

²Brood samples were not genotyped if they did not contain male brood or males were present in percentages lower than 5% in the sampled combs.

³Based on Equation (1).

Table 2. Managed stingless bee populations where diploid males have been reported.

Species	Location where colonies were kept ^a	Method ^b	Percentage of colonies with DMP (number of colonies) ^c	Estimated number of sex alleles ^d [95% CL]	Percentage of males that are workers' sons	Inferred effective population size (number of colonies) ^e	References
Large managed populations in natural range:							
<i>Melipona compressipes</i>	São Luis, MA, Br	C, PM	8.0% (49)	20.0 [11.1-50.0]	0-100% ^f	2298 (1532-1724)	Kerr (1987)
<i>M. scutellaris</i>	Igarassu, PE, Br	M, PM	2.6% (38)	25.9 [11.1-78.0]	23% ^g	3732 (2497)	this study
Medium-sized populations derived from natural range:							
<i>M. bicolor</i> ^h	São Paulo, SP, Br	M	14.0% (14)	10.0 [5.0-30.0]	37%	623 (420)	D.A.A. et al., unpublished data
<i>M. quadrifasciata</i> ⁱ	Ribeirão Preto, SP, Br	C, PM	20.0% (15)	8.0 [4.6-32.0]	64% ^j	409 (283)	Aidar & Kerr (2001)
<i>M. scutellaris</i> ^k	Uberlândia, MG, Br	C, PM	23.0% (124)	8.3 [6.4-11.9] ^l	23% ^g	441 (295)	Carvalho (2001)
<i>Scaptotrigona postica</i>	Ribeirão Preto, SP, Br	M	20.0% (10)	7.3 [3.7-22.0] ^m	37%	347 (231)	Paxton et al. (2003)
<i>Trigona carbonaria</i>	Sydney, Au	M	20.0% (5)	6.0 [3.0-12.0]	0%	237 (158)	Green & Oldroyd (2002)
Small isolated population outside natural range:							
<i>M. scutellaris</i>	São Simão, SP, Br	M, PM	25.0% (24)	3.8 [2.4-6.5]	-	-	this study

^aAbbreviations refer to Brazilian states: Maranhão (MA); Minas Gerais (MG); Pernambuco (PE); São Paulo (SP).

^bDiploid male production inferred based on: cytogenetics (C), microsatellite genotyping (M) or the proportion of males present in brood comb (PM).

^cObserved percentage of colonies in which diploid males were found (total number of colonies sampled).

^dEstimated number of sex alleles based on eqn. 2 (Laidlaw et al. 1956), under the assumption of monandry (see Methods), where possible correcting for the fact that queens that made a matched mating tend to have a shorter life expectancy.

^eBased on eqn. 4, assuming a mutation rate of $\mu = 1.6 \times 10^{-6}$ (Kerr 1986), and eqn. 6, respectively.

^fDate on worker male production are not available for *M. compressipes*, hence we estimated the nr. of breeding colonies for the entire range of possible values for worker male production (0-100%).

^gBased on data in Alves et al. (2009).

^hSpecies facultatively polygyne (i.e. occasionally more than one breeding queen is present in a colony).

ⁱFrom an unknown number of foundress colonies a population of 78 colonies was reached after 10 years of breeding in a meliponary in Ribeirão Preto city. From this population, 15 were randomly sampled to estimate the number of sex alleles and another 10 colonies were orphaned and taken to the Sea Mountains (Espírito Santo state), in order to allow new queens to mate with males from that region. After ten days, nine colonies returned to R. Preto and were sampled along with 16 colonies that stayed in the meliponary. Because 8% of sampled colonies produced diploid males (2 out of 25 colonies), the new number of sex alleles after this manipulation was estimated at 17.3.

^jBased on data in Tóth et al. (2002).

^kThe population started with 14 foundress colonies in 1988 and 8 colonies in 1990 and was kept outside its natural range (the colonies came from Lençóis, BA state). However 13, 3, 11 and 3 foreign mated queens from different locations in Bahia state were introduced in the study population in 1992, 1993, 1994 and 1995, respectively. This manipulation may have slightly inflated the estimated number of sex alleles and effective population size.

^lThe number of sex alleles reported by Carvalho (2001) was higher (23.9) than our estimate given that she considered some queens to be double-mated based on the proportion of males recovered from brood combs (3:1 female:male ratio as opposed to 1:1). Nevertheless, given that genetic studies have consistently found single mating in stingless bees (Peters et al. 1999; Tóth et al. 2004; Alves et al. 2009), these uneven ratios were probably merely the result of sampling error.

^mThe number of sex alleles reported by Paxton et al. (2003) was higher (10.0) than our estimate due to the use of another estimator, $n = 1/\text{DMP}$ (Cook & Crozier 1995), where DMP is the proportion of diploids that are male.

Fig. 1. Expected decline in the mean effective number of alleles present at the sex locus (red) and unlinked neutral *loci* (green) in a *Melipona scutellaris* bee population, starting from (a) an initial number of 2 foundress colonies containing 5 alleles and (b) 5 foundress colonies containing 12 alleles, assuming single mating, a constant population growth of 5 colonies per year, a constant mortality rate of 1/87 days and 1/175 days for queens that had or had not made a matched mating and with 23% of the males being workers' sons Alves *et al.* 2009. Lightly shaded areas represent the 95% confidence intervals, calculated as the 2.5% and 97.5% percentiles of the estimates obtained over 1,000 simulation runs.

DISCUSSION

Our results demonstrate that imposing a severe bottleneck in the stingless bee *M. scutellaris* resulted in a drastic decrease in the genetic variability and allelic richness, both at neutral *loci* and at the sex locus, and in a marked increase in the production of sterile diploid males. In particular, the estimated effective number of sex alleles decreased from 25.9 in the Igarassu source population to 3.8 in the bottlenecked S. Simão population, resulting in almost half of all queens making matched matings in the bottlenecked population. Surprisingly enough, however, given adequate management, the bottlenecked population could be successfully bred and maintained for over 10 years and grew at a constant rate of ca. 5 colonies per year up until the very end of our study. This shows that in stingless bees, breeding from a small stock of colonies may have less severe consequences than previously suspected (Kerr & Vencovsky 1982; Carvalho 2001; Zayed 2009). So far, only few examples are known in the social Hymenoptera of successful bottlenecked populations (e.g., in the fire ant *Solenopsis invicta* (Ross & Fletcher 1986), in the bumblebee *Bombus terrestris* (Schmid-Hempel *et al.* 2007) and the solitary bee *Lasioglossum leucozonium* (Zayed *et al.* 2007)), which may indicate that stingless bee populations are perhaps less sensitive to genetic bottlenecks and that they can tolerate lower numbers of sex alleles in their populations.

Why would this be so? Undoubtedly, one factor that helped the S. Simão population survive and reduced the negative impact of diploid male production was the applied artificial colony management, which involved providing abundant floral resources and artificial feeding and moving brood combs from strong to weak colonies (Nogueira-Neto 2002). Another important factor, however, which may contribute to diploid males having only a moderate effect on the viability of stingless bee populations is that in stingless bees, mother queens producing diploid males tend to be killed by the workers (Camargo 1979). In stingless bees the brood is reared in sealed, mass provisioned cells (Michener 1974) which results in adult workers being unable to eliminate diploid males at an early stage, unlike in honeybees where brood is reared in open cells (Woyke 1963). Nevertheless, in *M. quadrifasciata* it has been shown that queens that had made a matched mating are killed by the workers within 6 to 30 days after diploid males first begin to emerge (Camargo 1979). In *M. scutellaris*, our results also show that two queens which produced diploid males were replaced by another queen within an average time of ca. 40 days (range 11-67 days) after their first brood began to emerge (accounting for the fact that the developmental time from egg to adult in *M.*

scutellaris is nearly 48 days). The replacement of queens that had made a matched mating evidently limits the negative effects of the production of diploid males and of having small numbers of sex alleles in the population.

If we compare our results with those obtained for other native stingless bee populations (Table 2), we can see that they are also generally characterised by having relatively low numbers of sex alleles (6-9), small estimated effective population sizes (237-547) and a small corresponding number of effectively breeding colonies (158-368). Honeybees, by comparison, generally have much larger effective population sizes (ca. 2,000 for African honeybees, corresponding to ca. 1,000 breeding colonies (Equation (6), Estoup *et al.* 1995), thereby maintaining much higher numbers of sex alleles in their populations (ca. 17, Adams *et al.* 1977). The larger effective population size of honeybees compared to stingless bees is due to the fact that honeybees are bred on a much larger scale (De la Rúa *et al.* 2009) than most stingless bee species and that they are multiple mated (Estoup *et al.* 1994), which for a given number of breeding colonies will result in a higher effective population size compared to stingless bees where queens are single mated (Peters *et al.* 1999; Tóth *et al.* 2004). On the other hand, two stingless bees, *M. compressipes* and *M. scutellaris*, are also bred on a large scale in the Northeast of Brazil, with local beekeepers sometimes maintaining up to 1,500 colonies (Carvalho-Zilse *et al.* 2009). Consequently, in these two cases we also find large effective population sizes and high numbers of sex alleles (20-26, Table 2). In addition, the effective population size and the number of sex alleles is in these species also inflated by the common practise of exchanging nests, brood combs and mated queens among different meliponaries in different locations (Carvalho-Zilse *et al.* 2009; De la Rúa *et al.* 2009).

Our simulation results further show that population bottlenecks can potentially be a more potent cause of the loss of sex alleles in stingless bee populations than drift. This is because of the strong balancing selection at the sex locus, which favours rare alleles and disfavours common alleles, and which thereby keeps the different sex alleles in approximately equal frequencies, reducing the likelihood of drift (Figure 1; Kimura & Crow 1964; Yokoyama & Nei 1979; Zayed *et al.* 2007). This result implies that stingless bee conservation efforts and breeding programmes should be particularly concerned with avoiding severe population bottlenecks. For example, our calculations show that if one wanted to be 95% certain of sampling at least 6 sex alleles from a source population containing 25 sex alleles, one would have to start breeding from a foundress stock of at least

4 colonies, or 5 colonies if the source population contained a more moderate number of 8 sex alleles. On the other hand, if the aim is to maintain a certain number of sex alleles over very many generations, then drift too becomes important. Table 2 shows that in natural and small-scale managed populations, the number of sex alleles never appears to go below 6. If we take this figure as the minimum required to maintain viable natural populations (cf. Kerr & Vencovsky 1982), then Equation (3) (Yokoyama & Nei 1979) shows that with a mutation rate μ of 1.6×10^{-6} (Kerr 1986) one would need an effective population size of at least 238 to maintain this number sex alleles over many generations. Depending on the proportion of males that are workers' sons, this would correspond to an effective number of breeding colonies of between 159 and 179 (Equation (6)) (Owen & Owen 1989), or a colony density of at least 1 colony per 2 hectares (given a male flight range of ca. 1 km and a circular mating area of ca. 3 km², Carvalho-Zilse & Kerr 2004). This figure is relevant in a conservation context, since deforestation in Brazil is leading to increased fragmentation of suitable habitat, thereby threatening many natural stingless bee population (Brown & Albrecht 2001). It should be noted that this figure of 159-170 colonies is also higher than that provided by Kerr & Vencovsky (1982). This is because they had incorrectly assumed that worker reproduction is always present in stingless bees (which is not the case, Tóth *et al.* 2004), and more significantly, because they had made the erroneous assumption that every egg laying worker inside a colony should be counted as an independently breeding female, and that worker reproduction would therefore increase effective population size. More recent calculations have shown instead that worker reproduction tends to slightly reduce the effective population size, given that worker reproduction causes an extra round of gametic sampling, thereby leading to more drift (Crozier 1976; Owen & Owen 1989, see Equation (5)).

Overall, our study then leads to a dual conclusion. On the one hand, we show that given adequate management even severely bottlenecked stingless bee populations can be successfully maintained. On the other hand, it is clear that this conclusion might not apply to natural, unmanaged populations, and that for such populations quite a high number of at least ~150 effectively breeding colonies may be required to optimally maintain viable populations. These figures should provide useful guidelines for stingless bee breeding and conservation programmes.

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CAPÍTULO 3.

The queen is dead – long live the workers:
intraspecific parasitism by workers in the
stingless bee *Melipona scutellaris*

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The queen is dead – long live the workers: intraspecific parasitism by workers in the stingless bee *Melipona scutellaris*

ABSTRACT

Insect societies are well known for their high degree of cooperation, but their colonies can potentially be exploited by reproductive workers who lay unfertilised, male eggs, rather than work for the good of the colony. Recently, it has also been discovered that workers in bumblebees and Asian honeybees can succeed in entering and parasitizing unrelated colonies to produce their own male offspring. The aim of this study was to investigate whether such intraspecific worker parasitism might also occur in stingless bees, another group of highly social bees. Based on a large-scale genetic study of the species *Melipona scutellaris*, and the genotyping of nearly 600 males from 45 colonies, we show that ca. 20% of all males are workers' sons, but that around 80% of these had genotypes that were incompatible with them being the sons of workers of the resident queen. By tracking colonies over multiple generations we show that these males were not produced by drifted workers, but rather by workers that were the offspring of a previous, superseded queen. This means that uniquely, workers reproductively parasitize the next-generation workforce. Our results are surprising given that most colonies were sampled many months after the previous queen had died and that workers normally only have a life expectancy of ca. 30 days. It also implies that reproductive workers greatly outlive all other workers. We explain our results in the context of kin selection theory, and the fact that it pays workers more from exploiting the colony if costs are carried by less related individuals.

INTRODUCTION

Social insects such as ants, bees and wasps are well known for their high degree of cooperation, but the nonclonal structure of their colonies also sets the stage for various reproductive conflicts (Hamilton 1964; Trivers & Hare 1976; Ratnieks & Reeve 1992; Beekman & Ratnieks 2003; Ratnieks *et al.* 2006). One such conflict is queen-worker conflict over male parentage (Trivers & Hare 1976; Bourke 1988; Hammond & Keller 2004; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006b). Workers, though generally being unable to mate, are usually capable of laying unfertilized eggs, which develop into male offspring if successfully reared. In addition, they are generally selected to do so, since workers are always most related to their own sons ($r = 0.5$) (Hamilton 1964; Trivers & Hare 1976; Cole 1986; Bourke 1988; Wenseleers *et al.*

2004a). Hence, a queen-worker conflict over the production of males ensues. Several factors, however, may help to resolve queen-worker conflict over male parentage and keep successful worker reproduction at a low level (Beekman & Ratnieks 2003; Hammond & Keller 2004; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006a). First, the queen herself can counter worker reproduction by selectively eating any worker-laid eggs ("queen policing") (Oster & Wilson 1978; Ratnieks & Reeve 1992; Wenseleers *et al.* 2005a, b, reviewed in Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006a). Second, the workers may also cannibalize eggs laid by other workers ("worker policing", Ratnieks 1988; Ratnieks & Visscher 1989). This behaviour is seen particularly in species with multiple mated queens (such as honeybees, where worker policing was first discovered), due to the fact that in that situation workers are more related to the queen's sons (brothers, $r=0.25$) than to the sons of other workers (a mix of full- and half-nephews, $r<0.25$) (Ratnieks 1988; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006a,b; Ratnieks & Wenseleers 2008). By contrast, in species with a single-mated queen (such as some ants, bumblebees and stingless bees), workers are on average more related to the sons of other workers (full-nephews, $r=0.375$), than to the sons of the queen ($r=0.25$), and so collectively favour worker reproduction. Nevertheless, even in these species, worker reproduction may not always reach high levels, probably because of colony-level costs associated with worker reproduction, caused by the fact that reproductive workers in some species carry out less work in the colony (Cole 1986; Bourke 1988; Hillesheim *et al.* 1989; Wenseleers *et al.* 2004b) or because worker male production is traded off against worker production, thereby reducing future colony productivity (Ratnieks & Reeve 1992; Tóth *et al.* 2004; Wenseleers & Ratnieks 2006a; Ohtsuki & Tsuji 2009).

