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# Males are here to stay: fertilization enhances viable egg production by clonal queens of the little fire ant (*Wasmannia auropunctata*)

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**Abstract** Evolution of reproduction strategies is affected by both phylogenetic and physiological constraints. Although clonality may benefit females, it may not be selected if a male contribution is necessary to start egg laying and embryo development. In little fire ant, *Wasmannia auropunctata*, sexual populations employ a typical Hymenopteran system of reproduction. In clonal populations, however, queens and males are produced with only maternal and paternal genomes, respectively, whereas sterile workers are produced sexually. Although this system requires both sexes for worker production, previous work has shown that workers may also be produced clonally by the queens. If so, why are males maintained in this species? Our data suggest that fertilization is necessary to increase the hatching rate of eggs. Although clonal queens can indeed produce both workers and queens without mating, the hatching rate is far below the level necessary to maintain functional colonies. On the other hand, virgin queens from populations exhibiting the original Hymenopteran reproduction system also show low hatching rates, but produce only haploid male eggs. Reasons for the existence of *W. auropunctata* males have been disputed. However, our data suggest that physiological constraints, such as the requirement for insemination, must be considered in regard to evolution of reproduction systems, in addition to ecological data and theoretical considerations of fitness.

**Keywords** Hymenoptera · Parthenogenesis · Androgenesis · *Wasmannia auropunctata*

## Introduction

Organisms cannot always choose optimal reproductive strategies, because genetic or physiological constraints may be present. For example, in almost all obligately sexual animals, ova cannot initiate development without stimulation by sperm (Beukeboom and Vrijenhoek 1998). It is well known that in *Drosophila* species, seminal fluid proteins are important to initiate physiological changes in females that are necessary to start reproduction (Chen 1996; Wolfner 1997; Heifetz et al. 2000; Kubli 2003). During oogenesis, meiosis progresses until the middle of metaphase I or II; however, it is not resumed until insemination (Masui 1985; Page and Orr-Weaver 1997). In extreme cases, these developmental constraints have led to sperm-dependent parthenogenesis (pseudogamy), which is observed in a wide variety of animals (Beukeboom and Vrijenhoek 1998; Lehtonen et al. 2013). That is, sperm binding to the egg stimulates direct development, without a male genetic contribution. These studies suggest that organisms that have abandoned sex in favor of clonal reproduction have overcome a range of developmental constraints. Even though clonality may be beneficial to an organism for a variety of reasons (Williams 1975; Maynard 1978), it may never evolve if a male contribution is necessary to start egg laying and embryonic development. Therefore, the adaptiveness of sexual or clonal reproduction alone may be insufficient to explain its actual prevalence over the other reproductive strategies.

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In contrast to organisms with obligate sexual or clonal reproduction, hymenopterans (ants, bees, and wasps) employ both sexual and clonal reproduction systems to produce diploid females (queens or workers) and haploid males, respectively. Unmated individuals (virgin queens and workers) can also lay eggs (Fletcher and Ross 1985; Bourke 1988; Choe 1988; Vargo and Porter 1993; Hughes et al. 2008). However, all unfertilized eggs are haploid and develop into males. Thus, in most ants, males and mating are required for production of diploid offspring.

