Thelytokous Parthenogenesis in the Fungus-Gardening Ant *Mycocepurus smithii* (Hymenoptera: Formicidae)

Christian Rabeling¹, José Lino-Neto², Simone C. Cappellari¹, Iraçenir A. Dos-Santos², Ulrich G. Mueller¹, Mauricio Bacci, Jr.³

Introduction

Explaining the prevalence of sexual over asexual reproduction among organisms testifies to the evolutionary benefits of recombination, such as accelerated adaptation to changing environments and elimination of deleterious mutations. Documented instances of asexual reproduction in groups otherwise dominated by sexual reproduction challenge evolutionary biologists to understand the special circumstances that might confer an advantage to asexual reproductive strategies. Here we report one such instance of asexual reproduction in the ants. We present evidence for obligate thelytoky in the asexual fungus-gardening ant, *Mycocepurus smithii*, in which queens produce female offspring from unfertilized eggs, workers are sterile, and males appear to be completely absent. Obligate thelytoky is implicated by reproductive physiology of queens, lack of males, absence of mating behavior, and natural history observations. An obligate thelytoky hypothesis is further supported by the absence of evidence indicating sexual reproduction or genetic recombination across the species’ extensive distribution range (Mexico-Argentina). Potential conflicting evidence for sexual reproduction in this species derives from three *Mycocepurus* males reported in the literature, previously regarded as possible males of *M. smithii*. However, we show here that these specimens represent males of the congeneric species *M. obsoletus*, and not males of *M. smithii*. *Mycocepurus smithii* is unique among ants and among eusocial Hymenoptera, in that males seem to be completely absent and only queens (and not workers) produce diploid offspring via thelytoky. Because colonies consisting only of females can be propagated consecutively in the laboratory, *M. smithii* could be an adequate study organism a) to test hypotheses of the population-genetic advantages and disadvantages of asexual reproduction in a social organism and b) to inform kin conflict theory. For a Portuguese translation of the abstract, please see Abstract S1.

Abstract

The general prevalence of sexual reproduction over asexual reproduction among organisms testifies to the evolutionary benefits of recombination, such as accelerated adaptation to changing environments and elimination of deleterious mutations. Documented instances of asexual reproduction in groups otherwise dominated by sexual reproduction challenge evolutionary biologists to understand the special circumstances that might confer an advantage to asexual reproductive strategies. Here we report one such instance of asexual reproduction in the ants. We present evidence for obligate thelytoky in the asexual fungus-gardening ant, *Mycocepurus smithii*, in which queens produce female offspring from unfertilized eggs, workers are sterile, and males appear to be completely absent. Obligate thelytoky is implicated by reproductive physiology of queens, lack of males, absence of mating behavior, and natural history observations. An obligate thelytoky hypothesis is further supported by the absence of evidence indicating sexual reproduction or genetic recombination across the species’ extensive distribution range (Mexico-Argentina). Potential conflicting evidence for sexual reproduction in this species derives from three *Mycocepurus* males reported in the literature, previously regarded as possible males of *M. smithii*. However, we show here that these specimens represent males of the congeneric species *M. obsoletus*, and not males of *M. smithii*. *Mycocepurus smithii* is unique among ants and among eusocial Hymenoptera, in that males seem to be completely absent and only queens (and not workers) produce diploid offspring via thelytoky. Because colonies consisting only of females can be propagated consecutively in the laboratory, *M. smithii* could be an adequate study organism a) to test hypotheses of the population-genetic advantages and disadvantages of asexual reproduction in a social organism and b) to inform kin conflict theory. For a Portuguese translation of the abstract, please see Abstract S1.

**Citation:** Rabeling C, Lino-Neto J, Cappellari SC, Dos-Santos IA, Mueller UG, et al. (2009) Thelytokous Parthenogenesis in the Fungus-Gardening Ant *Mycocepurus smithii* (Hymenoptera: Formicidae). PLoS ONE 4(8): e6781. doi:10.1371/journal.pone.0006781

**Editor:** Corrie S. Moreau, Field Museum of Natural History, United States of America

**Received June 6, 2009; Accepted July 7, 2009; Published August 26, 2009**

**Copyright:** © 2009 Rabeling et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** Funding was provided by an Ernst Mayr Travel Grant in Animal Systematics (Museum of Comparative Zoology), the Green Fund (Harvard University), a NSF Doctoral Dissertation Improvement Grant (DEB-0808164), as well as research grants from the Section of Integrative Biology from the University of Texas at Austin to CR; a Dorothea Bennett Memorial Graduate Fellowship to SCC; the Fundação de Apoio à Pesquisa do Estado de São Paulo (2006/00185-7) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (476250/2008-0 and 310826/2006-3) to MB; the Conselho Nacional de Desenvolvimento Científico e Tecnológico to JLN and IAS; and the National Science Foundation (DEB-0639879) to UGM. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: rabeling@mail.utexas.edu

Asexuality is part of normal hymenopteran reproduction because males are produced via arrenotoky (males develop from unfertilized, haploid eggs) [13,16]. In contrast, females develop from fertilized, diploid eggs [17,18]. Thelytokous parthenogenesis, the production of diploid female offspring from unfertilized eggs, has been observed in a small number of eusocial hymenopteran species. So far, thelytoky has been convincingly demonstrated in the Cape honeybee, *Apis mellifera capensis* [19–21], and for seven distantly related species of ants (see Discussion).

*Mycocepurus smithii* is a common, but cryptic species of fungus-gardening ants, which is widely distributed throughout Latin America and obligately relies on a symbiotic fungus for food [22–24]. Recently, independent lines of evidence indicated that *M. smithii*, might be capable of thelytokous reproduction. First, the absence of males in three years of continuous fieldwork in Puerto Rico led Fernández-Marín et al. [25] to hypothesize that queens of *Mycocepurus smithii* might reproduce via thelytoky. A comparative
study of *Mycocepurus* populations in Brazil found that in contrast to *M. goeldii*, *M. smithii* nest entrances occurred in high densities and that nests seemed to be connected to each other, forming a colony network [26,27]. The connected nest systems of *M. smithii* suggested that colonies multiply by fission, potentially influenced by the reproductive biology of the species [26,27]. Additionally, Himler et al. [28] observed thelytoky of *M. smithii* in laboratory colonies from Panama. Males were not encountered in *M. smithii* nests during the *Mycocepurus* mating season in Peru, Brazil (Rabeling, pers. obs.), Puerto Rico [25] and Panama [28], even though sympatric, sexually reproducing *Mycocepurus* species engaged in nuptial flights, suggesting that *M. smithii* might be obligately asexual.