Recently, it has also been discovered that workers may not only exploit their own colony by laying male eggs, but that they may also parasitize and reproduce in other, unrelated colonies away from their natal nest (Beekman & Oldroyd 2008). For example, in the common bumblebee *Bombus terrestris*, workers may succeed in entering unrelated colonies, where they appear to reproduce earlier than normal, thereby insuring that their sons have a greater mating success than if they had produced such males during the normal male production period in their natal colony (Lopez-Vaamonde *et al.* 2004; Beekman & Oldroyd 2008). In addition, in two Asian honeybee species, *Apis florea* (Nanork *et al.* 2005) and *A. cerana* (Nanork *et al.* 2007), it has been shown that particularly queenless nests are prone to be parasitized by non-natal workers. The original interpretation was that this was due to workers from queenright colonies invading these nests, and that it represented a strategy to evade policing in their natal nest, since worker policing

is generally switched off in queenless nests (Nanork *et al.* 2005, 2007; Beekman & Oldroyd 2008). More recent data, however, rather seem to suggest that the majority of the worker parasites appear to come from other queenless hives nearby (Chapman *et al.* 2009). In both bumblebees and Asian honeybees, non-natal workers also tend to reproduce more than natal ones, presumably because any concomitant costs of worker reproduction are carried by the unrelated workforce of the parasitized nest, thus limiting any inclusive fitness costs (Beekman & Oldroyd 2008).

The aim of this study was to test whether intraspecific worker parasitism might also occur in stingless bees, another major group of eusocial bees, using the Brazilian species *Melipona scutellaris* as a model. Like honeybees, stingless bees form perennial, swarm-founded colonies, although more similar to bumblebees, their colonies are headed by a single once-mated queen (Peters *et al.* 1999). Furthermore, in both wild colonies and colonies kept in apiaries, genetic studies have demonstrated the occasional occurrence of workers that could not be attributed to the current queen, which indicates the presence of either unrelated drifters or workers derived from a superseded queen (Paxton *et al.* 1999a, 2003; Peters *et al.* 1999; Palmer *et al.* 2002). As yet, however, it has not been determined whether such workers might also successfully reproduce, and if so, whether they might do more so than the rest of the colony. In general, worker reproduction in stingless bees, though favoured on relatedness grounds (Tóth *et al.* 2002b, 2004), can vary from low to high, with anywhere between 0% and 95% of the males being workers' sons depending on the species (see e.g. Contel & Kerr 1976; Sommeijer *et al.* 1999; Drumond *et al.* 2000; Palmer *et al.* 2002; Tóth *et al.* 2002a, b, Tóth *et al.* 2003; Paxton *et al.* 2003; Koedam *et al.* 2005; Gloag *et al.* 2007, reviewed in (Hammond & Keller 2004; Tóth *et al.* 2004; Velthuis *et al.* 2005; Wenseleers & Ratnieks 2006a). Although the cause of this variation remains unknown, it could perhaps be due to interspecific differences in the colony-level cost of worker reproduction or due to the queen suppressing worker reproduction to varying extents (Tóth *et al.* 2004; Wenseleers & Ratnieks 2006a; Ratnieks & Wenseleers 2008).

Given the occurrence of worker reproduction in stingless bees (Hammond & Keller 2004; Tóth *et al.* 2004; Velthuis *et al.* 2005; Wenseleers & Ratnieks 2006a) and the documented presence of less-related or unrelated worker matrilineal colonies (Paxton *et al.* 1999a, 2003; Peters *et al.* 1999; Palmer *et al.* 2002), the specific aims of this study were to test whether in *Melipona scutellaris* such workers might be able to successfully reproduce, and if so, to what extent. By genotyping colony samples before and after queen supersedure events, we also test

for the first time if workers from a superseded queen could keep on producing male offspring, and if so, for how long. This allows us to determine whether workers might have the capacity to reproductively parasitize the next-generation workforce.

MATERIALS AND METHODS

Study species

Melipona scutellaris nests in cavities of tree trunks in the Atlantic rainforest and is widely distributed in the Northeast of Brazil (Camargo & Pedro 2007), where it is commonly kept by regional and traditional beekeepers for honey, pollen and wax (Cortopassi-Laurino *et al.* 2006). In addition, *M. scutellaris* is increasingly used for the pollination of various tropical crops (Heard 1999; Castro 2002). Colonies are perennial and swarm-founded, typically contain around 1,500 workers and are headed by one single-mated queen (Tóth *et al.* 2002b). Workers, gynes and males are reared individually in similar-sized cells which are filled with larval food and sealed by workers immediately after an egg is laid. For the present study, we used colonies of *M. scutellaris* maintained in free-foraging wooden nestboxes at Granja São Saruê (Igarassu, Pernambuco state, 7°50'3.74"S 34°54'22.87"W), at the Bee Laboratory at the University of São Paulo (São Paulo, S. Paulo state, 23°33'37.41"S 46°43'53.04"W) and at the Aretuzina Farm (São Simão, S. Paulo state, 21°26'25.97"S 47°34'54.65"W). Colonies were spaced ca. 1 to 10 metres apart, as is typical in most apiaries and also not uncommon in nature, where several nests can sometimes be found within a few metres of each other, either in the same tree or in different trees (Alves *et al.* 2005). During the time of our study colonies were managed only by occasionally providing them with food.

Brood sampling

Between January 2006 and October 2008, we sampled brood for genetic analysis from a total of 16, 9 and 12 colonies in Igarassu, S. Paulo and S. Simão, respectively (Table 1). This was done by collecting brood combs containing mature pupae and removing the cell cappings, after which 10 workers and any males present were preserved in absolute ethanol. The remainder of the brood was reintroduced into the natal nest. Brood samples that did not contain male brood were not retained for the present study. In S. Paulo and S. Simão, we also repeated the sampling of brood when the mother queen, which was individually marked, was

found to be replaced by a new one (Table 1). Such samplings were made at a median number of 206 days after the previous queen had died (range 77-737 days). Overall, we could collect male and worker brood produced by 45 distinct queens (Table 1).

Genetic analysis

For parentage analysis we genotyped 10 worker pupae and an average of 13 haploid male pupae (range 5-36, total 576) for each of our 45 colonies (Table 1) at three microsatellite *loci*, *T4-171* (Paxton *et al.* 1999b), *Mbi-254* and *Mbi-201* (Peters *et al.* 1998). These *loci* were found to be most variable in this species in a preliminary screening of 10 *loci*. To facilitate parentage reconstruction and where necessary we also noninvasively genotyped the mother queen from a wing tip sample (cf. Châline *et al.* 2004, Table 1). DNA was extracted using the Chelex method, whereby a single leg (for worker or male pupae) or wing tip sample (from mother queens) was frozen in liquid nitrogen and ground up using a plastic pestle, followed by an incubation at 95 °C for 15 min in 200 µL (50 µL for wing tip samples) of a 10% Biorad Chelex 100 resin solution. Samples were vortexed and centrifuged before use. Multiplex PCR reactions were carried out in a 10 µL reaction volume, and contained 0.5 µM of the forward and reverse primers of each locus, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 1 µL of crude DNA extract, 0.4 units of Silverstar Taq polymerase (Eurogentec, Seraing, Belgium) and enzyme buffer supplied by the manufacturer. PCR was performed following a touch-down programme (Bonckaert *et al.* 2008), with an initial denaturation for 3 min at 94 °C, followed by 20 cycles consisting of 30 s at 94 °C, 30 s at 58 °C, but decreasing 0.5 °C in each step, and 45 s at 72 °C; 10 cycles consisting of 30 s at 94 °C, 30 s at 46 °C, and 45 s at 72 °C; and a final 10-min extension step at 72 °C. After amplification, 1 µL of the PCR product was mixed with 8.8 µL formamide and 0.2 µL Genescan 500 LIZ size standard (Applied Biosystems, Lennik, Belgium), denatured, and loaded onto an ABI-3130 Avant capillary sequencer. Alleles were called using the supplied Gene Mapper software and checked by eye. Male genotypes that appeared to suggest they were the sons of drifted workers or sons of workers derived from a superseded queen (see below) were rePCRred, rescored and rechecked twice to eliminate the possibility of genotyping errors.

Analysis of male parentage

To infer male parentage we first reconstructed the genotype of each mother queen and that of her mate from the genotypes of the diploid brood she produced (worker pupae and

occasionally also diploid males, Table 1) (cf. Foster *et al.* 1999). This was straightforward, given that in all cases, genotypes were consistent with the mother queens being singly mated and with just a single matriline being present. In a few cases, we also had the genotype of the queen herself available, which further facilitated parentage reconstruction (Supplementary Table 1). As a null hypothesis, we assumed that males were either sons of the queen or of workers derived from the present queen. These two possibilities could be distinguished if the paternal allele at any one locus was different from the maternal alleles (e.g. parental genotypes of the type $ab \times c$ or $aa \times b$, but not $ab \times a$). In that case, a worker's son could be detected if he inherited a unique paternal allele that was not present in the queen (cf. Foster *et al.* 2001). However, if a male carried an allele not present in either the mother queen or that of its mate, then it was clear that such a male was either the son of a drifted worker or of a worker derived from a superseded queen. The latter two possibilities could be distinguished either by comparison to the known genotypes of workers produced by the previous, superseded queen (Table 1), or by counting the total number of alleles present among such males at each locus in any one colony. The idea is that reproduction by a single superseded matriline of workers should never lead to the presence of more than 3 alleles, whereas the reproduction by workers that had drifted from multiple colonies might. Estimates of the percentage of males that were sons of workers from the current or previous queen were corrected according to the statistical power with which such males could be detected in our dataset given the observed parental genotypes ($\bar{x}$ and $\bar{y}$, for details see Table 1, cf. Foster *et al.* 2001). Finally, we also calculated the probability that the son of a drifted worker would by chance be misclassified as the son of the current queen, a son of a worker of the current queen or a son of a worker of the superseded queen, by fortuitously having an identical multilocus genotype. This was done using the formula given in Nanork *et al.* (2005), using the population-specific allele frequencies.

RESULTS

Allelic diversity and statistical power to detect worker reproduction

Loci were reasonably polymorphic, with a total of 7, 8 and 8 alleles detected (Igarassu: 7, 5, 8; S. Paulo: 6, 8, 7; S. Simão: 4, 3, 3) and mean expected heterozygosities of 79%, 71% and 73% (Igarassu: 74%, 64%, 78%; S. Paulo: 77%, 72%, 73%; S. Simão: 51%, 64%, 61%) at *loci* T4-171, Mbi-254 and Mbi-201, respectively. The mean statistical power to detect sons of workers derived

from either the current queen or of the previous, superseded queen was $\bar{x}$ =56% and $\bar{y}$ =44%, respectively (Table 1), with the latter figure based on 13 colony samples for which we also had the genotypes available of workers produced by the previous, superseded queen (Table 1). The mean probabilities of the son of a drifted worker fortuitously having the same genotype as the son of the current queen, a son of a worker of the current queen or a son of a worker of the superseded queen, were small, 3.81% (Igarassu: 1.86%; S. Paulo: 2.02%; S. Simão: 6.90%), 3.86% (Igarassu: 3.21%; S. Paulo: 2.65%; S. Simão: 4.76%) and 3.57% (S. Paulo: 2.06%; S. Simão: 4.58%), respectively. Hence, the probabilities of males being erroneously misclassified were small.

Male parentage

Out of the 576 males genotyped, 61 out of 576 (10.59%) could not be assigned to the queen and were therefore definitely workers' sons (Table 1 and Supplementary Table 1). Only a small percentage of these workers' sons (14 out of 61), 22.95%, however, were consistent with being sons of workers of the current queen, and the remainder, 47 out of 61 (77.05%), were therefore either derived from drifted workers or from workers produced by an earlier, superseded queen. Restricting ourselves to the subset of 13 colonies for which we had the genotypes available of workers produced by the superseded queen, however, we can see that all the anomalous males (12 out of 12 in 4 colonies, Table 1) were consistent with being the sons of workers from a superseded queen as opposed to being sons of unrelated, drifted workers (Supplementary Table 1). In addition, for the remaining colonies where such anomalous male genotypes were found, we never sampled more than 3 alleles in males at any one locus in any of the colonies, again implying that such males were most likely produced by a single, superseded matriline of workers as opposed to by workers that had drifted from several other, unrelated colonies (Supplementary Table 1). For a few colonies, where only a few anomalous males were found, it remains possible, however, that these were the offspring of drifted workers. Assuming, nevertheless, that the majority of the anomalous males were the offspring of a superseded queen, and correcting our figures for non-detection, we estimate that 77.11% of the males were the queen's sons, 4.34% were the sons of the workers derived from the current queen and 18.54% were the sons of workers derived from a previous, superseded queen. Hence, using these corrected estimates, 81.03% of all workers' sons were the offspring of workers from superseded queens.

These figures are surprising given that for the São Paulo population, sons of workers deriving from the superseded queen could still be found in colonies SP-2b and SP-3b, despite the fact that these samples were taken 175 and 105 days after the previous queen had died, and that workers in *M. scutellaris* have a mean life expectancy of only 31 days (Oliveira & Kleinert-Giovannini 1991). Even accounting for the fact that some brood laid by the previous queen may have kept eclosing until ca. 40 days after her death (the development time from egg to adult in *Melipona*), and that the sampled brood was probably laid ca. 25 days before, we can see that reproductive workers must live a very long time, at least $175-40-25=110$ days, which is 3.5 times as long as that of a normal worker, and in fact not too far off from the life expectancy of queens in this species, 175 days (Wenseleers *et al.*, forthcoming).

There was no significant correlation between the inferred percentage of males that were workers' sons and the percentage of males present in the combs from which the samples were taken (Spearman $R=0.11$, $n=45$, $p=0.45$). This goes against the hypothesis that workers would reproduce only during periods in which the queen lays haploid eggs (Chinh *et al.* 2003; Sommeijer *et al.* 2003; Velthuis *et al.* 2005). In fact, in several cases (in colonies IG 08-a, IG 27-a, SP 03-b, SP 06-a and SS 06-b) we found evidence for worker reproduction even at times when fewer than 10% of the cells contained male pupae (Table 1). So although we concur with Chinh *et al.* (2003) and Velthuis *et al.* (2005) that stingless bee queens frequently lay male eggs in distinct batches (during "male producing periods"), it does not appear to be the case that workers only reproduce during such periods, since worker reproduction was found both in periods in which many and in which few males were reared.

Table 1. Male parentage in *Melipona scutellaris* colonies kept in Igarassu (IG), São Paulo (SP) and São Simão (SS), with information on the sampling date, the number of diploid and haploid individuals genotyped, the number of males which was inferred to be sons of workers from either the current queen, a superseded queen or unrelated drifters and the percentages of males present in the sampled brood combs.