Here, we focus on the peculiar reproductive strategy of the ant, *Wasmannia auropunctata*. Sterile workers develop from fertilized diploid eggs, whereas queens develop with nearly complete maternal genomes from unfertilized diploid eggs generated by fusion of two of the four meiotic products (Fournier et al. 2005a; Rey et al. 2011). Males never inherit maternal genomes, but are clones of their fathers (Fournier et al. 2005a). Two other ant species, *Vollenhovia emeryi* and *Paratrechina longicornis*, share this clonal reproduction with androgenesis, suggesting that this unusual system likely evolved several times independently (Kobayashi et al. 2008; Pearcy et al. 2011). Elimination of maternal genomes from inseminated eggs or embryogenesis without female genomes after insemination of non-nucleated eggs has been suggested as possible mechanism for androgenetic male production, although the cytological mechanism remains unknown (Fournier et al. 2005a; Foucaud et al. 2007). Two major hypotheses have been proposed to explain the advantage of this unusual reproductive system. First, because all three species that employ this strategy are invasive, this unusual system may avoid inbreeding at low population densities (Foucaud et al. 2007; Pearcy et al. 2011; Kjar and Suman 2007). Second, it may preserve high levels of heterozygosity of worker offspring, which could improve colony performance through improved colony immunity (Hughes and Boomsma 2004; Pearcy et al. 2004; Tarpy and Seeley 2006; Seeley and Tarpy 2007; Reber et al. 2008; Matsuura et al. 2009; Vargo et al. 2012). Although ecological advantages seem to explain the evolution of this unusual clonal reproduction system, exactly why these species maintain androgenetic males remains unexplained.

Among these three species, *W. auropunctata* is particularly suitable to answer this question. In addition to its unusual reproduction system, populations with the classical hymenopteran reproduction system have been reported (Foucaud et al. 2007). Foucaud et al. (2010b) suggested that this unusual reproduction system is derived from the classical hymenopteran reproduction system. This allows us to perform comparative studies using populations with both original and novel reproduction systems, to find traits that have been lost and/or acquired, and to search for transitions between the two reproductive systems. In this study, the unusual and classical hymenopteran reproduction systems will be hereafter referred to as "clonal reproduction" and "sexual reproduction" systems, respectively, following earlier work (Foucaud et al. 2010a). In addition, we will call colonies with clonal and sexual reproduction "clonal colonies" and "sexual colonies," respectively.

Although the clonal reproduction system has the advantages mentioned above, it should be noted that queens cannot increase their fitness through androgenetic male production. According to previous studies, queens may potentially evolve reproductive strategies to avoid male production for the following reasons. First, sex allocation data suggest that androgenetic male production is costly for queens because queens should try to achieve higher fitness through female-biased sex ratios among their offspring (Okamoto and Ohkawara 2010; Rey et al. 2013). Second, the study of Foucaud et al. 2010a, using inseminated *W. auropunctata* queens in laboratory colonies, showed that 4.7 % of workers were produced clonally, suggesting that the male genomic contribution is not strictly necessary for the production of workers. Finally, there are likely at least some costs associated with finding mates, which would be eliminated by a transition to pure clonality. Thus, it is possible for queens to produce only diploid female eggs developing into new queens or workers and to establish colonies composed of only females. However in *W. auropunctata*, obligate clonal colonies established by only unmated queens have never been reported. Thus, there should be other reasons that queens cannot be released from androgenetic male production, even in the short term.

We propose a hypothesis for the maintenance of males in *W. auropunctata* with clonal reproduction—that mating releases physiological constraints on queens and enhances oviposition. This is in line with positive effects of mating, such as earlier start of egg laying, higher fecundity, and longer life span that have been generally shown in ants (Schrempf et al. 2005). To support our hypothesis, we show that clonal queens can produce workers and queens asexually, but few eggs develop unless the queens have mated. By examining sexual colonies, this physiological constraint had already arisen in *W. auropunctata* before androgenetic male production evolved. Based on these results, we argue that a physiological constraint may be a reason that male elimination has not occurred even in the short term.

## Materials and methods

To test reproductive capacity of queens in W. auropunctata, we conducted sampling, colony maintenance, genotyping, ovipositional test and observation of embryos as follows.

1. Ant collection and establishment of laboratory colonies

   We collected sexual and clonal colonies from native populations near Kourou, French Guiana, in early March, 2012, and from a clonal colony on the agricultural farm of the University of Hawaii, Hilo (USA) in early July, 2012. After collection, colonies were kept in artificial plaster nests at 27–30 °C and 60–70 % humidity. Colonies were

provided with dry crickets, Okinawan brown sugar, and distilled water every other day.

