Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp

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***Wolbachia* are bacteria that live in the cells of various invertebrate species to which they cause a wide range of effects on physiology and reproduction. We investigated the effect of *Wolbachia* infection in the parasitic wasp, *Asobara tabida* Nees (Hymenoptera, Braconidae). In the 13 populations tested, all individuals proved to be infected by *Wolbachia*. The removal of *Wolbachia* by antibiotic treatment had a totally unexpected effect—aposymbiotic female wasps were completely incapable of producing mature oocytes and therefore could not reproduce. In contrast, oogenesis was not affected in treated *Asobara citri*, a closely related species that does not harbor *Wolbachia*. No difference between natural symbiotic and cured individuals was found for other adult traits including male fertility, locomotor activity, and size, indicating that the effect on oogenesis is highly specific. We argue that indirect effects of the treatments used in our study (antibiotic toxicity or production of toxic agents) are very unlikely to explain the sterility of females, and we present results showing a direct relationship between oocyte production and *Wolbachia* density in females. We conclude that *Wolbachia* is necessary for oogenesis in these *A. tabida* strains, and this association would seem to be the first example of a transition from facultative to obligatory symbiosis in arthropod–*Wolbachia* associations.**

Wolbachia are strictly intracellular bacteria infecting a number of invertebrates including mite, crustacean, filarial nematode, and especially insect (1, 2), where 16% of species could be infected (3, 4). Maternally transmitted through the cytoplasm of eggs, these endosymbionts form a monophyletic group relative to other α-proteobacteria, particularly to other *Rickettsia*, causing human diseases such as typhus, Rocky Mountain spotted fever, and Q fever (5). In arthropods, they are distinguished by their ability to modify host reproduction in a variety of ways: reproductive incompatibility in most species (6), thelytokous parthenogenesis in haplodiploid species (7, 8), male-killing in several insects (9), and feminization of genetic males in isopod crustaceans (10). All these effects are advantageous to *Wolbachia* and allow them to persist in host populations (6–10).

Wolbachia are of special interest in the study of the evolution of symbiosis, because they would seem not to fit current theory. Indeed, it is generally accepted that vertically transmitted microorganisms should tend to evolve toward a benign state, or even to be beneficial to their hosts, because their fitness is inextricably linked to host performance (11–13). In this context, it is perhaps not surprising that many symbionts provide new functions that give the host a sufficient fitness gain and that, through the course of evolution, the host finds itself dependent on its symbionts. Various examples support this scenario in eukaryotes where symbionts often are found to be a source of novel metabolic function that increases the host's capacity to exploit resources. Examples include photosynthesis, chemosynthesis, nitrogen fixation, synthesis of vitamins and essential amino acids, methanogenesis, cellulose degradation, and luminescence (14–18). Although *Wolbachia* would seem to follow this type of evolutionary pathway in filarial nematodes (19, 20), in

arthropods, *Wolbachia* are rarely found to be beneficial to their hosts. However, despite physiological costs (21, 22) or even virulence (23), they are able to maintain themselves in arthropod populations through induced modifications to host reproductive biology. Moreover, even if many studies have failed to detect any negative effect of infection (24, 25), a few have shown a slight enhancement of reproductive success in infected individuals (26, 27). Although *Wolbachia* engage in a wide range of interactions with their hosts, they have never been demonstrated to be obligatory to the latter; rather they are usually facultative (secondary symbionts) to their hosts, because cured (aposymbiotic) individuals are unaltered physiologically.

Here, we report that establishing such aposymbiotic lines is impossible in the wasp *Asobara tabida* Nees (Hymenoptera, Braconidae). Females from which *Wolbachia* is removed by antibiotics have no mature oocytes in their ovaries and therefore cannot reproduce. This totally unexpected effect is specific to oogenesis, because other adult traits including male fertility, locomotor activity, and size remain unaffected after antibiotic treatment. Our study describes and tests three hypotheses that could account for these observations: (*i*) antibiotics or (*ii*) bacterial endotoxins [lipopolysaccharides (LPS)] have a specific toxicity against oogenesis, or (*iii*) *Wolbachia* are specifically necessary for oogenesis in this species. Results strongly support the third hypothesis. To our knowledge, this is the first report of a microorganism being necessary for oogenesis in nature.

