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A New Record of Thelytoky in the Egg Parasitoid *Anagrus atomus* (Linnaeus) (Hymenoptera: Mymaridae)

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Abstract: Revertible thelytoky was observed for the first time in the mymarid wasp *A. atomus*. The thelytokous females develop faster than the arrhenotokous one with a shorter adult longevity. The production of males in the thelytokous species of *A. atomus* was induced by feeding antibiotics and high temperature treatments to their mother and male sexual functioning was determined. Rifampicin fed females produced 100% male offspring in the F_2 generation. The males induced by antibiotic treatment produced sperm, successfully mated with the females and produced female offspring.

Key words: *Anagrus atomus,* egg parasitoid, thelytoky, microorganisms, parthenogenesis, antibiotics

Introduction

The most common reproductive mode in Hymenoptera is arrhenotoky, a form of parthenogenesis in which male offspring arise from unfertilized, haploid eggs and female offspring from fertilized, diploid eggs. Two other modes of parthenogenesis, deuterotoky and thelytoky may occur in some hymenopterous lineages (Luck *et al*., 1993). With arrhenotoky, females control the sex of their offspring at oviposition by regulating the sperms' access to the egg. On the other hand, thelytokous females produce diploid female offspring from unfertilized eggs. Consequently, thelytokous lines can persist for multiple generations in the absence of males.

Two forms of thelytoky are recognized in Hymenoptera, revertible or microbe-associated thelytoky and non-revertible thelytoky (Stouthamer and Kazmer, 1994). In microbe-associated thelytoky, the symbionts involved in uniparental reproduction were first discovered in *Trichogramma spp*. (Stouthamer *et al*., 1990; Stouthamer and Werren, 1993). This form of thelytoky has also been reported in *Muscidifurax uniraptor* (Pteromalidae) (Stouthamer *et al*., 1993), *Encarsia formosa* (Aphelinidae) (Zchori-Fein *et al*., 1992), *Aphytis spp* (Aphelinidae) (Zchori-Fein *et al*., 1995) and in *Telenomus nawai* Ashmead (Scelionidae) (Arakaki *et al*., 2000).

The microbe-associated thelytoky reverted to arrhenotoky when the females were fed with antibiotics (Zchori-Fein *et al*., 1992, 1995; Arakaki *et al*., 2000; Majerus and Majerus, 2000). Transition from thelytoky to arrhenotoky in some parasitoid species may be caused by inactivation or damage to symbiotic microorganisms through exposure to high temperature (Stouthamer *et al*., 1990; Kajita, 1993; Arakaki *et al*., 2000). Microorganisms are also found to be involved in causing thelytoky in *Encarsia formosa* Gahan by an unknown mechanism, may be by the restoration of diploidy in the eggs to produce females (Zchori-Fein *et al*., 1992).

Anagrus atomus can reproduce parthenogenetically, where male and female offspring developed from unfertilized and fertilized eggs, respectively (MacGill, 1934) but thelytoky has not been reported so far in *A. atomus.* Some of the virgin females supplied from English Woodland Biocontrol produced mostly female offspring without fertilization in successive generations. This population was suspected to be infected by some of the unknown microorganisms. All studies so far on parasitoids have examined synovigenic species where antibiotic fed to the adults might be used to alter the contents of eggs or the associated fluids when eggs are laid. Because *A. atomus* is pro-ovigenic all eggs are fully formed on emergence. It seemed unlikely that antibiotics would be able to cure the thelytoky. Therefore the present study was designed to find out whether treatments with antibiotics and high temperature can revert the population from thelytoky to arrhenotoky.

Materials and Methods

Parasitoid and host culture: The thelytokous population of *A atomus* was maintained at 25°C with 85% r.h. in temperature controlled illuminated incubators in the laboratory of Imperial College at Wye, UK during 2000-2001. Leaf discs of 50mm in diameter containing leafhoppers egg were placed on agar medium in Petri-dish cages wfith the lower surface facing uppermost. The individual unmated female was introduced into each Petri-dish cage to parasitize leafhopper eggs for 24 hours. The petri-dish cages were put in a plastic box and kept inside an incubator at 25°C. The relative humidity inside the boxes was maintained at 85% r.h. using saturated KCl salt solution. Each Petri-dish was considered as one replication.