The existence of three unidentified *Mycocepurus* males collected by W. E. Kerr in Rio Claro, São Paulo State in Brazil [29] appears to challenge the hypothesis that *M. smithii* is obligately asexual. Until recently, only two *Mycocepurus* species were known from São Paulo State: *M. goeldii* and *M. smithii*. The males of *M. goeldii* had been previously described [30], and hence, by process of elimination, the males collected by Kerr were tentatively assigned to *M. smithii*. Interestingly, Kempf [22] described the morphology of these males in his taxonomic revision of the genus, but had the foresight to not name them, because the specimens were unassociated with a maternal colony, making it impossible to identify them with confidence. The ‘Kerr males’ therefore could belong to an undescribed species or to a described species not yet known from São Paulo State.

In order to test if thelytoky is widespread in queens of *M. smithii* and to investigate the behavioral ecological consequences of thelytoky in field populations, we studied the population biology, reproductive physiology of queens and workers, and the mating behavior of a *M. smithii* population in Brazil, and compared this population with two sexually reproducing, sympatric congeners. In addition, we provide taxonomic evidence for the identity of the ‘Kerr males’.

Materials and Methods
Population Biology of *Mycocepurus smithii*

Fieldwork was conducted from September 29th to October 26th 2008 on the Campus of São Paulo State University (UNESP) in Rio Claro, Brazil (22.3955°S, 047.5424°W; elevation 608 m). In the State of São Paulo, the nuptial flights of most attine ant species, including those of *Mycocepurus* [29], occur during late September and October, which mark the beginning of the rainy season. In order to maximize the chance of finding males in the nests of *M. smithii*, we focused collecting on the probable mating season.

Previous studies demonstrated that it might be difficult to recognize *M. smithii* nests as separate units because nest entrances may occur in high densities and lateral tunnels connect underground chambers [27]. We therefore selected a study site where nest entrances were spatially separated from other *M. smithii* populations and an entire nest aggregation could be studied through excavation, including all reproductively active and potentially inseminated queens. Once the target aggregation was identified, the study area was marked and the nest entrances mapped, resulting in a 3.5 m x 2 m study area with 10 nest entrances (Fig. 1). Throughout the excavation process, the study plot was frequently sprayed with water to increase the visibility of nest entrances (the ants open the nest entrances after the entrance hole has been closed by washed-in soil). These freshly excavated soil mounds are easier to recognize than older nest entrances, which sometimes erode over time during periods of drought. A pilot study conducted at the same site in 2006 demonstrated that

![Figure 1. Spatial distribution of nest entrances and fungus chambers of the studied *Mycocepurus smithii* population. Nest entrances are depicted as Xs, queenright chambers as solid black dots and all other fungus chambers as open black circles; size of circles does not represent the relative sizes of nest chambers. The x and y axes are labeled in cm. The nest excavation was continued 50 cm beyond the study plot limits in all four directions and no additional chambers were found. doi:10.1371/journal.pone.0006781.g001](http://www.plosone.org/article/doi/10.1371/journal.pone.0006781.g001)
recorded in a grid. The digging was continued 50 cm past the last excavated chamber.

All *Mycocepurus* specimens were stored in 95% ethanol for further examination and censusing. Voucher specimens of this study were deposited in the entomology collection of Maurício Bacci’s Molecular Evolution Laboratory at São Paulo State University (UNESP), the Museum of Comparative Zoology at Harvard University (MCZ), the Museu de Zoológia da USP (MZUSP), and in C. Rabeling’s collection.

To supplement the results of the *M. smithii* nest excavations and provide direct evidence for polydomy, different nest casting methods were tried, following methodologies described by Williams and Loﬁgren [31] and Tschinkel [32–34]. However, the tunnel diameter of *M. smithii* nests was as narrow as 1 mm, which did not permit plaster of Paris or molten zinc to trace the tunnels. Other casting techniques will be required in the future to illustrate that nests of *M. smithii* are connected via tunnels.

Reproductive Biology of *Mycocepurus* queens

All *Mycocepurus* specimens were collected in 95% ethanol and dissected in 0.2 M phosphate buffer. Female reproductive tracts were examined using an Olympus SZ40 dissecting scope and an Olympus CX31 microscope. Photographs of the female reproductive tracts were taken with a Nikon D200 SLR digital camera mounted on an Olympus SZH10 dissecting scope or an Olympus CX31 microscope. To test if queens were inseminated, the spermathecae were dissected, opened and examined under a microscope, to ensure that the content were sperm and no other kind of proteinaceous secretion. The developmental status of the ovaries was recorded as undeveloped (0), developing but without fully developed oocytes (1) and fully developed ovaries with mature oocytes present (2) (Table 1). The presence of yellow bodies (corpora lutea) at the base of the ovaries was also recorded. Yellow bodies are remnants of follicular cells and indicate if the respective female oviposited in the past. In case of a parthenogenetic, reproducitively active female, one would expect an empty spermatheca, fully developed ovaries with mature oocytes, and corpora lutea as an indicator of oviposition activity in the past. Reproductively inactive females could have both empty and sperm-filled spermathecae, depending on insemination status, but their ovaries are expected to be undeveloped with no yellow bodies. In contrast, a sexually reproducing female would have a sperm filled spermatheca, developed ovaries, and yellow bodies. To test if queens were the only reproducitively active individuals in the colonies a total of 50 workers were dissected. Five workers were randomly chosen from five queennigh (chambers 4, 30, 37, 45 & 59; see Table S1) and five queenless chambers (chambers 13, 21, 42, 47 & 50; see Table S1) and dissected following the methodology described above.