Colony and queen	Sampling date	No. of diploid individuals genotyped	No. of haploid males genotyped	No. of worker sons detected			Power to detected sons of workers of the		% of males in sampled combs
				from workers of the current queen	from workers of the previous queen	from workers previous queen or drifted workers*	current queen [†]	previous queen [‡]	
IG01-a	09/2006	10	14			3 (21.43%)	0.50		36.05%
IG03-a	01/2006	10	8				0.75		16.06%
IG05-a	09/2006	10	9				0.88		18.48%
IG06-a	09/2006	10	10				0.88		24.41%
IG08-a	01/2006	10	9			1 (11.11%)	0.50		8.36%
IG11-a	05/2007	10	9				0.75		45.08%
IG12-a	09/2006	10	9				0.75		28.42%
IG13-a	01/2006	10	9			8 (88.89%)	0.00		28.73%
IG16-a	01/2006	10	8				0.50		33.69%
IG19-a	01/2006	10	8				0.00		21.10%
IG20-a	05/2007	10	10				0.50		17.93%
IG26-a	09/2006	10	10	5 (50.00%)			0.88		21.99%
IG27-a	09/2006	10	10	1 (10.00%)			0.50		6.37%
IG30-a	09/2006	10	10				0.88		19.63%
IG36-a	05/2007	10	10				0.75		29.03%
IG39-a	05/2007	10	12				0.88		9.69%
SP01-a§	10/2006	10	5			1 (20.00%)	0.75		20.00%
SP01-d§¶	07/2008	10	11				0.00	0.44	10.93%
SP02-b§¶#	02/2007	10	13	1 (7.69%)	5 (38.46%)		0.75	0.58	90.00%
SP03-a	07/2006	10	36			10 (27.78%)	0.00		26.55%
SP03-b	12/2006	10	9		3 (33.33%)		0.50	0.25	2.94%
SP03-c§#	07/2007	10	24				0.88	1.00	13.22%
SP04-a	07/2007	10	27				0.00		28.21%
SP05-b¶	11/2006	10	30				0.50	0.00	39.59%
SP06-a	10/2008	10	6			4 (66.67%)	0.75		3.55%
SP08-a	12/2006	10	27			6 (22.22%)	0.75		18.74%
SP09-c#	09/2008	10	20				0.75	0.94	14.56%
SP10-b§¶	07/2008	10	33				0.50	0.00	18.55%
SS01-a	03/2006	12 ⁺⁺	7				0.75		66.31%
SS02-b¶#	02/2007	10	10				0.50	0.72	8.96%
SS04-a	04/2006	10	10	1 (10.00%)			0.50		13.77%
SS06-a	07/2006	10	8				0.75		15.85%
SS06-b	12/2006	10	9		1 (11.11%)		0.50	0.44	5.13%
SS08-a	03/2006	14 ⁺⁺	5	2 (40.00%)			0.88		61.89%
SS08-b	10/2006	10	10				0.75	0.00	17.02%

Colony and queen	Sampling date	No. of diploid individuals genotyped	No. of haploid males genotyped	No. of worker sons detected			Power to detected sons of workers of the		% of males in sampled combs
				from workers of the current queen	from workers of the previous queen	from workers previous queen or drifted workers*	current queen [†]	previous queen [‡]	
SS15-a	03/2006	10	11	3 (27.27%)			0.75		21.01%
SS28-a	08/2006	10	16				0.50		42.47%
SS36-a	03/2006	10	7				0.50		5.65%
SS36-b	11/2006	10	8		3 (37.50%)		0.00	0.25	69.69%
SS43-a	07/2006	10	10				0.50		19.41%
SS59-a	09/2006	10	23			1 (4.35%)	0.75		24.72%
SS59-b#	01/2007	10	20				0.75	0.63	38.06%
SS63-a	04/2006	17 ^{††}	7	1 (14.29%)		1 (14.29%)	0.50		55.39%
SS63-b	11/2006	10	9				0.50	0.44	10.00%
SS66-a	09/2006	10	10				0.00		14.95%
Total / average		$\Sigma = 463$	$\Sigma = 576$	$\Sigma = 14$	$\Sigma = 12$	$\Sigma = 35$	$\bar{X} = 0.56$	$\bar{Y} = 0.44$	

The letters in the colony codes refer to the succession of different mother queens. The statistical power with which we could unambiguously detect sons of workers of the current queen and sons of workers from a previous queen is also given. The reconstructed parental genotypes and male genotypes are given in Table S1.

* The possibility of males being sons of workers of the previous queen or of a drifted worker could only be distinguished with certainty if the genotype of the previous, superseded queen and that of her mate was known.

[†] Probability of a worker son inheriting a unique paternal allele at least on the loci studied, calculated using the formula given in Foster et al. (2001).

[‡] Probability that a son of a worker of the previous queen could be distinguished from a son of a worker of the current queen at one of the loci studied at least (calculated on a case by case basis).

§ Mother queen also genotyped.

¶ Genotype of the superseded queen and her mate determined in another study (Wenseleers et al., unpublished data; Table S1).

Queen was genetically unrelated to the previous, superseded queen (Wenseleers et al., unpublished data; Table S1).

^{††} The queen and her mate shared an allele at the sex locus. Diploid offspring genotyped comprised 10 workers plus 2, 4 and 7 diploid males respectively.

Supplementary Table 1. Reconstructed parental genotypes and male genotypes observed in *Melipona scutellaris* colonies kept at Igarassu (IG), São Paulo (SP) and São Simão (SS), with information on whether the males were inferred to be sons of workers of the current queen (WSC), sons of workers of the previous queen (WSP) or sons of either workers of the previous queen or of drifted workers (WSP/D). *n* is the number of males with a given multilocus genotype; the letters in the colony codes refer to the succession of different mother queens.

Colony and queen	Parental genotypes at locus			Males' genotypes at locus				Male parentage
	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>n</i>	
IG 01-a	104/110 x 104	197/211 x 207	150/150 x 150	100	197	140	3	WSP/D
				104	197	150	2	
				104	211	150	3	
				110	197	150	4	
				110	211	150	2	
IG 03-a	102/110 x 94	197/205 x 197	150/153 x 156	102	197	153	2	
				110	197	153	3	
				110	205	153	3	
IG 05-a	100/106 x 102	205/205 x 191 ^a	144/153 x 150	100	205	144	5	
				100	205	153	3	
				106	205	144	1	
IG 06-a	100/100 x 104 ^a	197/205 x 205	153/156 x 150	100	197	153	2	
				100	205	153	5	
				100	205	156	3	
IG 08-a	104/108 x 106	205/211 x 205	153/156 x 153	104	191	150	1	WSP/D
				104	205	153	1	
				104	205	156	1	
				104	211	153	1	
				108	205	153	1	
				108	205	156	2	
				108	211	153	2	
IG 11-a	102/104 x 104	197/205 x 207	159/159 x 153	102	197	159	1	
				102	205	159	4	
				104	197	159	2	
				104	205	159	2	
IG 12-a	104/106 x 102	191/197 x 205	144/153 x 144	104	191	144	1	
				104	191	153	1	
				104	197	144	2	
				106	191	144	2	
				106	191	153	1	
				106	197	144	1	
				106	197	153	1	
IG 13-a	104/104 x 104	205/205 x 197 or 197/197 x 205	153/153 x 153	100	207	147	1	WSP/D
				100	211	153	1	WSP/D
				102	207	147	2	WSP/D
				102	207	153	2	WSP/D
				102	211	153	2	WSP/D
				104	205	153	1	
IG 16-a	102/106 x 102	205/205 x 207 ^a	153/156 x 156	102	205	156	3	
				106	205	153	3	
				106	205	156	2	
IG 19-a	102/106 x 102	205/205 x 205	153/156 x 153	102	205	153	2	
				102	205	156	2

Colony and queen	Parental genotypes at locus			Males' genotypes at locus				Male parentage
	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>n</i>	
IG 20-a	102/104 x 106	197/205 x 197	150/156 x 150	106	205	153	1	
				106	205	156	3	
				102	197	156	2	
				102	205	150	2	
				102	205	156	1	
				104	197	150	2	
				104	197	156	1	
				104	205	150	1	
IG 26-a	100/106 x 102	205/205 x 197 ^a	150/153 x 147	104	205	156	1	
				100	197	150	1	
				100	197	153	1	
				100	205	150	1	
				100	205	153	1	
				102	197	150	1	
				102	205	153	1	
				106	197	153	1	
				106	205	150	1	
				106	205	153	2	
IG 27-a	102/102 x 104 ^a	197/197 x 197	140/153 x 153	102	197	140	4	
				102	197	153	5	
IG 30-a	102/104 x 106	197/197 x 205 ^a	153/156 x 147	104	197	153	1	WSC
				102	197	153	3	
				102	197	156	2	
				104	197	153	2	
IG 36-a	100/104 x 102	205/205 x 205	153/172 x 147	104	197	156	3	
				100	205	153	1	
				100	205	172	3	
				104	205	153	2	
IG 39-a	102/106 x 104	197/197 x 205 ^a	140/147 x 156	104	205	172	4	
				102	197	140	4	
				102	197	147	3	
				106	197	140	4	
SP 01-a ^b	102/108 x 102	205/222 x 207	147/153 x 159	106	197	147	1	WSP/D
				102	197	150	1	
				102	205	147	1	
				102	205	153	1	
				108	205	147	1	
SP 01-b ^{b,c} SP 01-c ^{b,c} SP 01-d ^b	102/108 x 104 104/108 x 102 102/108 x 108	205/207 x 197 197/205 x 191 191/205 x 205	153/159 x 153 153/153 x 150 150/153 x 150	108	205	153	1	
				102	191	153	1	
				102	205	150	1	
				102	205	153	3	
				108	191	150	3	
SP 02-a ^c SP 02-b ^b	102/104 x 104 104/108 x 104	197/207 x 205 205/205 x 207	144/153 x 153 153/153 x 150	108	191	153	1	
				108	205	150	2	
				104	205	150	1	
				104	205	153	4	
				102	197	153	1	
SP 03-a	102/104 x 104	205/207 x 207	147/159 x 159	104	197	153	4	WSP
				104	197	153	4	
				108	205	153	3	
				102	191	144	3

Colony and queen	Parental genotypes at locus			Males' genotypes at locus				Male parentage
	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>n</i>	
SP 03-b	102/104 x 102	207/207 x 207	147/159 x 153	102	205	144	2	WSP/D
				102	205	147	2	
				102	205	159	3	
				102	207	147	4	
				102	207	159	7	
				104	191	144	2	WSP/D
				104	205	153	3	WSP/D
				104	205	159	1	WSP
				104	207	147	3	
				104	207	159	6	
				102	207	147	1	
				102	207	159	2	
				104	205	159	3	
				104	207	147	2	
SP 03-c ^b	104/106 x 108	197/205 x 222	153/153 x 147	104	207	159	1	WSP
				104	197	153	5	
				104	205	153	5	
				106	197	153	6	
SP 04-a	100/102 x 100	197/205 x 205	147/159 x 159	106	205	153	8	WSP
				100	197	147	2	
				100	197	159	5	
				100	205	147	7	
				102	197	147	2	
				102	197	159	4	
				102	205	147	3	
				102	205	159	4	
SP 05-a ^{b,c}	100/102 x 100	207/207 x 205	159/159 x 159					
SP 05-b	100/102 x 102	205/207 x 207	159/159 x 159	100	205	159	3	WSP/D
				100	207	159	7	
				102	205	159	7	
				102	207	159	13	
SP 06-a	102/106 x 108	200/211 x 205	153/153 x 153	106	211	150	4	WSP/D
				106	211	153	2	
SP 07-a ^c	100/102 x 100	200/205 x 205	150/153 x 156					
SP 08-a	104/110 x 104	191/205 x 197	150/153 x 144	100	197	159	2	WSP/D
				102	197	147	1	WSP/D
				102	207	147	1	WSP/D
				102	207	159	2	WSP/D
				104	191	150	4	WSP/D
				104	191	153	4	
				104	205	150	2	
				104	205	153	4	
				110	191	153	3	
				110	205	150	1	
				110	205	153	3	
SP 09-a ^c	106/108 x 104	197/205 x 205	153/153 x 153					
SP 09-b ^{b,c}	104/106 x 108	197/205 x 222	153/153 x 147					
SP 09-c	102/108 x 102	205/207 x 203	147/159 x 140	102	205	147	6	WSP/D
				102	205	159	3	
				102	207	147	3	
				102	207	159	2	
				108	205	159	2	
				108	207	147	2

Colony and queen	Parental genotypes at locus			Males' genotypes at locus				Male parentage
	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>n</i>	
SP 10-a ^c	102/108 x 102	205/207 x 205	150/153 x 153	108	207	159	2	
SP 10-b ^b	102/108 x 102	205/205 x 207	150/153 x 153	102	205	150	5	
				102	205	153	8	
				108	205	150	9	
				108	205	153	11	
SS 01-a	106/106 x 106	197/205 x 211	159/159 x 153	106	197	159	2	
				106	205	159	5	
SS 02-a ^c	104/106 x 106	205/211 x 197	159/159 x 153					
SS 02-b	106/108 x 106	205/205 x 197 ^a	153/153 x 153	106	205	153	5	
				108	205	153	5	
SS 04-a	100/108 x 108	205/205 x 205	150/150 x 159 ^a	108	205	150	9	
				108	205	159	1	WSC
SS 06-a	104/108 x 106	205/211 x 197	150/153 x 150	104	205	153	1	
				104	211	150	2	
				108	205	153	3	
				108	211	150	2	
SS 06-b	106/108 x 108	197/211 x 211	150/150 x 153	106	211	150	2	
				108	205	150	1	WSP
				108	197	150	1	
				108	211	150	5	
SS 08-a	108/108 x 106	205/205 x 211	150/159 x 153	106	205	150	1	WSC
				106	211	150	1	WSC
				108	205	150	2	
				108	205	159	1	
SS 08-b ^b	106/108 x 106	205/211 x 197	150/153 x 159	106	211	153	4	
				108	205	150	2	
				108	205	153	1	
				108	211	153	3	
SS 15-a	108/108 x 106	197/197 x 205	150/153 x 150	106	197	150	3	WSC
				108	197	150	4	
				108	197	153	4	
SS 28-a	108/108 x 106 ^a	205/205 x 205	150/153 x 153	108	205	150	8	
				108	205	153	8	
SS 36-a	106/108 x 108	197/205 x 205	150/150 x 153 ^a	106	197	150	2	
				106	205	150	2	
				108	197	150	3	
SS 36-b	106/108 x 108	205/205 x 205	150/153 x 150	106	197	153	1	WSP
				106	205	150	1	
				108	197	153	2	WSP
				108	205	150	2	
				108	205	153	2	
SS 43-a	106/108 x 106	197/211 x 205	153/153 x 153	106	197	153	5	
				106	211	153	2	
				108	211	153	3	
SS 59-a	106/108 x 106	197/205 x 211	153/153 x 159 ^a	106	197	153	8	
				106	205	153	6	
				108	197	150	1	WSP/D
				108	197	153	5	
				108	205	153	3	
SS 59-b	106/106 x 106	197/205 x 211	153/153 x 150	106	197	153	13	
				106	205	153	7	
SS 63-a	106/108 x 106	197/205 x 211	153/159 x 153	106	197	150	1	WSP/D

Colony and queen	Parental genotypes at locus			Males' genotypes at locus				Male parentage
	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>T4-171</i>	<i>Mbi-254</i>	<i>Mbi-201</i>	<i>n</i>	
SS 63-b	106/106 x 106	205/211 x 205	153/153 x 159	106	197	153	2	WSC
				106	205	153	2	
				108	205	159	1	
				108	211	153	1	
				106	205	153	6	
				106	211	153	3	
SS 66-a	106/108 x 106	197/211 x 211	153/153 x 153	106	197	153	3	
				106	211	153	3	
				108	197	153	2	
				108	211	153	2	

^a Most likely parental genotypes, given that all males were of a single genotype at that locus, and that the genotypes at the other loci were consistent with these males being queen's sons. ^b Mother queen directly genotyped. ^c From these colonies no males could be sampled for analysis, hence they are not included in Table 1, but the parental genotypes were determined as part of another study (Wenseleers *et al.*, unpublished data).