Ten days after laying eggs by *A. atomus*, leaves were cut into small pieces containing an individual parasitized egg (those which turned red) and placed into 0.5 ml Eppendorf safe-locking microcentrifuge tubes with a piece of wet filter paper. These tubes were placed in a plastic sandwich box and kept in temperature controlled illuminated incubators at 25°C. The humidity inside the boxes was maintained in the same way mentioned above. The tubes were checked everyday for the emergence of adult *A. atomus* and the females were used as the test insects. The leafhopper *Hauptidia maroccana* were mass cultured on Primula (*Primula vulgaris* Huds.) in an insulated shipping container with a computer controlled environment. Temperature was kept constant at 21°C with 80-85% relative humidity, 16h light: 8h dark photoperiod and 4.2 watts/m^2 light intensity.

Test Procedure

Antibiotic treatments: Three different types of antibiotics Ampicillin, Rifampicin and Penicillin were used in the experiment. To prepare the antibiotic solution, antibiotics were mixed with distilled water at a rate of 1 mg ml^{-1} of distilled water and then the solution was mixed with honey at a rate of 1 mg ml^{-1} of honey (Kajita, 1993). Newly emerged females were fed with different antibiotic solution for 24 hours. The treated females were allowed to parasitize *H. maroccana* eggs in 50 mm diameter Petri-dish cages for 24 h and the eggs were kept in the incubators at 25°C until parasitoid emergence. Five replications were maintained for each antibiotic treatment. A control treatment with 5 replications was also maintained. The number of male and female offspring in the F1 was counted.

Five females were then randomly selected from the offspring (F1) of the treated females and were allowed to lay eggs for 24 h following the same procedure mentioned above. The number of male and female offspring in F2 was counted to calculate the sex ratio.

Temperature treatments: Parasitoid females were exposed to high temperature (31°C) at three different development stages:

Immature stages: Ten adult females were allowed to lay eggs for 24 hours. The parasitized eggs of *H. maroccana* by thelytokous females were subjected to high temperature treatment until adult emergence.

Pupal stages: Eight days after laying eggs when they are about to pupate, the parasitized eggs were exposed to high temperature for 48 h and then transferred again to 25°C until adult emergence. Ten replications were maintained in this case.

Adult female: Newly emerged females were exposed to high temperature for 3 days and fed with 50% honey solution. Treated females were allowed to oviposit for 24 h and the parasitized leafhopper eggs were incubated at 25°C until adult emergence. Ten replications were maintained

The sex ratio in F1 generation of the treated females was calculated on the basis of the emerging male and female wasps from each treatment. Five female wasps from F1 of each of the three treatments were selected randomly and allowed to parasitise *H. maroccana* eggs for 24 h. Each Petri-dish was individually marked and the offspring sex ratio in F_2 was calculated to determine whether high temperature had any effect.

Male functioning: The mating potential of the antibiotic–induced males was tested in two different ways:

Mating performance: An antibiotic-induced male along with a female was introduced into the 50 mm diameter Petri-dish cage. Behaviour of the male was observed under a binocular microscope and the mating performance was recorded. Twenty individuals from the antibiotic-induced males were used in the test to see their mating performance.

Viability of the sperm: Five females from the arrhenotokous (uninfected) culture were mated with antibiotic induced males. Those females along with the males were introduced into 50 mm diameter Petri-dish cages and allowed to parasitise *H. maroccana* eggs for 24 h. Each Petri-dish was considered as one replication. A control treatment was maintained using one unmated arrhenotokous female. The sex ratio of the emerging wasp from unmated and mated females was recorded.