Behavioral observations of nuptial flights

Under thelytoky and in the absence of males, the mating behavior is expected to degenerate, including the behavioral preparations of workers when modifying nest entrances for mating flights. Preliminary behavioral observations in previous years showed that the sexually reproducing *Mycocepurus goeldii* modifies its nest mounds in preparation of the nuptial flights of the alates (see results for details). These modified nest entrances serve as a proxy if a particular colony is preparing for a nuptial flight. Between September 29th and October 15th the nest entrances of ten *M. goeldii* colonies were monitored three times per day and compared to the ten entrances of *M. smithii* in the study plot.

Taxonomic identity of the ‘Kerr males’

For most *Mycocepurus* species only the worker caste is described. To find males that match the ‘Kerr males’, special emphasis was given to collecting workers, queens, and males from any *Mycocepurus* colonies. *Mycocepurus obsoletus* is one of the species known only from workers and we collected entire colonies in a Cerrado habitat at the margin of a gallery forest at the Reserva Ecológica do IBGE, 35 km south of Brasília, in the Federal District of Brazil (15.9436°S, 047.8767°W; elevation 1087 m). To collect reproductives, the entrances of several nests were flagged during the dry season in July 2008 and on November 12th 2008, with the first rains of the season, alate *Mycocepurus* males (n = 19) and queens (n = 15) were collected next to the previously marked nests. The external morphology of the males was compared to the ‘Kerr males’ under a Leica Z6 APO dissecting scope.

Additionally, these males were identified to species using molecular sequence information to ensure the males from Brasília are in fact *Mycocepurus obsoletus* males. The partial DNA of three single-copy nuclear markers (Elongation Factor 1-alpha F1 copy, Wingless and Long Wavelength Rhodopsin) and one mitochondrial gene (Cytochrome Oxidase I) were sequenced, resulting in 3512 base pairs, and compared to sequence information from *M. obsoletus* workers collected at the same site. The resulting molecular phylogeny will be presented in the context of an ongoing generic revision (C. Rabeling in prep.).

Results

Population Biology of *Mycocepurus smithii*

A total of ten *M. smithii* nest entrances were identified in the 7 m² study plot (1.43 entrances/m²) (Fig. 1). The excavation of this population revealed a total of 59 fungus chambers (see Table S1 for

| species                | n   | spermatheca     | ovary development (*) | corpora lutea |
|------------------------|-----|-----------------|-----------------------|--------------|
| *M. smithii* (dealate) | 12  | translucent/empty | 2                     | present      |
| *M. goeldii* (dealate) | 2   | opaque w/sperm   | 2                     | present      |
| *M. goeldii* (alate)  | 5   | translucent/empty | 0                     | absent       |
| *M. sp. nov.* (dealate)| 15  | opaque w/sperm   | 1 (n = 12); 2 (n = 3) | absent (n = 12); present (n = 3) |
| *M. sp. nov.* (alate) | 2   | translucent/empty | 0                     | absent       |

(*) The state of ovary development was divided in three categories: 0 = not developed, 1 = developing but without fully developed eggs, 2 = well developed with mature eggs present. In nests of *M. sp. nov.* more than one queen was found in a single chamber. Among those queens some were reproductively active, while others were inseminated, but the ovaries were still developing. The presence of corpora lutea indicates if a female oviposited.

doi:10.1371/journal.pone.0006781.t001
exact chamber contents). The entire population consisted of 851 workers and 12 queens, averaging approximately 14 workers per chamber and one queen in every 5th fungus chamber (see Table S1). The maximum number of workers per chamber was 43, the minimum one. All queenright chambers contained only a single reproductively active queen, however, the number of queens encountered was higher than the number of nest entrances in the study area. The brood was distributed in 21 (36%) chambers and consisted of 115 early instar larvae, 40 worker pupae, 51 queen larvae and 274 queen pupae. The sexual brood was restricted to ten chambers (17%) and in eight of them worker brood and sexual brood co-occurred. The maximum and minimum number of sexual brood per chamber was 80 and one, respectively. No male pupae brood co-occurred. The maximum and minimum number of sexual chambers (17%) and in eight of them worker brood and sexual consisted of 115 early instar larvae, 40 worker pupae, 51 queen encountered was higher than the number of nest entrances in the minimum one. All queenright chambers contained only a single reproductive activity of these females. Five alate, virgin queens (n = 2), which had empty spermathecae, undeveloped ovaries and absent corpora lutea.

The examined queens of *M. goeldii* and *M. sp. nov.* confirmed the predictions for sexually reproducing species: virgin, non inseminated queens had undeveloped ovaries and the absence of corpora lutea indicated that the respective female never oviposited. In contrast, the reproductively active females had sperm-filled spermathecae, developed ovaries and the presence of corpora lutea indicated oviposition. Some dealate queens, which were not reproductively active (indicated by the absence of corpora lutea), were also inseminated and had developing ovaries. None of the 50 examined *M. smithii* workers was reproductively active. Each worker had a pair of ovaries with a single ovariole per ovary. Ovarioles did not contain mature oocytes and yellow bodies were absent. All workers were lacking a spermatheca.

**Behavioral observations of nuptial flights**

Nest observations started on September 29th in Rio Claro, before the onset of the rainy season. At that time, nest entrances of *M. goeldii* and *M. smithii* consisted of loose, irregularly shaped soil mounds of 5–20 cm diameter, each with a single entrance. Rains started on October 2nd, stimulating *M. goeldii* workers to increase the number of nest entrances to about 20–30 entrances per nest mound. Consequently, the soil mound surrounding the entrances had a sponge like appearance. Several *M. goeldii* workers waving their antennae surrounded each entrance hole. Upon modification of the mound, males of *M. goeldii* could be observed moving in the passages of the sponge like nest mound, although the males did not surface. Queens were not observed. On October 3rd, cooler weather and rain showers caused temperatures to drop by 5–10°C. During this period, the nest entrances of *M. goeldii* stayed open or were reopened after rain had washed soil into the entrance tunnels. The workers of *M. goeldii* were still surrounding the entrance holes and alate males and queens could be seen in the nest mound tunnels. On the morning of October 7th, the temperature had increased to about 25°C, and the winds and the rain had stopped. At approximately 10.00 h, the males of *M. goeldii* colonies started the nuptial flight. First resting on the rim of each entrance crater, then spreading the wings and flying up vertically, perhaps gathering in the lower branches of a tree, as observed by Kerr [29]. The alate queens started flying slightly later at 11.30 h. The release of alates from each colony continued until 15.00 h. During the rest of the afternoon, workers closed the supernumerary nest entrances, until each colony had only a single entrance.