DISCUSSION

In line with most previous genetic and behavioural studies on male parentage in stingless bees (reviewed in Hammond & Keller 2004; Tóth *et al.* 2004; Velthuis *et al.* 2005; Wenseleers & Ratnieks 2006a), our results show that a significant fraction, 22.88%, of the males in *Melipona scutellaris* are sons of the workers rather than of the queen. This is as expected from inclusive fitness theory, given that single mating in stingless bees collectively favours worker reproduction (Ratnieks 1988; Tóth *et al.* 2002b, 2004). Surprisingly enough, however, our results also show that ca. 80% of the workers' sons had genotypes that were incompatible with them being the sons of workers of the present queen. Based on sampling of colonies over multiple generations, before and after queen supersedure events, and based on the fact that such males from any one colony never had more than 3 alleles at a given locus, we were able to show that these males were most likely produced by workers that were the offspring of a previous, superseded queen, rather than by workers that had drifted from other colonies. This represents the first demonstration of workers reproductively parasitizing the next-generation workforce for their own, selfish benefits.

That such a high percentage of the worker sons were produced by workers derived from a superseded queen is surprising, given that most colonies were sampled many months after the previous queen had died, and that workers in *Melipona scutellaris* normally only have a life expectancy of 31 days (Oliveira & Kleinert-Giovannini 1991; life expectancy of workers in 5 other *Melipona* species: 40-51 days, Giannini 1997; Biesmeijer & Tóth 1998). In fact, in one case we

estimated that reproductive workers had a life expectancy of ca. 110 days, which means they outlived the other workers by a factor of 3.5, and that they had a life expectancy which was almost comparable with that of the queen in this species, 175 days (Wenseleers *et al.*, forthcoming). The long life expectancy of laying workers is probably linked to the fact that reproductive workers usually do not carry out risky or energetically costly tasks, such as foraging, in order for them not to jeopardize their reproductive futures (Franks 1983; Hartmann & Heinze 2003; Wenseleers *et al.* 2004b).

The high percentage of males produced by workers derived from superseded queens (ca. 80%) was also surprising given that for colonies sampled at random points in time, such workers usually present only a very small fraction of the workforce. For example, the percentage of workers that had either drifted from other colonies or were descendants of a superseded queen was estimated at 3% across a sample of 12 species of stingless bees (Peters *et al.* 1999, excluding the facultatively polygyne *M. bicolor*), 10% in *M. beecheii* (Paxton *et al.* 1999a) and between 21% and 36% across three species of *Scaptotrigona* (Paxton *et al.* 1999a, 2003; Palmer *et al.* 2002). Overall, this probably means that, on a per capita basis, the workers from superseded queens specialise in reproduction. Partly this may be due to the fact that during the brief period of queenlessness following the death of the queen and preceding the establishment of a new laying queen, workers increase their rate of ovary activation as well as their egg-laying rate (Ferreira *et al.* 1989; Lacerda & Zucchi 1999; Kleinert 2005; Velthuis *et al.* 2005), and that this may give these workers a "head start" relative to the workers produced later by the newly mother queen. However, the greater reproductive rate of workers derived from a superseded queen is also consistent with kin selection theory, given that it pays workers more from exploiting the colony if costs are carried by less related individuals (Hamilton 1964; Wenseleers *et al.* 2004a,b; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006b). In stingless bees, worker reproduction is likely to be costly, first because workers might carry out less work (but see Cepeda 2006, who shows that reproductive workers in *M. bicolor* do engage in some brood care), and second because the exclusively male eggs laid by workers will end up replacing some of the female, worker-destined eggs laid by the queen (Ratnieks & Reeve 1992; Tóth *et al.* 2004). Hence, worker reproduction in stingless bees will inevitably be traded off against worker production, and come at a cost to future colony productivity (Ratnieks & Reeve 1992; Tóth *et al.* 2004). From the perspective of workers from a previous queen, however, this cost will in inclusive fitness terms be lower as it will be carried by more distantly related individuals

(nephews and nieces, $r=0.375$, as opposed to sisters, $r=0.75$), thereby causing them to be selected to exploit the colony more. These results are the first explicit demonstration that conflict over male parentage in insect societies is not just played out between the queen and workers and between the workers of one generation, but that the conflict may also spill over from one worker generation to the next. Future work will be required to test if such intergenerational worker reproduction conflict occurs more widely in other perennial insect societies.

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CAPÍTULO 4.

Intraspecific queen parasitism in the stingless bee *Melipona scutellaris* (Hymenoptera, Apidae)

D. A. Alves

Versão expandida do trabalho resumido e submetido:

Wenseleers T, **Alves DA**, Francoy TM, Billen J, Imperatriz-Fonseca VL
Intraspecific "cuckoo" queens in a highly eusocial bee. *Biology Letters*,
submetido

Intraspecific queen parasitism in the stingless bee *Melipona scutellaris* (Hymenoptera, Apidae)

ABSTRACT

Cuckoo bumblebee queens are famously known to take over and reproduce in the nests of other related species. Here we show for the first time that in the stingless bee *Melipona scutellaris* similar queen behaviour also occurs at the intraspecific level. In particular, we show based on microsatellite genotyping that upon loss of the mother queen, 25% of all colonies are invaded by unrelated queens that fly in from unrelated hives nearby. We argue that this remarkable case of intraspecific social parasitism is linked to the peculiar queen overproduction seen in *Melipona*, and allows virgin queens to obtain reproductive benefits in other colonies when there are no reproductive opportunities in their natal colony. Our results are the first demonstration that queens in highly eusocial bees can found colonies not only via supersedure or swarming but also via social parasitism.

INTRODUCTION

Bee colonies are well-known for their advanced cooperation (Hamilton 1964), but their societies can also be exploited by social parasites, who can benefit from the resources stored within the host nest, and get directly cared for by their hosts (Goulson 2003; Beekman & Oldroyd 2008). In social insects, social parasitism most frequently occurs between different species but more rarely it can also occur among members of the same species ("intraspecific social parasitism").

Bumblebees nicely illustrate both forms of social parasitism. Cuckoo bumblebees, of the subgenus *Psithyrus*, are interspecific workerless social parasites, and penetrate, take over and then go on to reproduce in the nests of other related species (Morse 1982; Goulson 2003). Since *Psithyrus* queens do not have pollen baskets and cannot produce wax, they are totally dependent on *Bombus* workers to rear their young. At the intraspecific level, a few studies have also reported case in which some late-emerging *Bombus* queens successfully usurp conspecific nests in the colony founding stage, after which they kill the original foundress queen and go on to use the nest for their own reproduction (Alford 1975; Paxton et

al. 2001; Goulson 2003; Carvell *et al.* 2008). Finally, it has recently been shown that bumblebee and Asian honeybee workers may also engage in intraspecific social parasitism, whereby workers penetrate and reproduce in alien, unrelated colonies of the same species (Lopez-Vaamonde *et al.* 2004; Nanork *et al.* 2005, 2007, reviewed in Beekman & Oldroyd 2008).

Unlike bumblebees and honeybees, as yet little is known about the possible occurrence of intraspecific social parasitism in stingless bees. Recently, however, Sommeijer *et al.* (2003a; 2003b) speculated that intraspecific queen parasitism might perhaps occur in the genus *Melipona*, after noting that in *M. favosa*, a significant proportion of the gynes left their natal nest, possibly with the aim of trying to reproduce in other colonies nearby. Indeed, *Melipona* forms an excellent model to study the possible occurrence of intraspecific queen parasitism. Like all other stingless bees, *Melipona* colonies are perennial (Michener 1974) and headed by a single once-mated queen (Peters *et al.* 1999). However, exceptional among highly eusocial bees, *Melipona* queens are reared continuously and in large numbers, with between 5% and 25% of all females developing as queens (Kerr 1950, 1969; Engels & Imperatriz-Fonseca 1990; Moo-Valle *et al.* 2001; Wenseleers & Ratnieks 2004; Santos-Filho *et al.* 2006). The reason for this high queen production has been explained by the fact that in *Melipona*, queens and workers, despite being morphologically distinct, develop in identical, sealed brood cells on a similar provision mass, and that females obtain greater reproductive benefits by developing as queens than as workers (Bourke & Ratnieks 1999; Ratnieks 2001; Wenseleers *et al.* 2003; Ratnieks & Wenseleers 2005). However, only few of these queens actually make it and only few go on to head a swarm or replace a failing mother queen – the vast majority are killed by the workers soon after emergence or are dispelled out of the colony (Silva *et al.* 1972; Koedam *et al.* 1995; van Veen *et al.* 1999b; Sommeijer *et al.* 2003b; Wenseleers *et al.* 2004). It is clear that this system of queen overproduction should be particularly conducive to the evolution of intraspecific queen parasitism, since queens without reproductive options in their own colony could obtain significant fitness benefits if they could succeed in taking other colonies nearby, which might well be possible if such colonies happened to be queenless.

The aim of this study is to carry out the first formal genetic test of whether or not intraspecific queen parasitism occurs in stingless bees, and whether queens could indeed succeed in entering unrelated hives to opportunistically rear their own brood. In order to do this, we carried out a long-term genetic study on the stingless bee *M. scutellaris*, whereby we

sampled female brood before and after queen replacement events to check if daughter queens were consistent with being the daughter of the previous queen or whether instead they were unrelated social parasites.

MATERIALS AND METHODS

Study organism

Melipona scutellaris nests in cavities of tree trunks in the Atlantic rainforest and is widely distributed in the Northeast of Brazil (Camargo & Pedro 2007), where it is commonly kept by regional and traditional beekeepers for honey, pollen and wax (Cortopassi-Laurino *et al.* 2006), and is also increasingly used for the pollination of various tropical crops (Castro 2002; Heard 1999). Colonies are perennial and swarm-founded, are headed by one singly-mated queen and typically contain around 1,500 workers (Peters *et al.* 1999; Tóth *et al.* 2004; Tóth *et al.* 2002). Between May 2006 and March 2007 we obtained 10 *M. scutellaris* colonies headed by newly mated queens from a beekeeper in Igarassu (Pernambuco state, Brazil, 7°50'3.74"S 34°54'22.87"W). Upon arrival, these colonies were put in free-foraging nestboxes in the bee laboratory at the University of São Paulo (23°33'37.41"S 46°43'53.04"W), where they were placed ca. 1 metre apart, with colonies 1 to 3 placed along the outside wall of the lab and colonies 4 to 10 along an adjoining wall, at a right angle to the first (the order of the colonies corresponds to that given in Fig. 1). In addition, we obtained data from 6 *M. scutellaris* colonies that were part of a larger population of 30 colonies kept at the Aretuzina Farm, São Simão (21°26'25.97"S 47°34'54.65"W). Colonies were spaced ca. 1 to 10 metres apart, as is typical in most apiaries and also not uncommon in nature, where several nests can sometimes be found within a few metres of each other, either in the same tree or in different trees (Alves *et al.* 2005). During the time of our study colonies were managed only by occasionally providing them with food, and no alien brood was ever introduced to boost colonies.

Queen replacement events and sampling scheme

a) Natural queen replacements

In order to know the mother queens' longevity and determine the timing of queen replacement events, we marked all mother queens with a coloured plastic tag (in colonies kept at

the laboratory) or white paint dot (in colonies kept at the farm) on their thorax, and regularly checked all queens over a total period of ca. 3 years (daily for the colonies kept in the lab and once every two weeks for the colonies kept at the farm). Before and after each queen replacement event, we sampled 10 worker pupae per colony and, whenever possible, also collected a wing tip of the mother queen (Table 1), which were preserved in absolute ethanol for later genotyping.

b) Artificial queen replacements

To further boost our sample size, between August and October 2008, we artificially induced eight queen replacement events in four colonies placed at the bee laboratory, whereby we manually removed the mother queen and waited for a new one to be accepted (Fig. 1, Table 1). Again, wing tips of all the newly adopted queens were preserved in absolute ethanol for later genotyping.

Genetic analysis

For parentage analysis we genotyped 10 worker pupae and either a wing tip sample from a live queen (cf. Châline *et al.* 2004) or a leg if she was killed or had recently died for each of our 16 colonies (Table 1) at three microsatellite *loci*, *T4-171* (Paxton *et al.* 1999), *Mbi-254* and *Mbi-201* (Peters *et al.* 1998). These *loci* were found to be most variable in this species in a preliminary screening of 10 *loci*. DNA was extracted using the Chelex method, whereby a single leg (from worker pupae or mother queen) or wing tip sample (from mother queens) was frozen in liquid nitrogen and ground up using a plastic pestle, followed by an incubation at 95°C for 15 min in 200µL (50µL for wing tip samples) of a 10% Biorad Chelex 100 resin solution. Samples were vortexed and centrifuged before use. Multiplex PCR reactions were carried out in a 10µL reaction volume, and contained 0.5µM of the forward and reverse primers of each locus, 0.2mM of each dNTP, 1.5mM MgCl₂, 1µL of crude DNA extract, 0.4 units of Silverstar Taq polymerase (Eurogentec, Seraing, Belgium) and enzyme buffer supplied by the manufacturer. PCR was performed following a touch-down programme (Bonckaert *et al.* 2008), with an initial denaturation for 3min at 94°C, followed by 20 cycles consisting of 30s at 94°C, 30s at 58°C, but decreasing 0.5°C in each step, and 45s at 72°C; 10 cycles consisting of 30s at 94°C, 30s at 46°C, and 45s at 72°C; and a final 10-min extension step at 72°C. After amplification, 1µL of the PCR product was mixed with 8.8µL formamide and 0.2µL Genescan 500 LIZ size standard (Applied Biosystems, Lennik, Belgium), denatured, and loaded onto an ABI-3130 Avant capillary sequencer. Alleles

were called using the supplied Gene Mapper software.

Reconstruction of parental genotypes

To test whether the genotypes of newly established queens were consistent with them being daughters of the previous queen or being unrelated parasites, we first reconstructed the genotype of each mother queen and that of her mate from the genotypes of her worker brood (cf. Foster *et al.* 1999). This was straightforward, given that in all cases, genotypes were consistent with the mother queens being singly mated. In a few cases, we also had the genotype of the queen herself available, which further facilitated parentage reconstruction (Table 1). An added bonus was that since *M. scutellaris* does not naturally occur in São Paulo state, all queens could be tracked down unambiguously to one of our laboratory colonies.