It is assumed that the antbiotic-fed females inject some of the antibiotics from the acid glands, which enters the developing embryo. Thus, the microorganisms inside the eggs were being cured and can produce male offspring in succeeding generations. An attempt was made to find out how does the antibiotic work in reverting the sex of infected wasps. Infected females parasitized twenty eggs of *H. maroccana* in a leaf disc. Twenty four hours after parasitization, females fed with antibiotics for 24 h were allowed to venomise ten of the parasitized eggs but the females were removed before oviposition and the same females were allowed to parasitize ten of the unparasitized egg. Thus the three types of eggs viz. 1) parasitized by infected female, 2) parasitized by infected female but venomized by Rifampicin treated female and 3) parasitized by Rifampicin treated female were obtained in the same leaf disc and the eggs were marked individually. The parasitized eggs were then put into the incubator. When they pupate, the leaf was cut into small pieces containing individual eggs and put inside 0.5 ml Eppendorf safe-locking microcentrifuge tubes along with a small piece of wet filter paper. The tubes were incubated and checked everyday for adult emergence. The number of emerging male and female parasitoids was counted.

Five females were then randomly selected from the offspring of each of the three groups and were allowed to parasitize for 24 h on different leaf disc. The parasitized eggs were incubated in the same procedure. The number of male and female offspring in F_2 was counted to calculate the offspring sex ratio.

Statistical analysis: Some characteristics of arrhenotokous and thelytokous females, viz. duration of development, potential fecundity, head width and hind tibia length were compared using Two-sample t test. Chi-squared test was performed to know whether different antibiotics and high temperature have any effect on the sex ratio of the emerging parasitoid offspring. In some cases, χ^2 analyses were invalid due to few data.

Results and Discussion

Characteristic differences between arrhenotokous and thelytokous *A. atoms*: The characteristic differences between thelytokous and arrhenotokous population of *A. atomus* is presented in Table 1. Significant difference was observed in development duration (P= 0.05, T=-1.98, df=76) between the two populations of *A. atomus*. The thelytokous female developed earlier (12.5 daysi than the arrhenotokous female (12.8 days). Significant difference was also observed between the longevity of thelytokous and arrhenotokous females ($P< 0.001$, T=-9.11, df=38). The arrhenotokous females survived twice as long as thelytokous females when provided with the same food (50% honey solution). On the other hand, two-sample t test did not show any significant differences between arrhenotokous and thelytokous population of *A. atomus* with regards to their potential fecundity and body size (head width and hind tibia length).

Effect of different antibiotic treatments on the sex ratio of the offspring of *A. atomus* in subsequent generations: Antiobiotic treatments to the thelytokous females did not show any significant differences in the sex ratio of *A. atomus* in F_1 generation (Table 2). But in F_2, χ^2 analyses showed significant differences in the sex ratio (P<0.001, df=3) (Table 3). Females whose parents had fed on Rifampicin produced 100% male offspring while without feeding with antibiotics they produced all female offspring. Ampicillin and Penicillin produced 13.73% and 14.75% males, respectively.

When the antibiotic fed females were allowed only to insert the ovipositor without laying eggs, females from those eggs produced 3.17% male offspring in F_2. On the other hand, females emerging from the eggs where the same antibiotic fed female successfully laid eggs, produced all male offspring in F_2. The infected females produced all female offspring (Table 4).

In the present study, antibiotic treatment showed a remarkable effect on male production in thelytokous *A. atomus*. Rifampicin was found to be most effective antibiotic in the induction of maleness producing 100% males in the F_2. However, the other two antibiotics, Ampicillin and penicillin produced 13.73% and 14.75% males, respectively. Untreated control females produced exclusively female progeny. This result is in agreement with the observations of Kajita (1993). He reported male occurrence in thelytokous *Encarsia formosa* Gahan when treated with Rifampicin and tetracycline hydrochloride. These two antibiotics inhibit RNA and protein synthesis, respectively. On the other hand, Ampicillin, penicillin G and sulfamethoxazole do not inhibit RNA and protein synthesis and therefore, did not show any effect of male production (Kajita, 1993). The thelytokous *Telenomus nawai* Ashmead produced 27.1% male progeny when treated with Tetracycline (Arakaki *et al.,* 2000).

Bacteria of the genus *Wolbachia* were identified as the causative agent of thelytoky in many parasitoid species that could be treated by feeding the female parents with antibiotics or by heat treatments (Stouthamer *et al.*, 1990). The parthenogenetic strain (thelytokous) of *Trichogramma* wasps successfully reverted to the production of male progeny after antibiotic treatment (Stouthamer *et al.*, 1990). Their subsequent works identified that cytoplasmically inherited bacteria of the genus *Wolbachia* were associated with that form of thelytoky (Stouthamer *et al.*, 1993).