Materials and Methods

Insect Biology, Strains, and Rearing. *A. tabida* is a common hymenopteran parasitoid wasp whose larvae feed on several *Drosophila* species. Female wasps oviposit into first or second instar *Drosophila* larvae, within which wasp larvae subsequently feed and develop (28). Every individual of *A. tabida* proves to be infected with three *Wolbachia* variants, each characterized by a partial sequence of the *wsp* gene (29). Thirteen *A. tabida* strains originating from different sites were checked for *Wolbachia* infection and were tested for effects of antibiotic treatment (Table 1). Complementary experiments were run on a single strain (Pierrefeu, France). Rearing and experiments were performed under a 12/12 light/dark cycle at 20°C with a relative humidity of 70% using a *Wolbachia*-free strain of *Drosophila melanogaster* as host (Lyon, France).

***Wolbachia* Detection and Localization.** *PCR procedure.* Detection of *Wolbachia* by PCR was conducted either on entire adults or on isolated thoraxes (isolated from liquid-nitrogen-frozen insects to

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Table 1. *Wolbachia* distribution in European *A. tabida* strains

Strain (collection site)	Country (department)	Infected females/total tested, no.	Infected males/total tested, no.
Cordes	France (81)	5/5	5/5
Evans	France (25)	7/7	5/5
Lablachère	France (07)	6/6	4/4
Malaucène	France (84)	5/5	5/5
Pierrefeu	France (83)	32/32	12/12
Plascassier	France (06)	5/5	5/5
Sospel	France (06)	5/5	5/5
St. Laurent	France (69)	5/5	5/5
St. Foy-lès-Lyon	France (69)	5/5	5/5
Villette	France (38)	5/5	5/5
Wervicq-Sud	France (59)	5/5	5/5
Hoge veluwe	The Netherlands	5/5	5/5
Kos	Greece	5/5	5/5

prevent hemolymph contamination). DNA extraction was adapted from Vavre *et al.* (30). PCRs were done by using a Geneamp 2400 (Perkin–Elmer). We used a set of internal primers specific for the *Wolbachia Fts Z* gene (31) that amplify 340 base fragments (forward primers: 5′-TTG CAG AGC TTG GAC TTG AA-3′ and reverse primers: 5′-CAT ATC TCC GCC ACC AGT AA-3′). PCR was done in a 25-μl final volume reaction containing 200 μM dNTP, 10 pm of primers, 0.5 units *Taq* DNA polymerase, and 2 μl of DNA solution. PCR conditions were 1 min at 95°C, then 35 cycles of 30 s at 95°C, 1 min at 55°C, and 90 s at 72°C. After the cycles, there was a 10-min elongation time at 72°C. Amplification products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized by using the BIO-PRINT (version 99) image analysis system (Bioblock Fischer, Illkirch, France).

Cytological analysis of ovaries and oocytes. Fixation of ovaries, DNA staining, and confocal analysis were adapted from Loppin *et al.* (32). Dissected ovaries were fixed in 1× PBS (pH 7.4)/3.7% formaldehyde for 30 min and rinsed in 1× PBS/0.1% Triton X-100. They were incubated for 1 h in a 2 mg/ml RNase A solution at 37°C, rinsed with PBS/0.1% Triton detergent, and incubated for 60 min in 5 μg/ml propidium iodide (PI) or 4′,6-diamino-2-phenylindole (1 μg/ml in PBS/Triton X-100 0.1%) at room temperature. Ovaries were washed in PBS/Triton 0.1% for 10 min and mounted in the same solution. Coverslips were sealed with nail polish before examination. Optical sections were made by using a confocal laser-scanning microscope (LSM 510, Zeiss). PI fluorescence was monitored by using the He–Ne laser 543-nm excitation line and a long-pass 585-nm filter. Images were further processed by using PHOTOSHOP 5.5 (Adobe).