RESULTS

Allelic diversity and statistical power to classify related and unrelated queens

Loci were reasonably polymorphic, with 6, 8 and 8 alleles detected and mean expected heterozygosities of 75.16%, 72.38% and 73.44% at *loci* T4-171, Mbi-254 and Mbi-201. All genotypes were consistent with the mother queens being mated to a single male, as is typical for stingless bees (Peters *et al.* 1999). The mean probability of erroneously classifying a queen as the daughter of the previous one when it was in fact a social parasite in the São Paulo population was very low, 0.0040 (it was 0 for 12 out of 14 inferred nonparasite queens, and 0.0313 and 0.0250 for queens 1-c and 3-d respectively; Table 1).

Queen replacement events

The results show that in 3 out of 10 (30%) and in 2 out of 6 (33,33%) of the natural queen replacements in S. Paulo and S. Simão, and in 1 out of 8 (12,5%) of the artificial queen replacements the newly established queens were indeed social parasites (Fig. 1, Table 1). Thus overall, one quarter (6/24) of the new queens has flown in from unrelated, alien hives.

In 50% (2/4) of the colonies in São Paulo the timing was such that the alien queens definitely must have come from queenright source colonies; for the other two the timing is ambiguous, and the queens could either have come from other queenright or queenless

colonies. Significantly, alien queens never came from an immediately neighbouring colony and in fact always came from opposite sides of the building (Fig. 1). Hence, queen parasitism could not have been a by-product of accidental drifting between nests.

Queen acceptance and life expectancy

For the colonies in São Paulo, the daily checks of all colonies allowed used to calculate that queens had a median life expectancy of 175 days (Kaplan-Meier analysis, $n=20$). In addition, the mean number of days for a new queen to be accepted was 15.33 days (range 0-46, $n=18$). For colonies in S. Simão, however, where colonies were checked only once every two weeks, detailed data on queen life expectancy could not be collected.

Fig. 1. Social parasitism in 10 *Melipona scutellaris* colonies. Coloured blocks show the tenure of any one queen; hatched areas are periods during which the colonies became queenless, after which a new queen was adopted. Asterisks indicate experimental queen removals. Vertical arrows indicate cases where genotyping showed the newly adopted queen to be unrelated.

Table 1. *Melipona scutellaris* colonies used in this study with data on sampling date, reconstructed parental genotypes, queen longevity, dates at which new queens became established and the identity of newly adopted queens and whether or not they were unrelated social parasites.

Colony and queen	Sampling date	Parental genotypes at locus			Queen longevity (days)	Date that queen became established	# of days before queen was accepted	Queen was a social parasite?	Colony origin of parasite queen
		T4-171	Mbi-254	Mbi-201					
a) Parental genotypes of São Paulo colonies over the period that natural queen replacements occurred									
SP1-a	10/2006	102/108 x 102	205/222 x 207	147/153 x 159	175	12/05/2006	-	-	
SP1-b ^{a,b}	12/2006	102/108 x 104	205/207 x 197	153/159 x 153	7	19/12/2006	46	no	
SP1-c ^{a,c}	02/2007	104/108 x 102	197/205 x 191	153/153 x 150	59	26/12/2006	0	no	
SP1-d ^a	07/2008	102/108 x 108	191/205 x 205	150/153 x 150	>525 ^e	23/02/2007	0	no	
SP2-a ^a	07/2006	102/104 x 104	197/207 x 205	144/153 x 153	115	12/05/2006	-	-	
SP2-b ^a	02/2007	104/108 x 104	205/205 x 207	153/153 x 150	>760 ^f	03/10/2006	29	yes	9-a
SP3-a	07/2006	102/104 x 104	205/207 x 207	147/159 x 159	111	12/05/2006	-	-	
SP3-b	08/2006	102/104 x 102	207/207 x 207	147/159 x 153	127	31/08/2006	0	no	
SP3-c	07/2007	104/106 x 108	197/205 x 222	153/153 x 147	>624 ^e	14/01/2007	9	yes	9-a
SP4-a	07/2007	100/102 x 100	197/205 x 205	147/159 x 159	>904 ^f	12/05/2006	-	-	
SP5-a	06/2006	100/102 x 100	207/207 x 205	159/159 x 159	122	12/05/2006	-	-	
SP5-b	11/2006	100/102 x 102	205/207 x 207	159/159 x 159	>750 ^f	13/10/2006	32	no	
SP6-a	10/2008	102/106 x 108	200/211 x 205	153/153 x 153	>603 ^f	09/03/2007	-	-	
SP 07-a	10/2008	100/102 x 100	200/205 x 205	150/153 x 156	>611 ^f	01/03/2007	-	-	
SP 08-a	12/2006	104/110 x 104	191/205 x 197	150/153 x 144	>681 ^e	28/10/2006	-	-	
SP 09-a	08/2006	106/108 x 104	197/205 x 205	153/153 x 153	169	12/05/2006	-	-	
SP 09-b ^a	01/2007	104/106 x 108	197/205 x 222	153/153 x 147	24	06/11/2006	9	no	
SP 09-c	09/2008	102/108 x 102	205/207 x 203	147/159 x 140	>683 ^f	19/12/2006	19	yes	1-a
SP 10-a	07/2006	102/108 x 102	205/207 x 205	150/153 x 153	74	12/05/2006	-	-	
SP 10-b ^a	04/2007	102/108 x 102	205/205 x 207	150/153 x 153	>735 ^e	28/07/2006	3	no	
b) Genotypes of queens that were newly adopted following experimental queen removal (São Paulo)									
SP 01-e ^a	10/2008	108/108	205/205	150/153	(>3) ^{e,g}	22/08/2008	21	no	
SP 01-f ^a	10/2008	108/108	191/205	150/150	(>7) ^{e,g}	10/09/2008	16	no	

Colony and queen	Sampling date	Parental genotypes at locus			Queen longevity (days)	Date that queen became established	# of days before queen was accepted	Queen was a social parasite?	Colony origin of parasite queen
		T4-171	Mbi-254	Mbi-201					
SP 01-g ^a	10/2008	102/102	205/205	159/159	(>35) ^{f,g}	27/09/2008	10	yes	5-b
SP 03-d ^{a,d}	10/2008	106/108	205/222	147/153	(>19) ^{f,g}	13/10/2008	14	no	
SP 08-b ^a	10/2008	104/110	197/205	144/150	(>1) ^{e,g}	01/10/2008	23	no	
SP 08-c ^a	10/2008	104/104	191/197	144/153	(>19) ^{f,g}	13/10/2008	11	no	
SP 10-c ^a	08/2008	102/102	205/207	153/153	(>1) ^{e,g}	28/08/2008	27	no	
SP 10-d ^a	08/2008	102/102	205/207	150/153	(>57) ^{f,g}	05/09/2008	7	no	
c) Parental genotypes of São Simão colonies that underwent natural queen replacements									
SS 02-a	07/2006	104/106 x 106	205/211 x 197	159/159 x 153					
SS 02-b	02/2007	106/108 x 106	205/205 x 197	153/153 x 153				yes	
SS 06-a	08/2006	104/108 x 106	205/211 x 197	150/153 x 150					
SS 06-b ^a	12/2006	106/108 x 108	197/211 x 211	150/150 x 153				no	
SS 08-a	03/2006	108/108 x 106	205/205 x 211	150/159 x 153					
SS 08-b ^a	10/2006	106/108 x 106	205/211 x 197	150/153 x 159				no	
SS 36-a	03/2006	106/108 x 108	197/205 x 205	150/150 x 153					
SS 36-b	11/2006	106/108 x 108	205/205 x 205	150/153 x 150				no	
SS 59-a	09/2006	106/108 x 106	197/205 x 211	153/153 x 159					
SS 59-b ^a	01/2007	106/106 x 106	197/205 x 211	153/153 x 150				yes	
SS 63-a	04/2006	106/108 x 106	197/205 x 211	153/159 x 153					
SS 63-b ^a	11/2006	106/106 x 106	205/211 x 205	153/153 x 159				no	

^a Maternal genotype obtained by direct genotyping of the mother queen (using either a wing tip sample from a live queen or a leg if she was killed or had recently died). ^b Since this queen laid eggs for only 7 days no brood could be sampled for genetic analysis. However, on 26/12/2006 the mother queen was found dead in the colony and so could be collected for genetic analysis. Given that the genotype of queen 1-d could not have originated from any other colony, we know via elimination that she was the daughter of 1-c, and this allowed us to indirectly infer the genotype of the male that queen 1-c had mated with. ^c Maternal genotype was consistent with it being the daughter of 1-b, but with a probability of ¼ it could also have been the daughter of 9-a. Given that at that time there were 8 alien colonies from which an unrelated queen could have originated, the probability of queen 1-c being erroneously classified as a daughter of 1-b is $1/4 \times 1/8 = 0.031$. ^d Maternal genotype was consistent with it being the daughter of 3-c, but with a probability of ¼ it could also have been the daughter of 9-b. Given that at that time there were 10 alien colonies from which an unrelated queen could have originated, the probability of queen 3-d being erroneously classified as a daughter of 3-c is $1/4 \times 1/10 = 0.025$. ^e Queen experimentally removed. ^f Queen still alive on 1/11/2008. ^g Since queens were experimentally removed or only recently became established the estimates were not used in the calculation of median life expectancy.

DISCUSSION

Our results convincingly demonstrate, based on data from two different localities, that *Melipona* queens without reproductive options in their own colony can leave the hive and enter and successfully take over unrelated, alien hives nearby. In fact, overall, our genetic data show that in one quarter of all cases, the death of the mother queen results in her being replaced by an unrelated queen that flies in from unrelated, alien hives. This provides formal support for Sommeijer et al.'s (2003a,b) hypothesis, and shows for the very first time that queens in highly eusocial bees can not only found colonies via queen supersedure or swarming but also by taking over and parasitizing mature non-natal colonies.

It is worth noting that our results are unlikely to be merely due to the accidental drifting of queens. Drifting workers are relatively common among stingless bees (Peters *et al.* 1999; Palmer *et al.* 2002), especially in bee yards, where colony entrances are in close proximity to each other. However, accidental drifting of queens is unlikely to occur, for several reasons. First, in 2 out of 4 of the colonies in São Paulo, we inferred that the alien queens definitely came from queenright source colonies, and such colonies would not normally be expected to send out queens for mating. Second, alien queens never came from an immediately neighbouring colony and in fact always came from opposite sides of the building (Fig. 1). Hence, our results are unlikely to be a by-product of queens accidentally drifting between nests. Although in *Apis mellifera*, queens occasionally drift to the wrong hive following their mating flights, this is a rare event, happening in only 4% of the cases, and it is always fatal as workers kill any alien queens (Perez-Sato *et al.* 2008). In addition, in contrast to what we found, such drifted queens are only ever found in hives immediately adjacent to the source colonies (Perez-Sato *et al.* 2008). Finally, at the behavioural level it has been observed in *M. favesa* that gynes who had visited male congregation sites repeatedly tried to enter unrelated hives and in fact, several were seen to successfully penetrate such colonies for some minutes (Sommeijer *et al.* 2003a).

That this remarkable system of social parasitism is observed exactly in *Melipona* is probably not a coincidence. Given the observed vast queen overproduction seen in this genus (Kerr 1950, 1969; Engels & Imperatriz-Fonseca 1990; Moo-Valle *et al.* 2001; Wenseleers & Ratnieks 2004; Santos-Filho *et al.* 2006), a great number of queens would find themselves without reproductive options in their natal colony and would end up being slaughtered by the

workers if they stayed there (Silva *et al.* 1972; Koedam *et al.* 1995; van Veen *et al.* 1999a; Wenseleers *et al.* 2004). Hence, escaping from their natal nest and attempting to penetrate other colonies, particularly queenless ones, could provide them with big fitness benefits. In fact, one would expect that workers could even gain inclusive fitness from chasing out queens out if such queens would have reproductive success elsewhere (Sommeijer *et al.* 2003b), and there is some evidence of this happening to some extent in *M. compressipes* (Kerr 1997). Overall, social parasitism is also aided by the relatively long time it takes for a queenless colony to requeen in *Melipona* (ca. 2 weeks, our results and Silva *et al.* 1972; van Veen *et al.* 1999b), which queens can use as a window to seize their chance to move in and parasitize a colony, as well as by the relatively short queen life expectancy (ca. half a year), which means that at any one time a significant proportion of the colonies in the population would find themselves queenless. From the perspective of the queenless colonies, accepting an unrelated social parasite queen, may also not entail a big cost, particularly not if it happens relatively rarely, and the cost of occasionally accepting a social parasite is almost certainly much smaller than the big cost that the colony would incur if the workers accidentally rejected their own daughter queen. Future work will have to determine exactly how widespread intraspecific queen parasitism is in *Melipona*, and whether it might also be more common in social insects as a whole, where due to its cryptic nature intraspecific queen parasitism has so far probably largely gone unnoticed.

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CAPÍTULO 5.

First discovery of a rare polygyne colony in the stingless bee *Melipona quadrifasciata* (Apidae, Meliponini)

Alves DA, Menezes C, Imperatriz-Fonseca VL, Wenseleers T (2010) First discovery of a rare polygyne colony in the stingless bee *Melipona quadrifasciata* (Apidae, Meliponini). *Apidologie*, aceito.

First discovery of a rare polygyne colony in the stingless bee *Melipona quadrifasciata* (Apidae, Meliponini)

Stingless bees are highly eusocial bees, and are characterised by having perennial colonies that are typically headed by one single-mated queen (Peters *et al.* 1999). The main exception to this pattern is found in *Melipona bicolor*, which is the only stingless bee species discovered so far to exhibit facultative polygyny, whereby several queens may coexist and share reproduction inside the colony for considerable periods of time (Bego 1989; Velthuis *et al.* 2006). Aside from that, there are, for a few stingless bee species, also some anecdotal reports of temporary, transient episodes of polygyny (e.g. in *Melipona scutellaris*, Carvalho-Zilse & Kerr 2004, *Plebeia droryana*, Silva 1972 and *P. wittmanni*, Witter & Wittmann 1997), which are usually associated with queen replacement events. Here we report on a novel case of occasional polygyny in the stingless bee *Melipona quadrifasciata*, in which an exceptionally high number (8) of egg-laying queens were found to coexist inside the same colony. In addition, and in contrast to some of the earlier studies demonstrating occasional polygyny in stingless bees, which were purely based on observations, we provide the first genetic data about the reproductive partitioning and relatedness among these different queens.

The polygyne *M. quadrifasciata* colony was first discovered by a beekeeper of stingless bees, Mr. Cleiton Geuster, in Luzerna, Santa Catarina state, where he maintained a total of 9 colonies of this species. In July 2009, the colony was found to be headed by a single mother queen, but one month later it underwent a queen supersedure event whereby the old queen died and 8 newly mated queens were adopted. Subsequently, the polygyne colony, containing around 1,000 workers, was transferred to the University of São Paulo, Ribeirão Preto for further study. Polygyny was maintained for a total of ca. 4 months, after which the colony adopted a new queen and reverted back to a monogynous state.