During this same time period, and until the end of the study three weeks later, the entrances of *M. smithii* nests remained unmodified, with some soil surrounding a single entrance hole. In addition, neither mating flights, nor modified nest mounds, nor males were ever observed throughout several years of field research in Latin America (Rabeling, field observations 2003–2008).

**Taxonomic identity of the ‘Kerr males’**

On November 12th, 19 males and 13 queens were collected when exiting the marked colonies of *Mycocepurus obsoletus*. The previous day, the first heavy rainstorm of the season soaked the soil...
for approximately 24 hours and stopped at 10.00 h of November 12th. Two hours later (12.00 h) alate males and queens were observed to lift off from the leaf litter for 10–50 cm before landing again in the litter. The nuptial flight lasted for approximately two hours from noon to 14.00 h and was interrupted by a brief rain shower; after the rain shower (15.00 h), no activity could be detected at the marked nests. It is unclear whether alates flew into the surrounding vegetation and formed mating aggregations, as has been reported for *M. goeldii* [29], or mated in the leaf litter.

The morphological comparison of the alate males to the males collected by Kerr in 1960 revealed that males from both collections, Brasília and Rio Claro, are almost identical (Fig. 3). Identifying characters for *Mycocepurus* males include the shape of the propodeal spine, the petiole and the wing venation (Fig. 3). Given the morphological variability observed within and between all species of *Mycocepurus* (Rabeling in prep), the ‘Kerr males’ appear to be specimens of *M. obsoletus*. A comparison of the male sequences to sequences of *M. obsoletus* workers from the same locality resulted in 100% sequence identity, confirming the identity of those males. Consequently, the ‘Kerr males’ are the males of *M. obsoletus* and not the males of *M. smithii*.

**Discussion**

Several lines of direct evidence (queen reproductive physiology) and indirect evidence (taxonomy & natural history) implicate obligate thelytokous parthenogenesis for a Brazilian population of the fungus-gardening ant species *M. smithii*. The presence of mature oocytes and yellow bodies indicate that dissected queens had oviposited and were reproductively active, whereas the empty spermathecae prove that queens were not inseminated. In combination, these characteristics indicate thelytokous partheno-
genetic propagation and support Fernández-Marín’s [25] hypothesis of asexuality in *M. smithii*, formulated to explain the absence of males in a Puerto Rican *M. smithii* population.

Three males from Rio Claro in Brazil [22,29] represented a challenge to the obligate asexuality hypothesis, because they were suspected to be males of *M. smithii*, indicating the potential for sex and recombination in this species. Our morphological study, however, indicates that the ‘Kerr males’ are the previously unknown males of *M. obsoletus*, not males of *M. smithii*. Previously, *M. obsoletus* was not known to occur in Rio Claro, but we now know that *M. obsoletus* has a Brazilian Cerrado distribution (Fig. 4), thus its occurrence in the Rio Claro area is certainly possible. In addition, *M. obsoletus* workers have been collected at two other relatively nearby localities in São Paulo State (Fig. 4; see also [35]), further increasing the likelihood of its occurrence at or near Rio Claro. Thus the ‘Kerr males’ seem to represent only the southernmost presently known distribution record of *M. obsoletus* (Fig. 4).

Matching the ‘Kerr males’ to *M. obsoletus* provides only circumstantial evidence for obligate thletoky, but it does remove the strongest potential counter evidence, i.e.: that the ‘Kerr males’ are actual males of *M. smithii*. While we cannot rule out the possibility that *M. obsoletus* and *M. smithii* males are morphologically identical; this is most unlikely, however, given the observed morphological variation within and between *Mycocepurus* species (Rabeling, pers. obs.).

Within eusocial Hymenoptera, arrhenotokous parthenogenesis is a widely distributed phenomenon [15,36] and the reproductive modes in ants are particularly diverse [37]. In contrast, thletoky parthenogenesis, the production of female offspring from unfertilized eggs, has been rarely confirmed in eusocial Hymenoptera, although the number of recognized thletoky species is increasing. So far, thletoky has been demonstrated convincingly for the South African honey bee subspecies *Apis mellifera capensis* [19–21] and seven ant species, which can be divided into three categories of asexual reproduction: queens produce queens and workers via thletoky, but workers are sterile and males seem to be absent (called here: type A thletoky); workers produce workers and potentially queens via thletoky (type B); or queens produce...
queens thelytokously and workers sexually, and workers produce queens thelytokously \( (\text{type C}) \). Males are known for all type B and type C asexual ant lineages, suggesting the potential for sex and recombination, but males remain unknown for type A asexuals, of which \( M.\ smithii \) is the first known example in the Formicidae.

Among asexual ant species, worker thelytoky \( (\text{type B thelytoky}) \) occurs in four distantly related ant species, the cerapachyine ant \( Cerapachys birou \) [38–40], the two myrmicine ants \( Messor capitatus \) [41] and \( Pristomyrmex punctatus \) [42–48], and the ponerine ant \( Platythyrea punctata \) [49–52]. The thelytokous production of queens by queens and sexual production of workers by queens \( (\text{type C}) \) has so far been documented in the formicine ant \( Cataglyphis cursor \) [53–56], and the two myrmicines \( Wasmannia auropunctata \) [57–59] and \( Vollheinovia emeryi \) [60–61].

At present, \( Mycocepurus obsoletus \) seems to be the only representative of \( \text{type A} \) asexual ant species. Natural history studies in Puerto Rico [25] and the Brazilian Amazon [26,27] suggested an unusual reproductive biology for \( M.\ smithii \). Fernández-Marín et al. [25] first proposed the hypothesis that \( M.\ smithii \) might reproduce thelytokously. This study shows that \( M.\ smithii \) queens from the Rio Claro, Brazil population are thelytokous. Himler et al. [28] dissected six queens from a Panamanian population, reaching the same conclusion. Based on these three geographic data points, Brazil, Panama, both confirmed, and Puerto Rico, still unconfirmed, it seems that thelytoky is widespread across \( M.\ smithii \)’s distribution range.