Antibiotic Treatments. *Larval treatment.* This mode of treatment is specific for larval parasitoid species and has been used successfully in various parasitoid species (30). Antibiotics were applied to parasitoids by means of the developing host larva. Infested *D. melanogaster* larvae were fed a standard diet (33) supplemented with antibiotics. These antibiotics are encountered by the endoparasitic larva while they are feeding on host tissues and hemolymph. Antibiotic concentration is indicated in mg/g of *Drosophila* standard diet. All 13 *A. tabida* strains were treated with 2 mg/g of standard diet and for one strain (Pierrefeu, France) we tested various concentrations ranging from 2 to 2.10^{-3} mg/g.

Comparison of antibiotics. To test antibiotic toxicity, we used four antibiotics differing in structure, mode of action, and effectiveness against *Wolbachia* rifampicin and tetracycline, which are efficient against *Wolbachia*, and ciprofloxacin and

gentamicin, which are not (6, 26, 34). These comparative treatments were performed on one *A. tabida* strain (Pierrefeu, France), and served as a control to the related species *Asobara citri*, which is naturally free of *Wolbachia*.

Reduction of bacterial endotoxin (LPS) action. In all Gramnegative bacteria, the outer leaflets of the outer-cell membrane contain LPSs that are released at bacterial death and could induce immunity responses in various vertebrate and invertebrate species (35). LPSs from *Wolbachia* have been detected only in the pathogenic filarial nematode *Brugia malayi*, where they could constitute a mediator of inflammatory pathogenesis in filarial disease (36). To reduce the possible action of these molecules during insect development, we administrated antibiotics orally to adults. In this classical method (6, 26, 34), newly emerged female wasps were fed a mixture of honey and 2% antibiotics for 5 days before they were provided with *Drosophila* larvae for parasitism. For each antibiotic, progeny of four to six treated females were maintained individually without further antibiotic treatment.

Measure of Adult Traits. *Oocyte load. A. tabida* females produce most of their complement of mature oocytes before adult emergence (37). For estimating oocyte load, newly emerged females were fed honey for 5 days to allow the completion of oocyte maturation. Ovaries were dissected in a physiological saline solution. Then, one ovary was transferred into a neutral red solution for 5 min and gently crushed between slide and coverglass to disperse its contents. Oocytes were counted by using a video system. Oocyte load was estimated as twice the number of oocytes in one ovary.

Male fertility. A. tabida is a haplodiploid species where eggs develop into diploid females if fertilized and into haploid males if not fertilized. Fertility of symbiotic and aposymbiotic males was estimated individually by the percentage of daughters among the offspring of the infected females they mated (visual control). The mated, infected females oviposited on an unlimited number of hosts for a period of 4 days.

Locomotor activity. Individual locomotor activity was monitored with a video-tracking and image analysis system, which allows continuous automatic measurement of 120 insects over several days (38). Individuals were isolated in experimental circular glass arenas without hosts, but with honey as food. The locomotor activity of each individual was quantified every 6 min as a binary datum (1 if a wasp moved for at least 2 sec and 0 if not), and hourly activity was calculated as the percentage of active recordings among the 10 hourly recordings. Symbiotic and aposymbiotic individuals were measured 3 days under a 12/12 light/dark cycle at 22°C. The average daily pattern of activity was determined for each individual, and the rate of locomotor activity was calculated as the mean percentage of active recordings.

Tibia length. Right posterior tibias were measured. Adults were dissected, and right forelegs were put onto a slide into water and were measured by using a micrometer.