To determine how the reproduction was partitioned among the different queens and to find out whether or not they were related (daughters of the previous queen, as opposed to unrelated social parasites), we collected female brood (n=168 female pupae) as well as wing tips from all eight physogastric queens for further genotyping. In addition, we also genotyped 22 foragers which given their age were most likely the offspring of the replaced mother

queen, and we sampled 10 worker pupae from each of the remaining eight colonies kept in the apiary. Subsequently, DNA was extracted using the Chelex method and samples were genotyped at four microsatellite markers, *T4-171* (Paxton *et al.* 1999), *Mbi-201* and *Mbi-278* (Peters *et al.* 1998) and *Mru-03* (Lopes *et al.* 2009), using multiplex PCR reactions and a touchdown PCR programme, as described previously (Alves *et al.* 2009). PCR amplification products were run on a 3130 Avant capillary DNA sequencer and alleles were identified using the supplied GeneMapper software. SPAGeDI was used to calculate the genetic relatedness among the workers (r) (Hardy & Vekemans 2002), following the estimator given in Queller & Goodnight (1989). The genetic relatedness among workers was then used to estimate the effective number of laying queens (N_e) inside the nest, $N_e = (3 - G)/(4r - G)$, where G is queen-queen relatedness (Pamilo 1991), as well as the maternity skew, using the index $S = (N_t - N_e)/(N_t - 1)$, where N_t is the total number of queens inside the colony (8). This skew index ranges between 0 (no skew) and 1 (one queen carries out all reproduction) (Pamilo & Crozier 1996).

Loci were reasonably polymorphic, with 5, 3, 2 and 7 alleles detected and mean expected heterozygosities of 41.08%, 62.18%, 38.52% and 77.67% at *loci* T4-171, Mbi-201, Mbi-278 and Mru-03, respectively. For 8 out of 9 of the studied colonies, the genotypes of the worker pupae were consistent with the colonies being headed by a single once-mated queen, as is typical for stingless bees (Peters *et al.* 1999). In addition, the genotypes of the newly established queens in the polygyne colony demonstrate that they were almost certainly full-sisters and therefore daughters of the previous queen (Table 1). The genotypes of 19 out of 22 of the foragers genotyped from this colony were consistent with them being derived from the superseded queen. A small number of foragers (3/22), however, had genotypes that were incompatible with either the genotype of the superseded queen or that of any of the currently established queens, which means they had probably drifted from other hives nearby (cf. Peters *et al.* 1999; Palmer *et al.* 2002). The average relatedness among workers in the polygyne colony, calculated from the genotypes of worker pupae, was low, $r = 0.30$, consistent with multiple laying queens being present. Indeed, the estimated effective maternity was 5, which was very high, although slightly lower than the observed absolute number of queens, 8. The reproductive skew estimated from these figures was $S = 0.43$, which was significantly higher than 0, expected if all queens would have shared reproduction equally.

Our study is the first to demonstrate occasional polygyny in *M. quadrifasciata*, one of the most intensely studied species of stingless bees. The high effective number of laying

queens (5) that we found in this rare polygyne colony was remarkable, given that even in *M. bicolor*, the average effective number of laying queens in polygyne colonies is only ca. 1.5 (D.A. Alves, unpublished data), and that in other stingless bees where occasional polygyny has been found, normally no more than two laying queens coexist at the same time (Silva 1972; Witter & Wittmann 1997; Carvalho-Zilse & Kerr 2004). We suggest that these occasional episodes of polygyny may occur when multiple virgin queens emerge and leave on their mating flights around the same time, thereby exploiting the short window during which newly mated queens may be accepted by the workers (Koedam *et al.* 1995; Wenseleers *et al.* 2004). In *Melipona*, this might not be so uncommon given the high levels of queen overproduction that are found in this genus (Kerr 1969; Santos *et al.* 2006), caused by larval caste self-determination (Bourke & Ratnieks 1999; Wenseleers & Ratnieks 2004). If several such queens can simultaneously seize the chance to start reproducing, this will evidently provide them with large individual fitness benefits. Nevertheless, it is unlikely to raise the productivity of the colony as a whole, given that in stingless bees, the cell building rate and not queen fecundity limits total colony productivity (Velthuis *et al.* 2006). Indeed, it may well be due to this tension between individual and colony-level interests that polygyny is not more common in stingless bees. Nevertheless, a better understanding of the processes leading to occasional episodes of polygyny might well end up providing novel insights into the evolution of more permanent polygyny in other species of social Hymenoptera.

Table 1. Genotypes of the physogastric queens of the polygyne *Melipona quadrifasciata* colony and the inferred genotype of their superseded mother and her mate.

Queen	Genotypes at locus			
	<i>T4-171</i>	<i>Mbi-201</i>	<i>Mbi-278</i>	<i>Mru-03</i>
SC 9-a	109/109	165/168	185/188	128/128
SC 9-b	109/109	165/168	185/188	118/128
SC 9-c	109/109	165/168	185/188	118/128
SC 9-d	109/109	162/168	185/188	118/128
SC 9-e	109/109	165/168	185/188	118/128
SC 9-f	109/109	162/168	185/188	118/128
SC 9-g	109/109	165/168	185/188	118/128
SC 9-h	109/109	165/168	185/188	128/128
<i>Inferred parental genotypes:</i>	<i>109/109 x 109</i>	<i>162/165 x 168</i>	<i>185/185 x 188 or 188/188 x 185</i>	<i>118/128 x 128</i>

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CONSIDERAÇÕES FINAIS

Esta tese proporcionou informações inéditas e atualizadas sobre a produção de machos e rainhas em pequenas populações de *Melipona* (**Capítulo 1**), um dos gêneros mais diversos de abelhas sem ferrão. Permitiu avaliar a diversidade genética em populações manejadas, sob condições de isolamento genético ou não, e as conseqüências da prolongada criação de uma população iniciada a partir de baixo número de colônias (**Capítulo 2**). Em termos de benefícios reprodutivos individuais, também foram reportados dois casos de parasitismo social (**Capítulos 3 e 4**) e um de poliginia ocasional (**Capítulo 5**), associados à produção de machos ou de rainhas.

Uma das principais contribuições (**Capítulo 2**) foi a demonstração que uma população de *M. scutellaris*, iniciada com poucas colônias e em condições de isolamento genético (em São Simão, SP), possui menor variabilidade genética, tanto no *locus* neutro quanto no sexual, quando comparada à população mantida na área natural de ocorrência da espécie (em Igarassu, PE). Dado que apenas 3,8 alelos sexuais estavam presentes na população de S. Simão, cerca de metade das rainhas que realizaram vôos nupciais eram fecundadas por machos que compartilhavam um mesmo alelo sexual (*matched mating*). Consequentemente, havia alta produção de machos diplóides, que representam um custo elevado para as colônias que os produzem e, finalmente, para a população. Sem dúvida, o manejo adequado e constante (como por exemplo, prover abundante flora apícola, alimentação artificial com solução açucarada, caixas com isolamento térmico, trocar favos de cria de colônias fortes para colônias fracas) possibilitou que os ninhos fossem multiplicados sucessivamente e a população fosse criada com sucesso por dez anos, minimizando os efeitos negativos da produção dos machos diplóides na viabilidade da população.

Outro fator, que provavelmente contribuiu para diminuir os efeitos dos machos diplóides, foi a substituição das rainhas-mãe que os produzem. Embora as operárias não matem a rainha-mãe logo após o aparecimento dos primeiros machos diplóides, ou seja, assim que eles emergem, elas o fazem alguns dias depois. Assim, uma nova rainha inicia as atividades de postura de ovos, havendo a possibilidade de que essa rainha não produza machos diplóides. E para corroborar esse mecanismo de compensação na produção desses machos, dois resultados foram fundamentais. O primeiro foi relativo à longevidade de rainhas que produzem machos diplóides, que vivem cerca da metade do tempo que vivem as rainhas que não os produzem (**Capítulos 2**). O outro resultado, de certa forma surpreendente, foi que as freqüências de rainhas nas colônias de S. Simão foram significativamente maiores que as mantidas em Igarassu, onde o número de alelos sexuais foi elevado, 25.9 (**Capítulo 1**). Assim,

produzir alto número de rainhas pode ser uma resposta para a alta taxa de trocas de rainhas que copularam com machos que compartilhavam mesmo alelo sexual e, portanto, produziram machos diplóides. Ou seja, criar muitas rainhas, embora seja um custo para a colônia, pois deixa-se de produzir algumas operárias (ie., para cada rainha produzida, uma operária deixa de ser criada), pode ser um mecanismo que limita os efeitos negativos do baixo número de alelos sexuais na população.

Também, o baixo número de alelos sexuais em *S. Simão* foi, provavelmente, o fator que influenciou as altas frequências de rainhas e machos. Isso porque tanto as operárias quanto os sexuais criados por colônias em *S. Simão* e em Igarassu eram de tamanhos corporais similares. Ou seja, as colônias investiram quantidades semelhantes de recursos na produção desses indivíduos e, portanto, o fator quantidade de recurso não pareceu ter sido a maior influência nessa diferença (**Capítulo 1**).

Mas independente da localidade em que colônias de *Melipona* estejam e quais sejam as influências, elas sempre produzem rainhas. Em maior ou em menor número, elas sempre estão presentes e, na grande maioria das vezes, são mortas pelas operárias. Mas se elas são mortas frequentemente, a pergunta pode ser: por que muitas rainhas são produzidas continuamente, se representam um gasto de recursos para as colônias? Até então, essa pergunta, em relação a termos de funcionalidade, era respondida em diferentes contextos: 1) as rainhas são produzidas para eventos de enxameagem; 2) as rainhas são produzidas para alguma substituição casual de suas rainhas-mãe, seja por motivo fisiológico (e.g. senilidade) ou mesmo quando essas produzem machos diplóides (**Capítulo 2**). Assim, até o momento, a alta taxa de rainhas era entendida como um verdadeiro estoque para eventualidades. Contudo, outra contribuição dessa tese é que as rainhas, caso escapem de serem mortas pelas operárias em suas colônias-natais, e não acompanhem um enxame ou não substituam suas mães, possuem outra estratégia reprodutiva (**Capítulo 4**). Quando não encontram oportunidades reprodutivas em seus ninhos-natais, elas acham-nas em outras colônias. Ou seja, muitas rainhas podem sair de suas colônias, provavelmente devem se acasalar com machos disponíveis nas proximidades, penetram em colônias órfãs, são aceitas pelas operárias dessas colônias e lá iniciam normalmente suas atividades de postura de ovos, agindo como parasitas sociais, uma vez que vão operárias não-aparentadas a ajudam na criação de sua prole. Dessa forma, no geral, em termos de aptidão, as rainhas parasitas obtêm benefícios diretos ao invadir outros ninhos e lá colocarem seus ovos, e as operárias

que as expulsam, ou as deixam, das colônias obtêm benefícios indiretos, pois suas irmãs tem oportunidades reprodutivas em outros ninhos.

Dado que rainhas penetram em ninhos não-natais e são bem-sucedidas em suas atividades de postura e como em uma colônia poligínica de *M. bicolor* uma das três rainhas era parasita social (Alves *et al.* in prep.), esperava-se que, no caso de poliginia em *M. quadrifasciata* (descoberta por um meliponicultor) alguma(s) das oito rainhas fisogástricas não tivesse(m) relação de parentesco com as demais (**Capítulo 5**). Outro complemento a essa hipótese foi que as oito rainhas se estabeleceram no mesmo período, em que a colônia estava órfã. Os resultados apontaram que todas as oito rainhas substituíram sua mãe no mesmo momento e, portanto, eram irmãs completas, embora nem todas (cinco) contribuíssem nas atividades de postura. Esse episódio ocasional de poliginia deve ser outra estratégia reprodutiva e, provavelmente deve ocorrer quando algumas rainhas virgens saem da colônia órfã para realizar o vôo nupcial e retornam, mais ou menos, no mesmo momento. Dessa forma, elas exploram o curto período de tempo que as operárias aceitam as rainhas recém-acasaladas. Se muitas rainhas podem aproveitar a chance de se reproduzirem, nem que seja por pouco tempo, elas obtêm amplos benefícios, em termos de aptidão direta.

Porém, não só as rainhas tem oportunidades reprodutivas na produção de cria. As operárias, embora impossibilitadas de se acasalarem, também as tem. Consequentemente, um conflito pela maternidade dos machos ocorre na arena do favo de cria, seja entre rainha–operária, seja entre operária–operária (**Capítulo 3**). Isso porque, ter um filho ($r = 0,50$) é mais vantajoso, em termos de relacionamento genético, do que ter um irmão ($r = 0,25$) ou sobrinho ($r = 0,375$). Apesar de as rainhas serem as maiores produtoras de machos em *M. scutellaris*, 20% deles são filhos de operárias. O mais surpreendente não foi esse resultado, mas sim que as operárias que mais produziram machos (80%) foram aquelas que eram filhas das rainhas que foram substituídas. Ou seja, as operárias filhas de uma dada rainha ainda permaneciam no ninho, mesmo após a substituição de sua mãe e a produção de novas operárias (sobrinhas), tendo uma longevidade maior do que a média das demais operárias, as que não se reproduziram. Essas operárias mais longevas parasitam reprodutivamente a força de trabalho da geração seguinte e obtêm mais benefícios em explorar a colônia quando os custos são arcados por indivíduos menos relacionados, que no caso, foram as suas sobrinhas.

Dessa forma, o conjunto de capítulos contribuiu para entender melhor a produção de sexuados em pequenas populações, além de prover informações úteis para a criação de abelhas sem ferrão, que pode ser aliada a ações voltadas para conservação. Além do mais, os resultados aqui obtidos mostram que há necessidade de reavaliar as metodologias e conceitos, utilizados até o momento, no melhoramento genético das abelhas sem ferrão e na manutenção de pequenas populações em meliponários. Também mostrou a importância do uso da biologia molecular como uma ferramenta que se torna cada vez mais necessária, devido ao seu amplo potencial em resolver questões de cunho evolutivo e ecológico, além de ser uma prática acessível, relativamente barata e altamente confiável, quando bem empregada e interpretada.

Assim, como as práticas de criação de abelhas podem influenciar profundamente a diversidade genética das populações naturais, além de transmissão de patógenos e hibridização, algumas recomendações são necessárias:

- a) escolher espécies que ocorrem na região onde o meliponário será mantido e prover boas e adequadas condições de manejo;
- b) evitar a troca de favos de cria, de rainhas fecundadas e mesmo de colônias entre regiões geográficas muito distintas, já que cada população está adaptada às condições ambientais locais. Além disso, a freqüente troca de material genético tem como consequência a homogeneização do conjunto (*pool*) genético entre diferentes regiões geográficas (Carvalho-Zilse *et al.* 2009; De la Rúa *et al.* 2009);
- c) iniciar um meliponário com, no mínimo, quatro colônias, para que a população tenha variabilidade genética (ao menos seis alelos sexuais) suficiente para minimizar as consequências de baixo número de alelos e a consequente produção de machos diplóides – esses cálculos foram baseados no caso da população-fonte (ie., de onde as colônias são obtidas) ter cerca de 25 alelos sexuais. Estudos anteriores em populações manejadas de outras espécies de abelhas sem ferrão (*M. bicolor* (Alves, dados não publicados), *M. compressipes* (Kerr 1987), *M. quadrifasciata* (Aidar & Kerr 2001), *M. scutellaris* (Carvalho 2001), *Scaptotrigona postica* (Paxton *et al.* 2003) e *Trigona carbonaria* (Green & Oldroyd 2002)), embora tenham mostrado que machos diplóides foram produzidos, nenhuma delas tinha menos de seis alelos sexuais, o que novamente, corrobora os cálculos realizados nesse trabalho.

d) avaliar se as colônias estão produzindo machos diplóides. Para isso, embora o uso de marcadores moleculares seja mais eficaz, a porcentagem de machos presentes nos favos de cria é um bom indicativo (Camargo 1979). Caso mais de um favo contenha porcentagem superior a 50% de machos entre as pupas presentes, essa é uma maneira adequada de verificar se a rainha-mãe está produzindo machos diplóides, já que os estes não diferem em tamanho corporal dos machos haplóides;

e) considerar a mobilidade das rainhas que escapam de suas colônias e penetram em ninhos órfãos, para os programas de melhoramento genético e organizar um modelo de organização espacial dos ninhos no meliponário a fim de selecionar as características desejáveis das colônias (e.g. maior produção de mel, maior estoque de pólen, resistência a doenças, maior produção de cria, produção de própolis, agressividade). Para revisão, sobre métodos de seleção para melhoramento genético em abelha sem ferrão, veja Kerr (2006).

