

Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/Trichogrammatidae) to revert to sex

(thelytoky/arrhenotoky/symbionts)

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ABSTRACT Completely parthenogenetic *Trichogramma* wasps can be rendered permanently bisexual by treatment with three different antibiotics or high temperatures. The evidence strongly suggests that maternally inherited microorganisms cause parthenogenesis in these wasps. Theories predict female-biased sex ratio in offspring under the influence of maternally inherited symbionts, but extreme sex ratios of 100% females were never considered because the lack of males would prevent the host's reproduction.

In recent discussion of the reasons for sex or its abandonment (1–3), surprisingly little attention has been given to possible roles of the biological agents for which sex creates the starkest alternatives. Agents transmitted solely in the female line, which includes mitochondria, plasmagenes, and, in some species, microbial symbionts, have encountered biological disaster when they find themselves passed to a male zygote: they then leave no descendants beyond the lifetime of their bearer. Mitochondria are considered to be under almost total control of the host genome; but what of the rest? The possibility that such agents might bias the sex ratio of sexual species in favor of females has sometimes been theorized (4, 5) and shown (6, 7), and the point has been made that their optimum is no males at all if this is still consistent with fertility (8). However, the idea that the agents might somehow cause uniparental reproduction has not been considered. We now report results strongly suggestive that this can happen. Treatment of adults of four species of thelytokous *Trichogramma* wasps with antibiotics specific for prokaryotes, or their subjection to high temperatures, reverts their thelytoky to arrhenotoky.

Thelytoky is the term used to describe female-to-female parthenogenesis in the Hymenoptera. This potentially permanent mode of reproduction is to be contrasted to arrhenotoky, which is the normal (and essentially sexual) system of reproduction in the Hymenoptera. Under arrhenotoky, males arise from unfertilized eggs and females from fertilized ones. The males are haploid, and the underlying cytogenetic system is called male haploidy. The offspring produced by virgin females is used to distinguish between thelytokous and arrhenotokous reproduction: thelytokous virgins produce female offspring, while arrhenotokous virgins produce male offspring. The “reverted” *Trichogramma* cultures mentioned above have returned permanently to arrhenotoky from thelytoky.

The genus *Trichogramma* has a cosmopolitan distribution and consists of >100 described species (9). These minute parasitic wasps (about 1 mm long) lay their own eggs within the eggs of moths and butterflies. At 24°C they complete their development from egg to adult in about 10 days. Among and within host species, the number of wasp eggs laid in a host egg

increases with its size. From hosts parasitized by arrhenotokous females, one to several females may emerge together with usually just one male. As a rule *Trichogramma* species reproduce by arrhenotoky; however, thelytokous reproduction also occurs. Some species are entirely thelytokous, others consist of both thelytokous and arrhenotokous populations, while in several arrhenotokous species an occasional thelytokous female is found. Males are normally rare in the offspring of thelytokous females, but if such females are exposed to temperatures >30°C during their larval development, some males will usually appear in their offspring (10, 11). The same phenomenon has been found in thelytokous forms of many different Hymenoptera species (12–17). Generally such temperature-induced males are considered to be nonfunctional (10, 12, 14). However, in several species the males are functional, and their sperm can successfully fertilize eggs of conspecific arrhenotokous females (17, 18) or thelytokous females (19).

MATERIALS, METHODS, AND RESULTS

The material in our study came from parasitized host eggs that were collected throughout North America and Hawaii for a taxonomic survey of *Trichogramma* species. Infrequently thelytokous females emerged from these field-collected eggs, and they were used to initiate thelytokous laboratory cultures of *Trichogramma pretiosum*, *Trichogramma deion*, *Trichogramma platneri*, and *Trichogramma chilonis* by using cabbage looper (*Trichoplusia ni*) eggs as hosts. Each culture was started with one thelytokous female. At the locations where thelytokous females of *T. deion* (Irvine, CA; Mountain Center, CA; Sanderson, TX; and Belle Fourche, SD), *T. platneri* (Riverside, CA), and *T. chilonis* (Kauai, HI) were collected, arrhenotokous females of the same species were also found. The collection of *T. pretiosum* (Kauai, HI) consisted of only thelytokous females, while *T. pretiosum* (Nuevo Leon, Mexico) was collected together with arrhenotokous conspecifics. Finally, the collection of *T. pretiosum* (Lara State, Venezuela) consisted of only one thelytokous female.