Effect of high temperature treatment (31°C) on the sex ratio of the offspring of *A. atomus* in subsequent generations: An exposure of *A. atomus* during immature stages, pupal stages and adult stages to high temperature caused the occurrence of male offspring in the F_2 generations. More male progeny (21.87%) was obtained when high temperature was used throughout immature

Table 1: Comparison of the characteristics of arrhenotokous and thelytokous population of *Anagrus atomus*

Characteristics	n	Arrhenotokous population*	n	Thelytokous population	Tow-sample t-test	
Development duration of female (days)	185	12.9±0.064a	40	12.5±0.097b	P=0.05,	T=-1.98, df=76
Potential fecundity (no.)	27	26.4±1.665a	24	25.4±0.92a	P=0.58,	T=-0.56, df=40
Head width (mm)	33	0.176±0.0016a	33	0.176±0.0012a	P=0.65,	T=-0.46, df=59
Hind tibia length (mm)	31	0.188±0.0016a	33	0.186±0.0017a	P=0.73,	T=-0.34, df=61
Adult longevity (days)	21	11.8±0.50a	20	5.7±0.43b	P<0.001,	T=-9.11, df=38

*Results obtained from another study, Means in rows, followed by the same lowercase letters are not significantly different from each other at $P \leq 0.05$. One-way ANOVA followed by Least Significant Difference test, n= number of insect as replicate

Table 2: Effect of antibiotic treatment on the sex ratio of unmated *Anagrus atomus* in F_1 generation

Treatment	Total emerged wasp (no.)	Male offspring (no.)	Female offspring (no.)	% Female mean ± S.E.	% Male mean ± S.E.
Control	71	3	68	95.77±2.39	4.22±2.39
Ampicillin	59	3	56	94.92±2.86	5.08±2.86
Penicillin	18	0	18	100.00±0.0	0.0±0.0
Rifampicin	59	0	59	100.00±0.0	0.0±0.0
χ^2 analysis	The 4x2 Chi-Square approximation was invalid due to 2 cells with expected counts less than 1.0 and 4 cells with expected counts less than 5.0.				

Table 3: Effect of different antibiotic treatments on the sex ratio of unmated *Anagrus atomus* in F_2 generation

Treatment	Total emerged wasp (no.)	Male offspring (no.)	Female offspring (no.)	% Female mean ± S.E.	% Male mean ± S.E.
Control	76	0	76	100.00±0.0a	0.0±0.0c
Ampicillin	51	7	44	86.27±4.82b	13.73±4.82b
Penicillin	61	9	52	85.25±4.54b	14.75±4.54b
Rifampicin	62	62	0	0.0±0.0c	100.00±0.0a
χ^2 analysis	χ^2= 186.12, df= 3, P<0.001				

Table 4: Comparison of offspring sex ratio of *Anagrus atomus* in F_2 produced by Rifampicin fed females, infected females and from hosts parasitized hosts by infected females but venomized by Rifampicin fed females

Treatment	Total emerged wasp (no.)	Male offspring (no.)	Female offspring (no.)	% Female mean ± S.E.	% Male mean ± S.E.
Females fed with Rifampicin	48	48	0	0.0±0.0b	100.0±0.0a
Venomized eggs laid by infected females	63	2	61	96.83±2.21a	3.17±2.21b
Control (infected)	76	0	76	100.0±0.0a	0.0±0.0b
χ^2 analysis	χ^2= 177.11, df= 2, P<0.001				

Table 5: Influence of high temperature treatment (31°C) on the sex ratio of offspring produced by *Anagrus atomus* in F_2 generation

	Total emerged wasp (no.)	Male offspring (no.)	Female offspring (no.)	% Female mean ± S.E.	% Male mean ± S.E.
High temperature throughout immature stages	64	14	50	78.13±5.17b	21.87±5.17a
High temperature treatment for 48 h at pupal stage	34	4	30	88.24±5.53b	11.76±5.53a
Adults treated with high temperature for 72 h	26	2	24	92.30±5.23b	7.70±5.23a
Control (at 25°C)	65	0	65	100.0±0.0a	0.0±0.0b
χ^2 test	χ^2= 16.598, df= 3, P<0.001				