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ANEXO

The queen is dead—long live the workers: intraspecific parasitism by workers in the stingless bee *Melipona scutellaris*

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Abstract

Insect societies are well known for their high degree of cooperation, but their colonies can potentially be exploited by reproductive workers who lay unfertilized, male eggs, rather than work for the good of the colony. Recently, it has also been discovered that workers in bumblebees and Asian honeybees can succeed in entering and parasitizing unrelated colonies to produce their own male offspring. The aim of this study was to investigate whether such intraspecific worker parasitism might also occur in stingless bees, another group of highly social bees. Based on a large-scale genetic study of the species *Melipona scutellaris*, and the genotyping of nearly 600 males from 45 colonies, we show that ~20% of all males are workers' sons, but that around 80% of these had genotypes that were incompatible with them being the sons of workers of the resident queen. By tracking colonies over multiple generations, we show that these males were not produced by drifted workers, but rather by workers that were the offspring of a previous, superseded queen. This means that uniquely, workers reproductively parasitize the next-generation workforce. Our results are surprising given that most colonies were sampled many months after the previous queen had died and that workers normally only have a life expectancy of ~30 days. It also implies that reproductive workers greatly outlive all other workers. We explain our results in the context of kin selection theory, and the fact that it pays workers more from exploiting the colony if costs are carried by less related individuals.

Keywords: Meliponini, reproductive conflict, social insects, social parasitism, stingless bees, worker reproduction

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Introduction

Social insects such as ants, bees and wasps are well known for their high degree of cooperation, but the nonclonal structure of their colonies also sets the stage for various reproductive conflicts (Hamilton 1964; Trivers & Hare 1976; Ratnieks & Reeve 1992; Beekman &

Ratnieks 2003; Ratnieks *et al.* 2006). One such conflict is queen-worker conflict over male parentage (Trivers & Hare 1976; Bourke 1988; Hammond & Keller 2004; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006a). Workers, although generally being unable to mate, are usually capable of laying unfertilized eggs, which develop into male offspring if successfully reared. In addition, they are generally selected to do so, as workers are always most related to their own sons ($r = 0.5$) (Hamilton 1964; Trivers & Hare 1976; Cole 1986; Bourke

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1988; Wenseleers *et al.* 2004b). Hence, a queen-worker conflict over the production of males ensues. Several factors, however, may help to resolve queen-worker conflict over male parentage and keep successful worker reproduction at a low level (Beekman & Ratnieks 2003; Hammond & Keller 2004; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006a). First, the queen herself can counter worker reproduction by selectively eating any worker-laid eggs ('queen policing') (Oster & Wilson 1978; Ratnieks & Reeve 1992; Wenseleers *et al.* 2005a, b; reviewed in Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006a). Second, the workers may also cannibalize eggs laid by other workers ('worker policing', Ratnieks 1988; Ratnieks & Visscher 1989). This behaviour is seen particularly in species with multiple mated queens (such as honeybees, where worker policing was first discovered), due to the fact that the workers are more related to the queen's sons (brothers, $r = 0.25$) than to the sons of other workers in that situation (a mix of full- and half-nephews, $r < 0.25$) (Ratnieks 1988; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006a, b; Ratnieks & Wenseleers 2008). By contrast, in species with a single-mated queen (such as some ants, bumblebees and stingless bees), workers are on average more related to the sons of other workers (full-nephews, $r = 0.375$), than to the sons of the queen ($r = 0.25$), and so collectively favour worker reproduction. Nevertheless, even in these species, worker reproduction may not always reach high levels, probably because of colony-level costs associated with worker reproduction, caused by the fact that reproductive workers in some species carry out less work in the colony (Cole 1986; Bourke 1988; Hillesheim *et al.* 1989; Wenseleers *et al.* 2004b) or because worker male production is traded off against worker production, thereby reducing future colony productivity (Ratnieks & Reeve 1992; Tóth *et al.* 2004; Wenseleers & Ratnieks 2006a; Ohtsuki & Tsuji 2009).

Recently, it has also been discovered that workers may not only exploit their own colony by laying male eggs, but that they may also parasitize and reproduce in other, unrelated colonies away from their natal nest (Beekman & Oldroyd 2008). For example, in the common bumblebee *Bombus terrestris*, workers may succeed in entering unrelated colonies, where they appear to reproduce earlier than normal, thereby insuring that their sons have a greater mating success than if they had produced such males during the normal male production period in their natal colony (Lopez-Vaamonde *et al.* 2004; Beekman & Oldroyd 2008). In addition, in two Asian honeybee species, *Apis florea* (Nanork *et al.* 2005) and *A. cerana* (Nanork *et al.* 2007), it has been shown that particularly queenless nests are prone to be parasitized by non-natal workers. The original

interpretation was that this was because of workers from queenright colonies invading these nests, and that it represented a strategy to evade policing in their natal nest, as worker policing is generally switched off in queenless nests (Nanork *et al.* 2005, 2007; Beekman & Oldroyd 2008). More recent data, however, rather seem to suggest that the majority of the worker parasites appear to come from other queenless hives nearby (Chapman *et al.* 2009). In both bumblebees and Asian honeybees, non-natal workers also tend to reproduce more than natal ones, presumably because any concomitant costs of worker reproduction are carried by the unrelated workforce of the parasitized nest, thus limiting any inclusive fitness costs (Beekman & Oldroyd 2008).

The aim of this study was to test whether intraspecific worker parasitism might also occur in stingless bees, another major group of eusocial bees, using the Brazilian species *Melipona scutellaris* as a model. Like honeybees, stingless bees form perennial, swarm-founded colonies; although more similar to bumblebees, their colonies are headed by a single once-mated queen (Peters *et al.* 1999). Furthermore, in both wild colonies and colonies kept in apiaries, genetic studies have demonstrated the occasional occurrence of workers that could not be attributed to the current queen, which indicates the presence of either unrelated drifters or workers derived from a superseded queen (Paxton *et al.* 1999a, 2003; Peters *et al.* 1999; Palmer *et al.* 2002). As yet, however, it has not been determined whether such workers might also successfully reproduce, and if so, whether they might do more so than the rest of the colony. In general, worker reproduction in stingless bees, although favoured on relatedness grounds (Tóth *et al.* 2002b, 2004), can vary from low to high, with anywhere between 0% and 95% of the males being workers' sons depending on the species (see e.g. Contel & Kerr 1976; Sommeijer *et al.* 1999; Drumond *et al.* 2000; Palmer *et al.* 2002; Tóth *et al.* 2002a, b, 2003; Paxton *et al.* 2003; Koedam *et al.* 2005; Gloag *et al.* 2007; reviewed in Hammond & Keller 2004; Tóth *et al.* 2004; Velthuis *et al.* 2005; Wenseleers & Ratnieks 2006a). Although the cause of this variation remains unknown, it could perhaps be because of interspecific differences in the colony-level cost of worker reproduction or due to the queen suppressing worker reproduction to varying extents (Tóth *et al.* 2004; Wenseleers & Ratnieks 2006a; Ratnieks & Wenseleers 2008).

Given the occurrence of worker reproduction in stingless bees (Hammond & Keller 2004; Tóth *et al.* 2004; Velthuis *et al.* 2005; Wenseleers & Ratnieks 2006a) and the documented presence of less related or unrelated worker matrilineal within colonies (Paxton *et al.* 1999a, 2003; Peters *et al.* 1999; Palmer *et al.* 2002), the specific

aims of this study were to test whether in *Melipona scutellaris* such workers might be able to successfully reproduce, and if so, to what extent. By genotyping colony samples before and after queen supersedure events, we also test for the first time if workers from a superseded queen could keep on producing male offspring, and if so, for how long. This allows us to determine whether workers might have the capacity to reproductively parasitize the next-generation workforce.

Materials and methods

Study species

Melipona scutellaris nests in cavities of tree trunks in the Atlantic rainforest and is widely distributed in the Northeast of Brazil (Camargo & Pedro 2007), where it is commonly kept by regional and traditional beekeepers for honey, pollen and wax (Cortopassi-Laurino *et al.* 2006). In addition, *M. scutellaris* is increasingly used for the pollination of various tropical crops (Heard 1999; Castro 2002). Colonies are perennial and swarm-founded, typically contain around 1500 workers and are headed by one single-mated queen (Tóth *et al.* 2002b). Workers, gynes and males are reared individually in similar-sized cells which are filled with larval food and sealed by workers immediately after an egg is laid. For this study, we used colonies of *M. scutellaris* maintained in free-foraging wooden nest boxes at Granja São Saruê (Igarassu, Pernambuco state, 7°50'3.74"S, 34°54'22.87"W), in the Bee Laboratory at the University of São Paulo (São Paulo, S. Paulo state, 23°33'37.41"S, 46°43'53.04"W) and at the Aretuzina Farm (São Simão, S. Paulo state, 21°26'25.97"S, 47°34'54.65"W). Colonies were spaced ~1–10 m apart, as is typical in most apiaries and also not uncommon in nature, where several nests can sometimes be found within a few metres of each other, either in the same tree or in different trees (Alves *et al.* 2005). During the time of our study, colonies were managed only by occasionally providing them with food.

Brood sampling

Between January 2006 and October 2008, we sampled brood for genetic analysis from a total of 16, 9 and 12 colonies in Igarassu, S. Paulo and S. Simão respectively (Table 1). This was carried out by collecting brood combs containing mature pupae and removing the cell cappings, after which 10 workers and any males present were preserved in absolute ethanol. The remainder of the brood was reintroduced into the natal nest. Brood samples that did not contain male brood were not retained for this study. In S. Paulo and S. Simão, we

also repeated the sampling of brood when the mother queen, which was individually marked, was found to be replaced by a new one (Table 1). Such samplings were made at a median number of 206 days after the previous queen had died (range 77–737 days). Overall, we could collect male and worker brood produced by 45 distinct queens (Table 1).

Genetic analysis

For parentage analysis, we genotyped 10 worker pupae and an average of 13 haploid male pupae (range 5–36, total 576) for each of our 45 colonies (Table 1) at three microsatellite loci, *T4-171* (Paxton *et al.* 1999b), *Mbi-254* and *Mbi-201* (Peters *et al.* 1998). These loci were found to be most variable in this species in a preliminary screening of 10 loci. To facilitate parentage reconstruction and wherever necessary, we also noninvasively genotyped the mother queen from a wing tip sample (cf. Châline *et al.* 2004; Table 1). DNA was extracted using the Chelex method, whereby a single leg (for worker or male pupae) or wing tip sample (from mother queens) was frozen in liquid nitrogen and ground up using a plastic pestle, followed by an incubation at 95 °C for 15 min in 200 µL (50 µL for wing tip samples) of a 10% Biorad Chelex 100 resin solution. Samples were vortexed and centrifuged before use. Multiplex PCR reactions were carried out in a 10 µL reaction volume, and contained 0.5 µM of the forward and reverse primers of each locus, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 1 µL of crude DNA extract, 0.4 units of Silverstar *Taq* polymerase (Eurogentec) and enzyme buffer supplied by the manufacturer. PCR was performed following a touch-down programme (Bonckaert *et al.* 2008), with an initial denaturation for 3 min at 94 °C, followed by 20 cycles consisting of 30 s at 94 °C, 30 s at 58 °C, but decreasing 0.5 °C in each step and 45 s at 72 °C; 10 cycles consisting of 30 s at 94 °C, 30 s at 46 °C and 45 s at 72 °C; and a final 10-min extension step at 72 °C. After amplification, 1 µL of the PCR product was mixed with 8.8 µL formamide and 0.2 µL Genescan 500 LIZ size standard (Applied Biosystems), denatured, and loaded onto an ABI-3130 Avant capillary sequencer. Alleles were called using the supplied GeneMapper software and checked by eye. Male genotypes that appeared to suggest they were the sons of drifted workers or sons of workers derived from a superseded queen (see below) were rePCR'd, rescored and rechecked twice to eliminate the possibility of genotyping errors.