Backcrossing Experiments. To determine if chromosomal factors might affect the inheritance of thelytoky in *Trichogramma*, we created females that had the genome of a thelytokous strain and the cytoplasm of a field-collected arrhenotokous strain of the same species and determined their mode of reproduction (i.e., arrhenotoky vs. thelytoky). If thelytoky were inherited chromosomally, these females would be expected to produce only female offspring. To create such females, we crossed males from a thelytokous strain (produced by exposing thelytokous females to temperatures >30°C) with females from an arrhenotokous strain

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kept at 24°C. The resulting hybrid females (averaging 7 females per generation for *T. deion* and 8 for *T. pretiosum*) were mated again with males from the thelytokous strain. We continued these backcrosses for five generations with *T. deion* and for nine generations with *T. pretiosum*. Since cytoplasm is inherited through the maternal line, all of the cytoplasm in these backcrossed females arose from the arrhenotokous strain. On average, the percentage of the genome stemming from the thelytokous line in females of the *n*th generation of backcrossing is given by the relationship $1 - (0.5)^n$; this percentage in generations 5 and 9 is 96.9% and 99.8%, respectively. We tested the mode of reproduction of the backcrossed females every generation by supplying virgin backcrossed females (averaging 23 virgins for *T. deion* and 17 for *T. pretiosum*) with host eggs: all female offspring indicated the females reproduced thelytokously, whereas all male offspring indicated they reproduced arrhenotokously.

The backcrossed females remained arrhenotokous, showing that thelytoky is not inherited in a simple way through the chromosomes of the wasp. (In generation 4 of *T. deion*, 45 virgins were tested, and the offspring of 1 of these females consisted of 2 males and 2 females. This female was probably not a virgin when used in this test. The all-male offspring of 36 virgins in the next generation of backcrossing corroborates this.) Therefore, extrachromosomal inheritance is probable.

Antibiotic Experiments. To test if thelytoky is caused by extrachromosomally inherited microorganisms, we allowed newly emerged wasps to feed on honey mixed with one of several antibiotics for 1 day before providing them with host eggs. Populations were exposed to antibiotics for several generations by repeating this procedure. In those females fed honey mixed with the antibiotics tetracycline hydrochloride, sulfamethoxazole, and rifampicin (100 mg of antibiotic per 1 ml of honey), males appeared in the offspring of generation 1 or 2 and were present in all subsequent generations. Mixtures of 1% tetracycline in honey proved effective in other experiments. Male offspring did not occur where thelytokous females were fed the antibiotics gentamycin, penicillin G, and erythromycin. We initiated three to five isofemale lines (lines started with one female) with mated females from each population that had been exposed for several generations to tetracycline or high temperatures (Table 1). The new lines were maintained without antibiotics

at the normal rearing temperature of 24°C. We tested the mode of reproduction of these lines between generations 2 and 5 after they were initiated. Those reproducing by arrhenotoky in this test were maintained and remained arrhenotokous when tested again around generations 10 and 20 (Table 1). Therefore, we had permanently changed the mode of reproduction from thelytoky to arrhenotoky by exposing thelytokous females of these four *Trichogramma* species to either antibiotics or high temperatures. The lines where the treatment failed to revert thelytoky to arrhenotoky often produced some male offspring in the first few generations after the treatment had ended, but by generation 5, only female offspring were produced. One exception to this were two lines of *T. deion* (SD) that consisted of a mixture of thelytokous and arrhenotokous individuals when tested in generation 7 after the treatment had ended. Three new isofemale lines were subsequently started with mated arrhenotokous females from one of the two original lines tested again for their mode of reproduction 12 generations later and were found to be arrhenotokous.