2. Identifying reproductive systems with microsatellite analysis

To identify the reproductive systems of collected colonies, four to ten queens per colony were genotyped with microsatellite markers used in Fournier et al. (2005b) (Table S1). Genotyping was performed on a single leg removed from each queen. Because we removed legs from all queens used in this experiment, it should not affect the difference of ovipositional behavior between virgin and inseminated queens. DNA extractions were performed using a QIAamp DNA micro kit, following the manufacturer's instructions (Qiagen). Legs were incubated overnight at 56 °C with animal tissue lysis (ATL) buffer and proteinase K. We selected 11 pairs of polymorphic microsatellite loci that have been used successfully in previous studies (Fournier et al. 2005b). These included Waur-418, Waur-2164, Waur-1gam, Waur-730, Waur-3176, Waur-716, Waur-275, Waur-225, Waur-680, Waur-1166, and Waur-521. PCR was performed in 10 μL volumes containing 0.4 μL of 1 mM primer mix each, 1 μL of diluted DNA, 0.8 μL of ExTaq 10× buffer, 1 μL of 2.5 mM dNTP, 0.05 μL of ExTaq enzyme, and 6.75 μL of RNAse-free water. PCR conditions were as follows: an initial denaturation cycle at 94 °C for 3 min, followed by 35 cycles at 94 °C for 20 s, annealing at 57 °C for 30 s, and a cycle at 72 °C for 30 s. Thereafter, a final extension step at 72 °C for 5 min was performed. Fluorescent PCR fragments were visualized by capillary electrophoresis on an ABI 3100xl Genetic Analyzer (Applied Biosystems). Genotypes were scored manually using GeneMarker (Hulce et al. 2011).

Colonies that mix both clonal and sexual reproduction systems have almost never been collected (Foucaud et al. 2007), so this sample size was adequate to determine the reproductive mode of a colony. In addition, rates of recombination in clonal queens are low (Rey et al. 2011). Thus, for purposes of this study, if colonies contained queens that were genetically identical at all loci, they were designated as clonal. In contrast, colonies where each queen genotype was unique were classified as sexual colonies.

3. Oviposition by clonal and virgin sexual queens

Experimental colonies from French Guiana and Hawaii were established soon after collection and maintained until the end of the experiments, in June, 2013. To check the reproductive potential and capacity for worker production without insemination, five experimental colonies containing single virgin queens (alate queens or queen pupa) isolated from four original clonal colonies were kept with 50–100 workers. To examine the oviposition behavior without fertilization in *W. auropunctata* employing the classical hymenopteran reproduction system, seven experimental colonies, each containing a virgin queen, were

established from five original sexual colonies.

In one clonal experimental colony, we found four discolored eggs produced by a virgin queen. These eggs developed until the late embryo stage, but were isolated from the egg pile, and were not tended by workers. New offspring produced by virgin queens were collected and genotyped using the same methods of DNA extraction and PCR, mentioned above.

4. Embryonic development of eggs produced by virgin and inseminated queens

Observations of oviposition by virgin queens showed that few of the eggs hatched and developed into adults. To observe embryonic development in un-hatched eggs produced by virgin queens, egg piles were randomly collected from experimental colonies. To assess differences in embryo development between virgin and inseminated queens, we also established five clonal and three sexual colonies containing a single inseminated queen and collected produced eggs. All collected eggs were kept for 1 week with 20–30 workers in artificial nests. To observe the stage of embryo development, eggs were treated with 100 % bleach for 2 min and rinsed in 0.1 % PBT (PBS and 0.1 % Triton X-100). After removal of the chorion, eggs were fixed in 4 % paraformaldehyde (PFA) and an equal amount of heptane for 30 min. Yolks were removed using the protocol for *Drosophila* (Tautz and Pfeifle 1989). After fixation and exclusion of the yolk, eggs were washed 5× with PBT and mounted on VECTASHIELD Mounting Medium with DAPI. Samples were observed with an Olympus confocal microscope (FV1000-IX81).