Firm evidence capable of identifying the cytological mechanism of thelytoky \( (\text{i.e.}, \text{apomixis versus automixis}) \) in \( M.\ smithii \) is currently lacking. A comprehensive genetic population study in combination with cytological evidence is necessary to distinguish between meiotic and mitotic parthenogenesis. Himler et al. [28] tested queens and workers of 12 Panamanian colonies for allelic variation at a single variable genetic locus and found queens and offspring to be genetically identical, concluding that \( M.\ smithii \) propagates clonally. However, during automixis with central fusion, two haploid nuclei are produced during the first meiotic division, which subsequently fuse to form the diploid zygote. Gene recombination does not necessarily occur and thus, the genetic signature of apomictically and automictically produced offspring can be very similar if not identical [15,56,62,63]. A single genetic locus is hence insufficient to distinguish between mitotic and meiotic parthenogenesis.

\( Wolbachia \) bacteria induce parthenogenetic reproduction in some parasitoid wasps [64]. Several hymenopteran species have been tested for \( Wolbachia \) infections, but \( Wolbachia \) could never be demonstrated to induce parthenogenesis in any thelytokous eusocial Hymenoptera species [28,41,52,65]. This supports the hypothesis that a sex determining system based on heterozygosity may form a limitation to \( Wolbachia \)-induced parthenogenesis [65]. \( Wolbachia \) endosymbionts induce the production of homozygous diploid offspring through gamete duplication [66,67], which results in the exclusive production of diploid males. In ants and eusocial bees, females are required to be heterozygous at the sex-determining locus, a condition difficult to achieve when parasitized by a gamete duplicating endosymbiont [21,65]. Because thelytoky seems to have evolved independently of \( Wolbachia \) in ants and bees, it also seems unlikely that thelytoky of \( M.\ smithii \) queens was induced by \( Wolbachia \). The absence of \( Wolbachia \) in \( M.\ smithii \) supports this expectation and additional antibiotic treatment could not ‘cure’ \( M.\ smithii \) from parthenogenesis [28]. The wide geographic distribution could suggest an ancient origin of asexuality in \( M.\ smithii \). Low variability in DNA sequence, however, does not support an ancient asexuality hypothesis, but rather argues for a recent origin of thelytoky followed by a rapid population expansion (Rabeling, unpublished). The widespread geographic distribution also suggests that geographic parthenogenesis, the occurrence of parthenogenetically reproducing lineages towards the margin of a distribution range [68,69], unlikely applies to \( M.\ smithii \), because both São Paulo State and Panama are located well within the continuous distribution range, which extends from Northern Mexico to Argentina [22,23; Rabeling, in prep].

Theory predicts that morphological structures degenerate if not maintained by natural selection. Panamanian \( M.\ smithii \) queens apparently lack the so-called mussel organ, and Himler et al. [28] therefore argued for degenerate mating apparatus, which is thought to support an ancient origin of asexuality in \( M.\ smithii \). The mussel organ is a sclerotized structure in the reproductive tract of leaf cutter ant queens, which is thought to protect the female genitalia tissue from damage inflicted by sclerotized hooks of the male’s penis [70]. In an obligately asexual species both the spermatheca and the mussel organ are expected to lose their function. Given that soft tissues like the spermatheca were present and not reduced in size in \( M.\ smithii \) queens when compared to two

---

**Figure 4. Geographic distribution of *Mycocepurus obsoletus*.** Thick black lines depict the boundaries of Brazilian States, open circles represent collections based on worker specimens, the solid black dot depicts the collection site of the ‘Kerr males’: Rio Claro in São Paulo State. From north to south, *M. obsoletus* has been recorded in the States of Pará, Tocantins, Goiás, the Federal District, Minas Gerais and São Paulo. Besides Rio Claro, *M. obsoletus* has been collected in two other localities in São Paulo State: a) Reserva Ecológica de Jataí near Luis Antônio [23–25.V.1997, Silvestre & Silva col.; see [35], which is located approximately 100 km north of Rio Claro, and b) Ibitinga [25.I.1964, K. Lenko col.], which is approximately 150 km northwest of Rio Claro. doi:10.1371/journal.pone.0006781.g004
sexual congener, it seems premature to argue for ancient asexuality based on the absence (or non-detection) of the mussel organ. Ideally, a comparative functional-morphological study should first establish a) whether the mussel organ is present in closely related, sexually reproducing *Mycopsis* species, because *Mycopsis* and *Alto* shared the most recent common ancestor some 50 Million years ago and are therefore only distantly related to each other [71]; b) whether the mussel organ in *M. smithii* is homologous to the respective structure in *A. colombica* for which this organ was first described [70]; and c) to what degree sclerotization varies with age, because weakly sclerotized structures are often translucent in recently eclosed arthropods.

High relatedness is expected in colonies of *M. smithii* whether queens reproduce via apomorphic or automic phyloxy. Kin conflict theory predicts conflict between individual kin or groups of kin (i.e., castes) within a colony, because individuals or castes differ in relatedness, reproductive success or both [72]. In a society of highly related or even clonal organisms kin conflict over reproduction is expected to be reduced if not entirely absent. However, Hamilton's rule [73,74] does not account for low frequency mutations with strong selective effects and therefore, relatedness alone is not always a good predictor of kin conflict over reproduction [75,76]. Once the genetic underpinnings of reproduction in *M. smithii* societies are understood, this ant should be an interesting study organism for kin selection theory.

Behaviors specific to sexual reproduction might degenerate rapidly in parthenogenetic lineages [77]. In *M. smithii*, modifications of the nest related to nuptial flight activity were never observed during six field-seasons (2003–2008), even though alate queens were present in *M. smithii* nests. At the same time sympatric sexual species showed modified nest structures and performed nuptial flights. The observation that *M. smithii* queens were still in the pupal stage whereas sexually reproducing species performed nuptial flights suggests that *M. smithii* queens may be produced over a wider time interval than in sympatric, sexual congener. In the absence of males, it is unnecessary for *M. smithii* queens to depart in a synchronized population wide nuptial flight. Consequently, it appears unnecessary to produce young queens in synchrony and to open the nest entrance for mating flights, which could possibly increase the risk of predation and parasitism.