DISCUSSION

The backcrossing experiments showed that thelytoky was not inherited as a simple chromosomal trait; therefore, extrachromosomal inheritance of thelytoky became likely. Recently, several sex ratio-distorting factors have been discovered in insects—for instance, the parasitic wasp *Nasonia vitripennis* (4) and various *Drosophila* (5) species. Females carrying these factors produce abnormal sex ratios in their offspring. These sex-ratio distorters are inherited extrachromosomally and are in some cases microorganisms that are passed on from mother to offspring through the cytoplasm of the egg (5). These microorganisms could either be killed by treatment with tetracycline or by exposure to heat. We exposed our thelytokous cultures to several antibiotics and high rearing temperatures. The three antibiotics that caused a reversion from thelytoky to arrhenotoky all differ in their mode of action (20). Rifampicin inhibits prokaryotic DNA-dependent RNA polymerase, tetracycline affects the protein synthesis on microbial ribosomes, whereas sulfamethoxazole inhibits the folate synthesis in microorganisms. Microorganisms need to synthesize their own folate and cannot use

Table 1. Species name and collection site of initially thelytokous *Trichogramma* that were treated for several generations to either temperatures > 30°C (T) or the antibiotic tetracycline hydrochloride (A)

| Species and collection site | Treatment | Generations treated, no. | Lines started, no. | Arrhenotokous lines in 1st test, no. | Tested in generation |
|-----------------------------|-----------|--------------------------|--------------------|--------------------------------------|----------------------|
| <i>T. pretiosum</i> | | | | | |
| Nuevo Leon, Mexico | T | 23 | 5 | 5 | 4, 12, 24 |
| | A | 4 | 4 | 4 | 3, 12, 28 |
| Lara State, Venezuela | A | 3 | 4 | 3 | 1, 17 |
| Hawaii | T | 13 | 4 | 2 | 4 |
| | A | 7 | 4 | 2 | 3, 10, 22 |
| <i>T. deion</i> | | | | | |
| Irvine, CA | A | 4 | 3 | 3 | 5, 15 |
| Mountain Center, CA | T | 9 | 4 | 4 | 2, 12, 22 |
| | A | 6 | 4 | 4 | 2, 12, 23 |
| Belle Fourche, SD | A | 5 | 4 | 1 | 2, 7, 10, 13, 22 |
| Sanderson, TX | T | 4 | 4 | 3 | 2, 11, 24 |
| | A | 5 | 5 | 5 | 5, 22 |
| <i>T. platneri</i> | | | | | |
| Riverside, CA | A | 7 | 4 | 4 | 2, 10, 26 |
| <i>T. chilonis</i> | | | | | |
| Hawaii | A | 8 | 4 | 4 | 5, 10, 23 |

At the end of the treatment, lines were started with individual mated females. These lines were tested for their mode of reproduction after several generations. After the first test, only the arrhenotokous lines were maintained. In subsequent tests they remained arrhenotokous.

performed folate, while animals only use preformed folate. The mode of action of the ineffective antibiotics cannot be used to better identify the presumed microorganism for several reasons. We could not determine (i) if the honey mixed with the ineffective antibiotics was ingested by the wasps and (ii) if ingested, whether the antibiotic reached the microorganisms.

The treatment failed to lead to arrhenotoky in a few lines started from a treated culture (Table 1). The males that were produced in these "failed" lines during the first few generations after the treatment ended can be explained by an incomplete removal of the microorganisms. With a lower microorganism titer once the treatment has ended, the females are still thelytokous but produce some male offspring. Over generations, the microorganism titer would increase again and result in only female offspring.