## Results

### Microsatellite genotyping

Microsatellite analysis indicated that the colony collected in Hawaii and three of the colonies collected in French Guiana were clonal, with all queens sharing identical genotypes at all successfully amplified loci. Five other colonies collected in French Guiana were classified as sexual (Table S1). These results were consistent with previous studies by Foucaud et al. (2007) and Mikheyev et al. (2009) from these populations.

### Castes of offspring produced by virgin queens

In clonal colonies, five experimental virgin queens produced 34 workers and seven new queens (Table 1). All genotyped larvae and adults produced by virgin queens from clonal colonies were genetically identical to their mothers (Table S2: see the electronic supplementary material). The caste ratio of workers and queens differed significantly among the five

**Table 1**   Number of offspring produced by experimental colonies composed of virgin queens in *Wasmannia auropunctata*

| Sexual/clonal | Colony | Test colony | Number of offspring | | | | |
|---|---|---|---|---|---|---|---|
| | | | Male | Female | Worker | Diploid egg | Haploid egg |
| Clonal | FG7 | FG7-1 | 0 | 2 | 12 | 0 | 4 |
| | | FG7-2 | 0 | 1 | 14 | 0 | 0 |
| | FG24-3 | FG24-3 | 0 | 0 | 5 | 0 | 0 |
| | FG3-7 | FG3-7 | 0 | 0 | 2 | 0 | 0 |
| | HU | HU | 0 | 4 | 1 | 0 | 0 |
| Sexual | FG1-1 | FG1-1 | 1 | 0 | 0 | 0 | 0 |
| | FG3-6 | FG3-6-1 | 2 | 0 | 0 | 0 | 0 |
| | | FG3-6-2 | 5 | 0 | 0 | 0 | 0 |
| | FG4-2 | FG4-2 | 27 | 0 | 0 | 0 | 0 |
| | FG4-6 | FG4-6-1 | 1 | 0 | 0 | 0 | 0 |
| | | FG4-6-2 | 1 | 0 | 0 | 0 | 0 |
| | FG4-10 | FG4-10-1 | 1 | 0 | 0 | 0 | 0 |

All seven sexual colonies produced only male offspring, whereas five clonal test colonies produced diploid females (worker or queen offspring). Haploid eggs were detected from one clonal colony (FG7-1); however, adult males were never detected from clonal test colonies

experimental colonies ($p<0.001$, Fisher's exact test). The first adult offspring were produced 10 months after the start of the experiment. Egg hatching was observed approximately once a month, whereas non-developing eggs were produced continuously until the end of the experiment. The average time between appearances of egg hatching was 33.4 days±12.8 SD. Additionally, the four discolored eggs developed until the late embryo stage, but were isolated from the egg pile, were collected from one clonal colony. These had half of the maternal genome, although no haploid larvae or adult males with the maternal genome were observed in clonal colonies.

In contrast to the clonal colonies, all 38 offspring produced by 15 virgin sexual queens were males that inherited half of the maternal genomes (Table S3: see the electronic supplementary material). Female offspring were never detected during experiment. The first adult offspring were produced 4 months after the start of the experiment and the average time between appearances of egg hatching was 40.2 days±24.8 SD. In this experiment, developed eggs and undeveloped eggs were indistinguishable.

### Differences in embryonic development between virgin and inseminated queens

Confocal images of eggs suggest that undeveloped eggs produced by virgin queens from clonal and sexual colonies remained at very early stages of embryonic development, whereas eggs produced by inseminated queens nearly all reached the hatching stage (Fig. 1). Percentages of developed eggs produced by virgin queens were significantly lower than those produced by inseminated queens in both clonal and sexual experimental colonies (clonal $p<0.0001$, sexual $p<0.0001$, Fisher's exact test analyzed by R version 3.0.1)

(Fig. 2). Whether periodic production of developed eggs resulted from physiological factors in the queens or from the developmental period of the eggs could not be determined.