Analysis of male parentage

To infer male parentage, we first reconstructed the genotype of each mother queen and that of her mate

Table 1 Male parentage in *Melipona scutellaris* colonies kept in Igarassu (IG), São Paulo (SP) and São Simão (SS), with information on the sampling date, the number of diploid and haploid individuals genotyped, the number of males which was inferred to be sons of workers from either the current queen, a superseded queen or unrelated drifters and the percentages of males present in the sampled brood combs

Colony and queen	Sampling date	No. diploid individuals genotyped	No. haploid males genotyped	No. worker sons detected (%)			Power to detected sons of workers of the		% of males in sampled combs
				From workers of the current queen	From workers of the previous queen	From workers previous queen or drifted workers*	Current queen†	Previous queen‡	
IG 01-a	Sep. 2006	10	14			3 (21.43)	0.50		36.05
IG 03-a	Jan. 2006	10	8				0.75		16.06
IG 05-a	Sep. 2006	10	9				0.88		18.48
IG 06-a	Sep. 2006	10	10				0.88		24.41
IG 08-a	Jan. 2006	10	9			1 (11.11)	0.50		8.36
IG 11-a	May 2007	10	9				0.75		45.08
IG 12-a	Sep. 2006	10	9				0.75		28.42
IG 13-a	Jan. 2006	10	9			8 (88.89)	0.00		28.73
IG 16-a	Jan. 2006	10	8				0.50		33.69
IG 19-a	Jan. 2006	10	8				0.00		21.10
IG 20-a	May 2007	10	10				0.50		17.93
IG 26-a	Sep. 2006	10	10	5 (50.00)			0.88		21.99
IG 27-a	Sep. 2006	10	10	1 (10.00)			0.50		6.37
IG 30-a	Sep. 2006	10	10				0.88		19.63
IG 36-a	May 2007	10	10				0.75		29.03
IG 39-a	May 2007	10	12				0.88		9.69
SP 01-a§	Oct. 2006	10	5			1 (20.00)	0.75		20.00
SP 01-d§¶	Jul. 2008	10	11				0.00	0.44	10.93
SP 02-b§¶**	Feb. 2007	10	13	1 (7.69)	5 (38.46)		0.75	0.58	90.00
SP 03-a	Jul. 2006	10	36			10 (27.78)	0.00		26.55
SP 03-b	Dec. 2006	10	9		3 (33.33)		0.50	0.25	2.94
SP 03-c§**	Jul. 2007	10	24				0.88	1.00	13.22
SP 04-a	Jul. 2007	10	27				0.00		28.21
SP 05-b¶	Nov. 2006	10	30				0.50	0.00	39.59
SP 06-a	Oct. 2008	10	6			4 (66.67)	0.75		3.55
SP 08-a	Dec. 2006	10	27			6 (22.22)	0.75		18.74
SP 09-c**	Sep. 2008	10	20				0.75	0.94	14.56
SP 10-b§¶	Jul. 2008	10	33				0.50	0.00	18.55
SS 01-a§	Mar. 2006	12++	7				0.75		66.31

Table 1 Continued

Colony and queen	Sampling date	No. diploid individuals genotyped	No. haploid males genotyped	No. worker sons detected (%)			Power to detect sons of workers of the		% of males in sampled combs
				From workers of the current queen	From workers of the previous queen	From workers previous queen or drifted workers*	Current queen†	Previous queen‡	
SS 02-b¶**	Feb. 2007	10	10				0.50	0.72	8.96
SS 04-a	Apr. 2006	10	10	1 (10.00)			0.50		13.77
SS 06-a	Jul. 2006	10	8				0.75		15.85
SS 06-b§	Dec. 2006	10	9		1 (11.11)		0.50	0.44	5.13
SS 08-a§	Mar. 2006	14††	5	2 (40.00)			0.88		61.89
SS 08-b§	Oct. 2006	10	10				0.75	0.00	17.02
SS 15-a§	Mar. 2006	10	11	3 (27.27)			0.75		21.01
SS 28-a	Aug. 2006	10	16				0.50		42.47
SS 36-a	Mar. 2006	10	7				0.50		5.65
SS 36-b	Nov. 2006	10	8		3 (37.50)		0.00	0.25	69.69
SS 43-a	Jul. 2006	10	10				0.50		19.41
SS 59-a	Sep. 2006	10	23			1 (4.35)	0.75		24.72
SS 59-b§**	Jan. 2007	10	20				0.75	0.63	38.06
SS 63-a	Apr. 2006	17††	7	1 (14.29)		1 (14.29)	0.50		55.39
SS 63-b§	Nov. 2006	10	9				0.50	0.44	10.00
SS 66-a	Sep. 2006	10	10				0.00		14.95
Total/average	$\Sigma = 463$	$\Sigma = 576$	$\Sigma = 14$	$\Sigma = 12$	$\Sigma = 35$	$\bar{x} = 0.56$	$\bar{y} = 0.44$		

The letters in the colony codes refer to the succession of different mother queens. The statistical power with which we could unambiguously detect sons of workers of the current queen and sons of workers from a previous queen is also given. The reconstructed parental genotypes and male genotypes are given in Table S1.

*The possibility of males being sons of workers of the previous queen or of a drifted worker could only be distinguished with certainty if the genotype of the previous, superseded queen and that of her mate was known.

†Probability of a worker son inheriting a unique paternal allele at at least on the loci studied, calculated using the formula given in Foster *et al.* (2001).

‡Probability that a son of a worker of the previous queen could be distinguished from a son of a worker of the current queen at one of the loci studied at least (calculated on a case by case basis).

§Mother queen also genotyped.

¶Genotype of the superseded queen and her mate determined in another study (Wenseleers *et al.*, unpublished data; Table S1).

**Queen was genetically unrelated to the previous, superseded queen (Wenseleers *et al.*, unpublished data; Table S1).

††The queen and her mate shared an allele at the sex locus. Diploid offspring genotyped comprised 10 workers plus 2, 4 and 7 diploid males respectively.

from the genotypes of the diploid brood she produced (worker pupae and occasionally also diploid males, Table 1) (cf. Foster *et al.* 1999). This was straightforward, given that in all cases, genotypes were consistent with the mother queens being singly mated and with just a single matriline being present. In a few cases, we also had the genotype of the queen herself available, which further facilitated parentage reconstruction (Table S1, Supporting Information). As a null hypothesis, we assumed that males were either sons of the queen or of workers derived from the present queen. These two possibilities could be distinguished if the paternal allele at any one locus was different from the maternal alleles (e.g. parental genotypes of the type $ab \times c$ or $aa \times b$, but not $ab \times a$). In that case, a worker's son could be detected if he inherited a unique paternal allele that was not present in the queen (cf. Foster *et al.* 2001). However, if a male carried an allele not present in either the mother queen or that of its mate, then it was clear that such a male was either the son of a drifted worker or of a worker derived from a superseded queen. The latter two possibilities could be distinguished either by comparison to the known genotypes of workers produced by the previous, superseded queen (Table 1), or by counting the total number of alleles present among such males at each locus in any one colony. The idea is that reproduction by a single superseded matriline of workers should never lead to the presence of more than three alleles, whereas the reproduction by workers that had drifted from multiple colonies might. Estimates of the percentage of males that were sons of workers from the current or previous queen were corrected according to the statistical power with which such males could be detected in our data set given the observed parental genotypes ($\bar{x}$ and $\bar{y}$, for details see Table 1, cf. Foster *et al.* 2001). Finally, we also calculated the probability that the son of a drifted worker would by chance be misclassified as the son of the current queen, a son of a worker of the current queen or a son of a worker of the superseded queen, by fortuitously having an identical multilocus genotype. This was carried out using the formula given in Nanork *et al.* (2005), using the population-specific allele frequencies.

Results

Allelic diversity and statistical power to detect worker reproduction

Loci were reasonably polymorphic, with a total of 7, 8 and 8 alleles detected (Igarassu: 7, 5, 8; S. Paulo: 6, 8, 7; S. Simão: 4, 3, 3) and mean expected heterozygosities of 79%, 71% and 73% (Igarassu: 74%, 64%, 78%; S. Paulo:

77%, 72%, 73%; S. Simão: 51%, 64%, 61%) at loci *T4-171*, *Mbi-254* and *Mbi-201* respectively. The mean statistical power to detect sons of workers derived from either the current queen or of the previous, superseded queen was $\bar{x} = 56\%$ and $\bar{y} = 44\%$ respectively (Table 1), with the latter figure based on 13 colony samples for which we also had the genotypes available of workers produced by the previous, superseded queen (Table 1). The mean probabilities of the son of a drifted worker fortuitously having the same genotype as the son of the current queen, a son of a worker of the current queen or a son of a worker of the superseded queen, were small, 3.81% (Igarassu: 1.86%; S. Paulo: 2.02%; S. Simão: 6.90%), 3.86% (Igarassu: 3.21%; S. Paulo: 2.65%; S. Simão: 4.76%) and 3.57% (S. Paulo: 2.06%; S. Simão: 4.58%) respectively. Hence, the probabilities of males being erroneously misclassified were small.

Male parentage

Out of the 576 males genotyped, 61 (10.59%) could not be assigned to the queen and were therefore definitely workers' sons (Table 1 and Table S1). Only a small percentage of these workers' sons (14 out of 61), 22.95%, however, were consistent with being sons of workers of the current queen, and the remainder, 47 out of 61 (77.05%), were therefore either derived from drifted workers or from workers produced by an earlier, superseded queen. Restricting ourselves to the subset of 13 colonies for which we had the genotypes available of workers produced by the superseded queen, however, we can see that all the anomalous males (12 out of 12 in four colonies, Table 1) were consistent with being the sons of workers from a superseded queen as opposed to being sons of unrelated, drifted workers (Table S1). In addition, for the remaining colonies where such anomalous male genotypes were found, we never sampled more than three alleles in males at any one locus in any of the colonies, again implying that such males were most probably produced by a single, superseded matriline of workers as opposed to workers that had drifted from several other, unrelated colonies (Table S1). For a few colonies, where only a few anomalous males were found, it remains possible, however, that these were the offspring of drifted workers. Assuming, nevertheless, that the majority of the anomalous males were the offspring of a superseded queen, and correcting our figures for nondetection, we estimate that 77.11% of the males were the queen's sons, 4.34% were the sons of the workers derived from the current queen and 18.54% were the sons of workers derived from a previous, superseded queen. Hence, using these corrected estimates, 81.03% of all workers' sons were the offspring of workers from superseded queens.

These figures are surprising given that for the São Paulo population, sons of workers deriving from the superseded queen could still be found in colonies SP 02-b and SP 03-b, despite the fact that these samples were taken 175 and 105 days after the previous queen had died, and that workers in *M. scutellaris* have a mean life expectancy of only 31 days (Oliveira & Kleinert-Giovannini 1991). Even accounting for the fact that some brood laid by the previous queen may have kept enclosing until ~40 days after her death (the development time from egg to adult in *Melipona*), and that the sampled brood was probably laid ~25 days before, we can see that reproductive workers must live a very long time, at least $175 - 40 - 25 = 110$ days, which is 3.5 times as long as that of a normal worker, and in fact not too far off from the life expectancy of queens in this species, 175 days (Wenseleers *et al.*, unpublished data).

There was no significant correlation between the inferred percentage of males that were workers' sons and the percentage of males present in the combs from which the samples were taken (Spearman $R = 0.11$, $n = 45$, $P = 0.45$). This goes against the hypothesis that workers would reproduce mainly during periods in which the queen lays haploid eggs (Chinh *et al.* 2003; Sommeijer *et al.* 2003; Velthuis *et al.* 2005). In fact, in several cases (in colonies IG 08-a, IG 27-a, SP 03-b, SP 06-a and SS 06-b), we found evidence for worker reproduction even at times when fewer than 10% of the cells contained male pupae (Table 1). So although we concur with Chinh *et al.* (2003) and Velthuis *et al.* (2005) that stingless bee queens frequently lay male eggs in distinct batches (during 'male producing periods'), it does not appear to be the case that workers mainly reproduce during such periods, as worker reproduction was found both in periods in which many and in which few males were reared.

Discussion

Consistent with most previous genetic and behavioural studies on male parentage in stingless bees (reviewed in Hammond & Keller 2004; Tóth *et al.* 2004; Velthuis *et al.* 2005; Wenseleers & Ratnieks 2006a), our results show that a significant fraction, 22.88%, of the males in *Melipona scutellaris* are sons of the workers rather than of the queen. This is as expected from inclusive fitness theory, given that single mating in stingless bees collectively favours worker reproduction (Ratnieks 1988; Tóth *et al.* 2002b, 2004). Surprisingly enough, however, our results also show that ~80% of the workers' sons had genotypes that were incompatible with them being the sons of workers of the present queen. Based on sampling of colonies over multiple generations, before and after queen superseding events, and based on the fact

that such males from any one colony never had more than three alleles at a given locus, we were able to show that these males were most probably produced by workers that were the offspring of a previous, superseded queen, rather than by workers that had drifted from other colonies. This represents the first demonstration of workers reproductively parasitizing the next-generation workforce for their own, selfish benefits.

That such a high percentage of the worker sons were produced by workers derived from a superseded queen is surprising, given that most colonies were sampled many months after the previous queen had died, and that workers in *M. scutellaris* normally only have a life expectancy of 31 days (Oliveira & Kleinert-Giovannini 1991; life expectancy of workers in five other *Melipona* species: 40–51 days, Giannini 1997; Biesmeijer & Tóth 1998). In fact, in one case, we estimated that reproductive workers had a life expectancy of ~110 days, which means they outlived the other workers by a factor of 3.5, and that they had a life expectancy which was almost comparable with that of the queen in this species, 175 days (Wenseleers *et al.*, unpublished data). The long life expectancy of laying workers is probably linked to the fact that reproductive workers usually do not carry out risky or energetically costly tasks, such as foraging, in order for them not to jeopardize their reproductive futures (Franks & Scovell 1983; Hartmann & Heinze 2003; Wenseleers *et al.* 2004b).

The high percentage of males produced by workers derived from superseded queens (~80%) was also surprising given that for colonies sampled at random points in time, such workers usually present only a very small fraction of the workforce. For example, the percentage of workers that had either drifted from other colonies or were descendants of a superseded queen was estimated at 3% across a sample of 12 species of stingless bees (Peters *et al.* 1999; excluding the facultatively polygyne *Melipona bicolor*), 10% in *Melipona beecohii* (Paxton *et al.* 1999a) and between 21% and 36% across three species of *Scaptotrigona* (Paxton *et al.* 1999a, 2003; Palmer *et al.* 2002). Overall, this probably means that, on a per capita basis, the workers from superseded queens specialize in reproduction. Partly, this may be due to the fact that during the brief period of queenlessness following the death of the queen and preceding the establishment of a new laying queen, workers increase their rate of ovary activation as well as their egg-laying rate (Ferreira *et al.* 1989; Lacerda & Zucchi 1999; Kleinert 2005; Velthuis *et al.* 2005), and that this may give these workers a 'head start' relative to the workers produced later by the newly mother queen. However, the greater reproductive rate of workers derived from a superseded queen is also consistent with kin selection theory, given that it pays workers more

from exploiting the colony if costs are carried by less related individuals (Hamilton 1964; Wenseleers *et al.* 2004a, b; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006b). In stingless bees, worker reproduction is probably to be costly, first because workers might carry out less work (but see Cepeda (2006) who shows that reproductive workers in *M. bicolor* do engage in some brood care), and second because the exclusively male eggs laid by workers will end up replacing some of the female, worker-destined eggs laid by the queen (Ratnieks & Reeve 1992; Tóth *et al.* 2004). Hence, worker reproduction in stingless bees will inevitably be traded off against worker production, and come at a cost to future colony productivity (Ratnieks & Reeve 1992; Tóth *et al.* 2004). From the perspective of workers from a previous queen, however, this cost will in inclusive fitness terms be lower as it will be carried by more distantly related individuals (nephews and nieces, $r = 0.375$, as opposed to sisters, $r = 0.75$), thereby causing them to be selected to exploit the colony more. These results are the first explicit demonstration that conflict over male parentage in insect societies is not just played out between the queen and workers and between the workers of one generation, but that the conflict may also spill over from one worker generation to the next. Future work will be required to test if such intergenerational worker reproduction conflict occurs more widely in other perennial insect societies.

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This work forms part of D.A.A.'s PhD research on the ecological and genetic basis of social behaviour in stingless bees, and the conservation biology and viability of small populations. V.L.I.F. is interested in the behaviour, evolution and conservation of stingless bees and is the head of the bee laboratory at the University of São Paulo. T.M.F. is interested in evaluating the genetic and morphological biodiversity in bee populations for their conservation and uses honey bees, stingless bees and orchid bees as his main model systems. P.S.S.F. works on the ecology and evolution of stingless bees. P.N.N. works on the ecology and breeding of stingless bees, with emphasis on their conservation, behaviour and sustainable use. J.B. is the leader of the Laboratory of Entomology at the University of Leuven and has a longstanding interest in the social organization of insect societies. T.W. is an evolutionary biologist interested in the origin and evolution of social behaviour, and uses wasps, stingless bees and honey bees as his main model systems.

Supporting information

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

Table S1 Reconstructed parental genotypes and male genotypes observed in *Melipona scutellaris* colonies kept at Igarassu (IG), São Paulo (SP) and São Simão (SS), with information on whether the males were inferred to be sons of workers of the current queen (WSC), sons of workers of the previous queen (WSP) or sons of either workers of the previous queen or of drifted workers (WSP/D)

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