Two alternative hypotheses can be posed to explain these results. First, high temperatures and certain antibiotics may signal to the female that the environment is about to change and that arrhenotokous reproduction may be favored. A situation similar to that has been shown in aphids, where there is a transition from asexual to sexual reproduction induced by low temperatures and day length (21). The permanently arrhenotokous cultures that were established after treatment are difficult to explain by this hypothesis. Why would these cultures remain arrhenotokous when they are put under conditions that previously favored thelytoky? A second alternative is that the exposure to high temperatures or certain antibiotics may cause some chromosomal damage that induces a change from thelytoky to arrhenotoky. What kind of mechanism would cause such a change is not known, but if such a change occurs, it does not lead to gross chromosomal rearrangements because the males from "reverted" lines are completely compatible in crosses with females from field-collected arrhenotokous lines (22). Additionally, experiments (23) in which the three effective antibiotics were tested for their effects on longevity and fecundity showed that whereas 10% mixtures of tetracycline had a negative influence on both longevity and fecundity, possibly consistent with the chromosomal-damage hypothesis, neither sulfamethoxazole nor rifampicin had a significant influence on these traits. Therefore, if some chromosomal change occurs, this change must be very specific and does not have any negative influence on these life-history traits.

These two alternative hypotheses are less well supported by the data than the microbial hypothesis. Symbiotic microbes are often extremely fastidious, and they have only been successfully cultured in a few cases, so that Koch's postulates could be satisfied (24, 25). Although we have not yet satisfied Koch's postulates and therefore have not proven the microbial involvement in thelytoky, the backcrossing and antibiotic experiments strongly suggest that microbial symbionts are involved in causing thelytoky.

The advantage to vertically transmitted symbionts of causing a transition to either spanandrous arrhenotoky (arrhenotoky in which few males are produced) or thelytoky was pointed out above. Symbionts with ovarial transmission or other elements (excluding mitochondria) that pass only in the female line are known to be associated with a majority of cases of male haploidy and parahaploidy (26–30), but their possible role in causing transition to these modes of reproduction has not been reviewed. Here it is merely pertinent to note that induction of male haploidy opens the way, via arrhenotokous control of the sex of offspring by the mother, to strong female biases of sex ratio, especially under conditions of local mate competition. These biases are common (31) and, while they are fairly well explained by current evolutionary stable strategy (ESS) theory concerning the interests of the host genome (32–34), they also converge with the adaptations of a symbiont when inbreeding is close. When

broods are large and sex ratios are very female-biased (8), further selection to eliminate the male will be weak. However, this is hardly the case with *Trichogramma*, where brood sizes are constrained by host egg sizes and are often small (one to two eggs per host egg): a change in *Trichogramma* from arrhenotoky to thelytoky gains a substantial fraction of immediate fitness. Data on the offspring production by thelytokous and "reverted" arrhenotokous cultures show (23) that thelytokous females produce the same number of offspring per host egg as arrhenotokous individuals. Therefore, thelytokous females produce more female offspring per parasitized egg than arrhenotokous females.

There are also other possible interesting evolutionary connections of our phenomenon. In many species of thelytokous wasps, males are induced by exposing females to temperatures >30°C (10–17). We suspect that microorganisms cause thelytoky in these wasps also, although permanent reversion to arrhenotoky was not shown. In some cases thelytokous females produce male offspring in response to exposure to high temperatures during their larval development; however, when such adult females are subsequently exposed to lower temperatures, they will start to lay female offspring again (13), indicating that the high temperatures might affect the transmission of the microorganism or its effect on thelytoky, without actually killing it. The temperature sensitivity of thelytoky suggests that microorganisms may be the agents involved in the cyclic thelytoky characteristic of many invertebrates (1). These invertebrates alternate biparental and uniparental generations. The correlation of sex with season in alternation suggests a possibility of the inactivation of the microorganisms' influence, at least in the origin of these systems. While the evidence in our case suggests elimination of a maternally inherited symbiont, evolution of a temporary controlled incapacitation without elimination in a potentially powerful adaptation for the host, affording entry to facultative or cyclical parthenogenesis. In two of the best known groups of insects showing such modes, coccids and aphids, maternally transmitted symbionts, sometimes of several kinds, are already known to be universal in the females. Although they have not yet been shown to have causative roles in gametogenesis or sex determination, they are closely associated with the events and, in at least two cases, fail to enter male eggs or embryos (35, 36).

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