Table 6: Comparison of the offspring sex ratio from arrhenotokous females mated with arrhenotokous and antibiotic induced males of *Anagrus atomus*

	Total emerged wasp (no.)	Male offspring (no.)	Female offspring (no.)	% Female mean ± S.E.	% Male mean ± S.E.
Arrhenotokous unmated	62	62	0	0.00±0.00	100.00±0.00
Arrhenotokous, mated with unifected male	87	19	68	78.17±4.43	21.83±4.43
Arrhenotokous, mated with antibiotic induced males	72	9	63	87.50±3.90	12.50±3.90
χ^2 test	χ^2= 16.598, df= 3, P<0.001				

Values in columns followed by the same letters are not significantly differet from each other in χ^2 analysis. P value calculated from χ^2 analysis

S.E. Calculated from the equation:

$$S.E. = 100\sqrt{pq/n}$$

Where
p = Proportion of male
q = 1-p

development. On the other hand, all female progeny was obtained from the control treatment (Table 5).
The exposure of thelytokous *T. nawai* during the pupal stage to a high temperature (35°C) was reported to produce 16.4% males whereas in the control treatment, only 0.1% males were produced (Arakaki *et al.*, 2000). This might be due to the high temperature having a harmful effect on the development of reproductive organs in the pupal stages.

Sexual functioning of the antibiotic induced males of *A. atomus*: In the present study, antibiotic induced males showed 100% mating success when allowed to mate with the arrhenotokous females. Result in Table 6 shows that antibiotic induced males produced viable sperms and successfully transferred to the offspring. The unmated arrhenotokous females produced 100% male offspring.

Whereas, the females from the same population produced 78.17 and 87.5% female offspring when they were mated with arrhenotokous and antibiotic induced males, respectively.

Stouthamer *et al.* (1990) reviewed the functionality of heat-induced males in thelytokous species of *Trichogramma* and reported them as non-functional. Even when the females of *Encarsia formosa* were fed with antibiotics, the males obtained from those females were not sexually functional (Zchori-Fein *et al.*, 1992). In another study, Zchori-Fein *et al.* (1995) reported successful induction of male offspring from the uniparental lines of two species of the genus *Aphytis* when treated with Rifampicin. Those males produced viable sperms, which successfully transferred to the female spermatheca. However the sperms were not viable as they failed to produce any female after fertilization. Kajita (1993) reported that the males induced from treatment with Rifampicin and tetracycline hydrochloride showed mating behaviour in *Encarsia formosa* Gahan. Arakaki *et al.* (2000) observed functional males in *T. nawai* from the antibiotic treated thelytokous females. Males could successfully fertilized arrhenotokous females and produced 19.1% males. This appears to be the result of successful mating and fertilization. Arrhenotokous wasps usually show few male offspring after mating. On the other hand, *Wolbachia*-infected females exclusively produced female progeny after mating with the tetracycline-induced males, suggesting that the eggs developed parthenogenetically and sperm did not participate in egg development (Arakaki *et al.*, 2000).

Thelytoky in a biological control agent may confer disadvantages such as low genetic variability, difficulties in studying the genetics and, thus, limitations in genetic improvement (Zchori-Fein *et al.*, 1992). However, the result from antibiotic and heat treatment to the thelytokous population strongly suggests that some unknown microorganisms infecting the thelytokous *A. atomus* are the causative agents of parthenogenesis, which can be treated by antibiotic treatments or high temperature exposure even the males mated successfully with the females and successful insemination also occurred. Thus the transition from thelytoky to arrhenotoky in *A. atomus* might be caused by inactivation or damage to symbiotic microorganisms through exposure to high temperature or feeding with antibiotics. The thelytoky observed in these studies may not be seen as detrimental. All parasitoids produced were females and hence capable of attacking host eggs. While longevity is reduced it is still long enough for the pro-ovigenic species to lay all of its egg load. The size and general activity did not seem to be impaired. Further studies are needed to confirm what microorganisms are involved in thelytoky in this parasitoid.

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