Alternative to a nuptial flight, young queens could establish themselves in a new chamber that is part of the maternal colony. Such colony expansion would be consistent with the observation that multiple reproductively active *M. smithii* queens inhabit the same colony [25,27]. However, the ability of independent nest foundation seems to persist in *M. smithii*, as infrequently alate *M. smithii* queens can be found in the leaf litter (Rabeling, pers. obs.) and Fernández-Marín et al. [25,78] observed clausal nest founding of single dealate queens in Puerto Rican *M. smithii*.

Asexual lineages are capable of colonizing new habitats and exploiting new food sources more efficiently than sexually reproducing relatives [2,4,5]. Interestingly, *M. smithii* relies on multiple, genetically distinct lineages of fungi for food [26,79,80]. Surveys of fungal diversity cultivated by attine ants suggested that multiple, genetically distinct lineages or species in 74 *T. septentrionalis* [81]. However, this trend does not necessarily apply to all species, as was demonstrated by a comprehensive survey of microbial diversity associated with the North American attine species, *Trachymyrmex septentrionalis* [82]. This study identified four genetically distinct fungal lineages or species in 74 *T. septentrionalis* nests. For *M. smithii*, fewer samples were screened (n = 20), but these few samples already revealed twice the number of genetically distinct cultivars, which moreover spanned almost the entire diversity of lower attine cultivars [26,79]. No firm conclusion can be drawn from these preliminary results, but the observed pattern is consistent with the hypothesis that asexuals exploit new food sources effectively and colonize new habitats rapidly. A similar effect can be observed in cyclical parthenogenetic animals like water fleas (Daphnia), aphids, flies or some gall wasps living in seasonal environments: during the summer, food sources are abundant and reproduction switches to parthenogenesis, whereas later in the season, when resources become scarce, populations adopt sexual reproduction [4, pp. 57–62]. Because *M. smithii* inhabits both tropical and subtropical habitats, the need for sex to persist in seasonal environments may not be pressing, but a comprehensive comparison of *Mycopsis* cultivars grown by asexual and sexual hosts will have to test this hypothesis more thoroughly.

Several naturalists have discussed the possibility that *M. smithii* colonies are polydomous, i.e., single colonies occupy more than one nest [22,25,27,83,84]. Conflicting reports stem from frequent observations of the patchy distribution and high nest densities characteristic of *M. smithii* populations. Small areas often contain multiple nest entrances, as much as six entrances per square meter [26], whereas adjacent areas may have none at all. So far, only two studies have investigated the nest architecture systematically. Fernández-Marín et al. [25] concluded that *M. smithii* colonies had a single nest entrance in Puerto Rico, whereas Rabeling et al. [27] found that in the Brazilian Amazon large nest systems were interconnected through lateral tunnels, potentially enabling the exchange of workers and brood, which would argue for polydomy. In the Rio Claro *M. smithii* population, some nest chamber clustered underneath 2 or 3 spatially separated nest entrances (6 chambers below entrances #5 and #6 and 12 chambers below entrances #8, #9 and #10; Fig. 1) and cluster contained only a single reproductively active queen. In addition, a queenright chamber was located in equal distance to four nest entrances (#4, #5/6 and #7; Fig. 1) and this chamber had no detectable entrance above it. These patterns suggest that nests are connected underground and workers move freely between chambers, sharing the same entrances, further supporting polydomy. The alternative hypothesis could be that additional, potentially inseminated queens of the population escaped during excavation and that some entrances remained inactive and thus remained undetected during the study period of a month. Because of high levels of relatedness are expected for a strictly thelytokous species, it might be that *M. smithii* might not only be polydomous but also uniclonial.

Eidmann [83] first hypothesized that *M. smithii* colonies were polygynous, because “in every of the numerous examined fungus chambers” he found “several old, dealate queens” (p. 86). Unfortunately, Eidmann never tested his hypothesis by dissecting the queens. Fernández-Marín et al. [25] further substantiated Eidmann’s hypothesis, suggesting that *M. smithii* colonies might be secondarily polygynous, because pleometrosis, the foundation of colonies by multiple queens, does occur in some colonies. Dissections confirmed that multiple queens from the same nest were reproductively active, as indicated by the presence of developed ovaries and yellow bodies. However, it remains unclear if the dissected queens were found in the same fungus chamber or if they could have been reproductive females from different colonies. In addition, Amazonian *M. smithii* colonies had several dealate queens in the same fungus chamber, but their reproductive status was not confirmed through dissections [27].

In the Rio Claro population, only a single queen was found per fungus chamber (n = 10). The presence of several queenright
chambers in one fungus chamber cluster below a single entrance suggests that one polydomous colony can contain multiple reproductively active queens, supporting functional polygyny. Other clusters of fungus chamber, however, inhabited only a single reproductively active queen. In case these clusters are not part of the same polydomous nest, the Rio Claro M. smithii population would comprise a mosaic of monogynous and polygyneous colonies, which would be consistent with Fernández-Marín et al.’s [25] hypothesis of secondary polygyny. Again, a genetic population study will have to test if queens and worker offspring share the same genetic signature or if multiple mothers produced the workers of a single colony.

Conclusions

Three types of thelytokous reproduction have been documented in eight distantly related ant species. Mycocepurus smithii appears to be obligately asexual based on its reproductive physiology, and the absence of males and mating behavior. A population genetic study of M. smithii should show if this fungus-gardening ant species relies exclusively on thelytoky or if cryptic sex does occur. Wolbachia endosymbionts seem unlikely as parthenogenesis-inducing agent in eusocial Hymenoptera in general. Ancient asexuality might be suggested by the wide geographic distribution, but rendered unlikely by presence of fully developed soft tissue (i.e., spermatheca) in the mating apparatus of M. smithii queens and low genetic variability between populations. For all thelytokous ant species we lack the kind of evolutionary ecology framework that might indicate how long asexuality has persisted in the respective species and what factors might have influenced the rise of thelytokous populations from a sexually reproducing ancestor. The fact that M. smithii cultivates multiple lineages of fungi is consistent with the hypothesis that asexual efficiency colonizers of novel habitats and food sources. Due to the expected high relatedness within M. smithii colonies this species is likely to inform kin conflict theory. Careful natural history observations and behavioral experiments coupled with population genetic and phylogenetic studies will certainly answer some of these questions and further increase the already broad spectrum of reproductive diversity in eusocial Hymenoptera and beyond.