## Discussion

### Effect of insemination on embryo development

There are clear differences in developmental patterns between eggs produced by virgin and inseminated queens from both
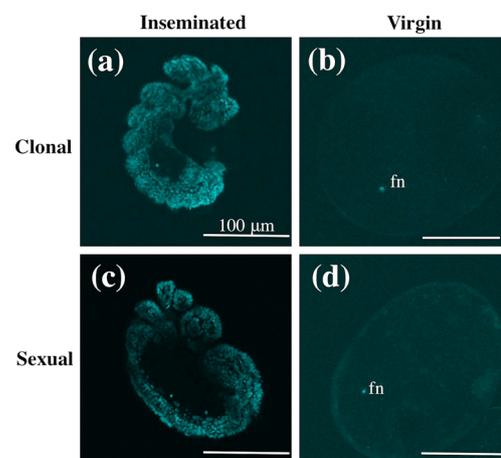


**Fig. 1** Differences in egg development between virgin and inseminated queens in *Wasmannia auropunctata*. An embryo 1 week after being collected from a colony composed of inseminated queens of **a** clonal and **c** sexual reproductive states. Eggs are ready to hatch so that larval segmentation can be observed. Embryos 1 week after being collected from a colony composed of virgin queens in **b** clonal and **d** sexual reproductive states. Only female nuclei were observed (*fn*). *Scale bar* 100 μm. Samples were stained by DAPI
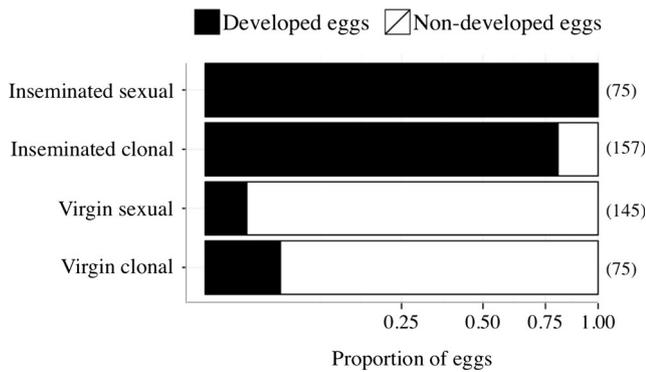
**Fig. 2** Proportion of developed and undeveloped eggs produced by inseminated sexual, inseminated clonal, virgin sexual, and virgin clonal queens *Wasmannia auropunctata*. Values given in *parentheses* are the total number of eggs stained with DAPI. These values were significantly different between virgin queens and inseminated queens from clonal colonies ($p<0.0001$, Fisher's exact test) and between virgin queens and inseminated queens from sexual colonies ($p<0.0001$, Fisher's exact test). Most eggs produced by virgin clonal and sexual queens were undeveloped. In contrast, most eggs produced by inseminated clonal and sexual queens developed

clonal and sexual colonies. Our data suggest that mating is necessary for queens to start viable egg production and to promote embryo development. In other words, queens cannot initiate colonies composed only of female offspring and achieve fitness through only diploid, uninseminated egg production. Since virgin queens from sexual colonies failed to lay high proportions of viable eggs (Fig. 2), this physiological constraint had already arisen in *W. auropunctata* before androgenetic male production evolved. Thus, this physiological constraint likely did not result from a sexual conflict originating with clonal reproduction.

Previous studies have shown that newly mated queens produce their first adult offspring after 1 month and produce reproductive individuals after a couple of months (Foucaud et al. 2010a). However, larvae were not observed for the first 4 and 10 months, respectively, in colonies of virgin sexual and clonal queens. It is known that initiating egg laying is one of the positive effects of mating in other ant species (Schrempf et al. 2005). Likewise, mating appears to accelerate the onset of egg laying in *W. auropunctata*.

Although some of the eggs produced by virgin queens developed into adults, this occurred much less frequently than in eggs laid by inseminated queens (Fig. 2). Stained embryos from non-developing eggs produced by virgin queens showed only single nuclei, while eggs produced by inseminated queens nearly all reached the hatching stage (Fig. 1). Usually, in invertebrates including Hymenoptera, female gametogenesis is arrested in the ovaries during the first meiotic division (Masui 1985; Page and Orr-Weaver 1997; Yamamoto et al. 2008). Thus, images of non-developing eggs possibly show queen "pro-nuclei" in the middle of gametogenesis (Fig. 1). Interestingly, non-viable egg production has also been observed in *P. longicornis* by preliminary tests (Morgan Pearcy,

personal communication) and *V. emeryi* (Misato Miyakawa, unpublished) with clonal reproduction, as in *W. auropunctata*.