Supporting Information

Abstract S1

Found at: doi:10.1371/journal.pone.0006781.s001 (0.03 MB DOC)

Table S1

Found at: doi:10.1371/journal.pone.0006781.s002 (0.16 MB DOC)

Acknowledgments

We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) for permission to conduct fieldwork in Brazil. We are grateful to Beto Brandão (MZSP), Stefan Cover (MCZ), Ted Schultz (NINH) and Heraldo Vasconcelos (UFU) for contributing specimens to our study. We also thank the staff of the Reserva Ecológica do IBGE for support during fieldwork. We would like to thank Natalia Biani, Stefan Cover, Barrett Klein, Susan Mikheyev, Jon Seal, Kazuki Tsuji, and Phil Ward for commenting on earlier versions of this manuscript, and four anonymous reviewers for constructive criticism. This study is a chapter of Christian Rabeling’s doctoral dissertation at the University of Texas at Austin.

Author Contributions

Conceived and designed the experiments: CR. Performed the experiments: CR, JLN, IADS. Analyzed the data: CR. Contributed reagents/materials/analysis tools: CR, JLN, SCC, UGM, MBJ. Wrote the paper: CR. Performed fieldwork: CR. SCC. Critically revised the manuscript: CR, JLN, SCC, IADS. Contributed analysis tools: CR, JLN, SCC, UGM, MBJ. Performed the experiments: CR, JLN, IADS. Contributed reagents/materials/analysis tools: CR, JLN, SCC, UGM, MBJ.

References

1. Agrawal AF (2006) Evolution of Sex: Why Do Organisms Shuffle Their Genotypes? Current Biology 16: R696-R704.
2. Bell G (1982) The Masterpiece of Nature: The Evolution and Genetics of Sexuality. University of California Press: 635 p.
3. Barton NH (1996) The geometry of adaptation. Nature 395: 751-752.
4. Maynard Smith J (1978) The evolution of sex. Cambridge University Press: 236 p.
5. Williams GC (1975) Sex and evolution. Princeton University Press: 210 p.
6. Barton NH, Charlesworth B (1998) Why Sex and Recombination? Science 281: 1896-1900.
7. Kundrathov AS (1993) Classification of hypotheses on the advantage of amphimixis. Heredity 74: 372-387.
8. West SA, Lively CM, Read AF (1999) A pluralist approach to sex and recombination. Journal of Evolutionary Biology 12: 1003-1012.
9. Hamilton WD (1980) Sex vs. non-sex vs. parasite. Oikos 33: 292-296.
10. Hamilton WD, Axelrod R, Tanese R (1980) Sexual reproduction as an adaptation to resistant parasites. Proceedings of the National Academy of Sciences USA 77: 3566-3567.
11. Muller HJ (1964) The Relation of Recombination to Mutational Advance. Mutation Research 106: 2-9.
12. Kundrathov AS (1982) Selection against harmful mutations in large sexual and asexual populations. Genetical Research 40: 325-332.
13. Birky WC (2004) Bellowid rotifers revisited. Proceedings of the National Academy of Sciences USA 101: 2651-2652.
14. Meselson M, Mark Welch D (2007) Stable Heterozygosity? Science 318: 202-203.
15. Crozier RH (1975) Hymenoptera. In: John B, ed. Animal Cytogenetics 3: Insecta (7), Gruberer Bontrager, Berlin, pp 1-95.
16. Cook JM, Crozier RH (1995) Sex determination and population biology in the Hymenoptera. Trends in Ecology and Evolution 10: 281–286.
17. Cook JM (1993) Sex determination in the Hymenoptera: a review of models and evidence. Heredity 71: 421–435.
50. Heinze J, Hölldobler B (1995) Thelytokous parthenogenesis and dominance

52. Schilder K, Heinze J, Gross R, Hölldobler B (1999b) Microsatellites reveal clonal

49. Hartmann A, Wantia J, Heinze J (2005) Facultative sexual reproduction in the

48. Tsuji K (1995) Reproductive Conflicts and Levels of Selection in the Ant

45. Sasaki T, Tsuji K (2003) Behavioral property of unusual large workers in the ant

43. Hasegawa E, Sanada S, Satoh T, Obara Y (2001) Microsatellite loci and genetic

41. Grasso DAT, Wenseleers T, Mori A, Le Moli F, Billen J (2000) Thelytokous

42. Dobata S, Sasaki T, Mori H, Hasegawa E, Shimada M, Tsuji K (2009) Cheater

39. Ravary F, Jaisson P (2004) Absence of individual sterility in thelytokous colonies of the ant Pristomyrmex punctatus. Entomological Science 7: 247–255.

37. Keller L (2007) Uncovering the biodiversity of genetic and reproductive systems. Theoretical Biology 7: 1–16.

35. Silvestre R, Silva RR (2001) Guilds of ants from the Estação Ecológica Jatía, of the ant Cerapachys biroi. Insectes Sociaux 49: 147–159.

32. Tschinkel WR (2003) Subterranean ant nests: trace fossils past and future? Palaeogeography, Palaeoclimatology, Palaeoecology 192: 321–333.

31. Williams DF, Lodge CS (1988) Nest casting of some grounddwelling Florida ant species using dental labstone. In: Trager JC, ed. Advances in Myrmecology, Brill Academic Publishers. pp 433–443.

30. Ford A (1908) Ameisen aus São Paulo (Brasilien), Paraguay etc. gesammelt von Prof. Herrn v. Beiring, Dr. Lutz, Dr. Fiebrig, etc. Verh. K.K. Zool. Bot. Ges. Wien 58: 340–418.