One note of caution concerns the fact that we could not directly measure the number of eggs laid by queens. We first attempted to exclude queen after eggs were laid, but this proved unsuccessful, as workers in orphan colonies consumed the eggs. Since we could not track the fate of individual eggs, the numbers observed represent some balance between the rates of egg production and consumption, rather than overall fecundity. However, the net result was biologically the same, with virgin queens generating fewer persistent eggs.

## Caste of offspring produced by virgin queens

Interdependence of the sexes for worker production has been cited as the principal reason for the ongoing maintenance of males and queens, even in the absence of regular genetic exchange (Fournier et al. 2005a; Foucaud et al. 2006; Foucaud et al. 2010a). However, queens from clonal colonies can potentially produce both workers and queens without mating. This means that neither genes nor methylation of genes by males is strictly necessary for worker and queen development. Physiological constraints may prevent the evolutionary elimination of males in clonal *W. auropunctata*, despite the queens' ability to produce clonal workers. In contrast to offspring produced by virgin clonal queens, all offspring produced by sexual virgin queens were males that inherited half of the maternal genome, as typically observed in other Hymenoptera.

Although four haploid eggs that possessed half the maternal genome were detected in an experimental clonal colony, we did not observe any adult males or even larvae, suggesting that offspring produced by arrhenotokous parthenogenesis are non-viable. Thus, viable haploid egg production may have been lost during the transition from sexual to clonal reproduction. However, the sample size at the level of queen clones studied and the number of non-developing eggs observed may be too small to generalize reproductive traits of virgin clonal queens to all *Wasmannia* populations, which may vary in viable haploid egg production. Studies on other populations may yet uncover exceptions to the pattern we observed.

## Hypothetical mechanisms of non-viable egg production

A molecular mechanism that arrests viable egg production was reported in the two largest subfamilies of ants (Khila and Abouheif 2008, 2010). In these ants, workers have capacity to produce haploid eggs, but production of viable eggs is lower than in queens. Khila and Abouheif found that mislocalization or mis-patterning of maternal mRNA (*vasa*) and proteins (*nanos*) reduced workers' ability to produce viable eggs. It is known that correct localization and patterning of these maternal determinants in embryos is necessary for germ

line formation and differentiation and for functional embryo development in *Drosophila* (Lehmann and Nüsslein-Volhard 1991; Styhler et al. 1998). Therefore, it is possible that similar mechanisms exist in virgin queens of *W. auropunctata* and that queens acquire the capacity for viable egg production after mating. It is known that seminal fluid proteins can promote physiological changes inducing oviposition in females of many insects, including Hymenoptera (Avila et al. 2011; Collins et al. 2006; Perez-Staples et al. 2008; Baer et al. 2009). Therefore, it appears that in the little fire ant, similar mating-induced physiological mechanisms affect patterns of oocyte development and increase the rate of viable egg production.

Although maintenance of androgenetic male production may have colony level or long-term advantages, the fitness cost encumbering clonal queens when producing unrelated sons is obvious. Our data suggest that physiological constraints, such as a requirement for male-derived factors, may prevent male elimination in the short term. Recent reciprocal crosses showed that androgenesis is a maternal trait, rather than a sperm-dependent phenomenon in *W. auropunctata*, since new clonal male lineages are produced by clonal queens by mating with males from sexual populations (Rey et al. 2013). Thus, *W. auropunctata* males, rather than being competitors of queens, actually increase individual queen fitness by enhancing the hatching rate. More generally, our data suggest that physiologic constraints also must be considered in regard to the evolution of reproduction systems, in addition to ecological data and theoretical considerations of fitness.

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