29. Tscharntke T (1999) The nest architecture of the Florida harvester ant, Pogonomyrmex badius. Journal of Insect Science 4: 21. Available: www.insectscience.org/4.21. Accessed 2009 June 9.

28. Tscharntke WR (2003) The nest architecture of the ant Camponotus socius. Journal of Insect Science 3: 5. Available: www.insectscience.org/3.5. Accessed 2009 June 9.

27. Silvestre R, Silva RR (2001) Guia de formigas da Estação Ecológica Jatía, Luís Antônio-SP, sugestões para a aplicação do modelo de gilhas como biomarcadores ambientais. Biotemas 14: 37–69.

26. Bourke AFG (1988) Worker reproduction in the higher eusocial Hymenoptera. The Quarterly Review of Biology 63: 291–311.

25. Keller L (2007) Uncovering the biodiversity of genetic and reproductive systems: Time for a more open approach. American Society of Naturalists E. O. Wilson Award Winner Address. American Naturalist 169: 1–9.

24. Ravary F, Jaisson P (2002) The reproductive cycle of thelytokous colonies of the ant Pogonomyrmex badius (Forel, Formicaceae). Insectes Sociaux 49: 61–67.

23. Ravary F, Jaisson P (2004) Absence of individual sterility in thelytokous colonies of the ant Pogonomyrmex badius (Forel, Formicaceae). Insectes Sociaux 51: 61–73.

22. Tsuji K, Yamauchi K (1995) Production of females by parthenogenesis in the ant Cerapachys biroi. Insectes Sociaux 42: 333–336.

21. Grasso DAT, Wenseleers T, Mori A, Le Moli F, Billen J (2000) Thelytokous worker reproduction and lack of Wolbachia infection in the harvesting ant Messor capitatus. Ethology, Ecology and Evolution 12: 309–314.

20. Dobata S, Sasaki T, Mori H, Hasegawa E, Shimada M, Tsuji K (2009) Cheater genotypes in the parthenogenetic ant Pristomyrmex punctatus. Proceedings of the Royal Society London Series B 276: 567–74.

19. Hasegawa E, Sanada S, Satoh T, Obara Y (2001) Microsatellite loci and genetic polymorphism among colony members in the parthenogenetic ant Pristomyrmex punctatus. Entomological Science 4: 399–402.

18. Iwas T, Kobayashi K, Kubota M, Ogata K, Imai HT, Crossley RH (1984) The reproductive cycle of the queenless ant Pristomyrmex punctatus. Insectes Sociaux 31: 87–102.

17. Sasaki T, Tsuji K (2005) Behavioral property of unusual large workers in the ant Pristomyrmex punctatus. Journal of Ethology 21: 145–151.

16. Tsuji K (1988) Obligate parthenogenesis and reproductive decision of labor in the Japanese queenless ant Pristomyrmex punctatus. Behavior, Ecology and Sociobiology 23: 247–255.

15. Tsuji K (1990) Reproductive division of labour related to age in the Japanese queenless ant Pristomyrmex punctatus. Animal Behavior 39: 843–849.

14. Tsuji K (1995) Reproductive Conflicts and Levels of Selection in the Ant Pristomyrmex punctatus. Contextual Analysis and Partitioning of Covariance. American Naturalist 146: 586–608.

13. Hartmann A, Wantia J, Heinze J (2005) Facultative sexual reproduction in the parthenogenetic ant Platygryra punctata. Insectes Sociaux 52: 155–162.

12. Heinze J, Hölldobler B (1995) Thelytokous parthenogenesis and dominance hierarchies in the ponerine ant, Platygryra punctata. Naturwissenschaften 82: 40–41.

11. Schilder K, Heinze J, Hölldobler B (1999a) Colony structure and reproduction in the thelytokous parthenogenetic ant Platygryra punctata (F. Smith) (Hymenoptera, Formicidae). Insectes Sociaux 46: 150–158.

10. Schilder K, Heinze J, Gross R, Hölldobler B (1999b) Microsatellites reveal clonal structure of populations of the thelytokous ant Platygryra punctata (F. Smith) (Hymenoptera, Formicidae). Molecular Ecology 8: 1497–1507.

9. Cagniant H (1973) Apparition d’ouvriers a partir d’œufs pondus par des ouvrières chez la fourmi Cataglyphis cursor Fonscolomb (Hymenoptera: Formicidae). Comptes Rendus de l’Académie des Sciences Paris 277: 2197–2198.

8. Cagniant H (1979) La parthenogenèse thylétique et archénotique chez la fourmi Cataglyphis cursor Fonsc. (Hym. Form.). Cycle biologique en élevage des colonies avec reine et des colonies sans reine. Insectes Sociaux 26: 51–60.

7. Peary M, Aron S, Doums C, Keller L (2004) Conditional use of sex and parthenogenesis in a worker-dominated queen production in ants. Science 306: 1780–1783.

6. Peary M, Hardy O, Aron S (2006) Thelytokous parthenogenesis and its consequences on inbreeding in an ant. Heredity 96: 377–382.

5. Foucaud J, Jourdan H, Le Breton J, Loiseau A, Konghoudoux D, Estoup A (2006) Rare sexual reproduction events in the clonal reproduction system of introduced populations of the little fire ant. Evolution 60: 1646–1657.

4. Foucaud J, Fournier D, Orivel J, Delahie JHC, Loiseau A, et al. (2007) Sex and Clonality in the Little Fire Ant. Molecular Biology and Evolution 24: 2465–2473.

3. Fournier D, Estoup A, Orivel J, Foucaud J, Jourdan H, et al. (2005) Clonal reproduction by males and females in the little fire ant. Nature 435: 1230–1234.

2. Kobayashi K, Hasegawa E, Ohkawa K (2000) Clonal reproduction by males of the ant Vollenhovia emeryi (Wheeler). Entomological Science 11: 167–172.

1. Ohkawara K, Nakayama M, Satoh A, Trinell A, Heinze J (2006) Clonal reproduction and genetic differences in a queen polymorphic ant Vollenhovia emeryi. Biological Letters 2: 539–543.

PloS ONE | www.plosone.org 10 August 2009 | Volume 4 | Issue 8 | e6781