Results and Discussion

Infection Rate and Localization of *Wolbachia* in *A. tabida*. For all strains, all females and males tested proved PCR-positive for the *Wolbachia*-specific *Fts Z* gene, suggesting complete infection of this species (Table 1). Confocal microscopy clearly demonstrated that *Wolbachia* are present in oocytes and are concentrated particularly at the posterior cytoplasm (Fig. 3 *C–E*), a region that already has been shown to be the preferential site of *Wolbachia* in other Hymenoptera (genera *Nasonia* and *Trichogramma*; refs. 39 and 40). Because posterior cytoplasm generally contains germ-cell determinants in insects (41), presence in this particular locality has been interpreted as an adaptation to enhance bacterial transmission to host progeny (1). All isolated thoraxes
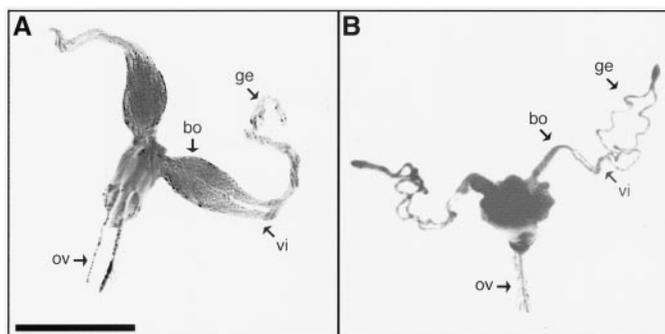
Fig. 1. Genital apparatus of *Asobara tabida* females at emergence. (*A*) Apparatus from untreated control female. Note the presence of numerous mature oocytes in the basal region of ovarioles (bo). (*B*) Apparatus from cured female (larval treatment, rifampicin, 2 mg/g). Note the total absence of oocyte in ovarioles. ge, germarium; ov, ovipositor; vi, vitellarium. [Bar = 1 mm (for *A* and *B*).]

(females and males) proved PCR-positive ($n = 19$), demonstrating the presence of bacteria in tissues other than the germ line. This finding is consistent with recent studies on several insect species (42).

Comparison of Symbiotic and Aposymbiotic Individuals. We first determined the effect of *Wolbachia* by using rifampicin. Treatment of larvae (2 mg/g) cured the wasps of their *Wolbachia*, because all emerging adults proved PCR-negative (10–12 individuals tested for each strain). Surprisingly, newly emerged aposymbiotic females had no mature oocytes in their ovaries (Fig. 1) and did not produce progeny. Females from all strains shown in Table 1 were cured and observed to have no oocytes (20 or more females were dissected per strain). To evaluate the possible effect of the treatment on traits other than oogenesis, we measured additional traits of antibiotic-treated individuals. Male fertility was unaltered; all proved successful in fertilizing females and in producing the same offspring sex ratios as the control group. Moreover, we failed to detect changes in any of the measured traits in treated females—their locomotor activities (a good indicator of overall physiological state) and sizes were found to be unchanged (Table 2). Moreover, females even totally destitute of oocytes conserve apparently normal oviposition behaviors (F.D., personal observation).

These results contrast with those observed on filarial nematodes, where elimination of *Wolbachia* by tetracycline treatment decreases host fitness in several nonspecific ways, including survival, developmental success, and reproduction (19, 20). The effect observed in our study therefore seems to be very specific to oogenesis and may result from any one of three effects: (*i*) the

specific toxicity of rifampicin to *A. tabida* oogenesis; (*ii*) the release of bacterial endotoxins by killed bacterial cells that specifically and totally inhibit oogenesis of *A. tabida* during development; and (*iii*) the necessity of *Wolbachia* to *A. tabida* oogenesis.

Test of Rifampicin Toxicity. To test rifampicin toxicity, we investigated the effects of other antibiotics. Tetracycline, which also removes *Wolbachia*, also totally inhibits oocyte production. Other antibiotics that do not act against *Wolbachia* (ciprofloxacin and gentamicin) have no effect on oogenesis. Moreover, none of these antibiotics has any effect on oogenesis in *A. citri*, a related species that is naturally free of *Wolbachia* (17 individuals all tested PCR-negative; Table 3).

Rifampicin and tetracycline, which prove efficient against *Wolbachia*, differ in their structures and modes of action; rifampicin inhibits prokaryotic DNA-dependent RNA polymerase, whereas tetracycline affects protein synthesis on bacterial ribosomes (43). Given that two antibiotics differing in their structures and functions totally inhibit oocyte production in one insect species and not in a related one, we conclude that the lack of oocytes in treated *A. tabida* females does not result from antibiotic toxicity.

Test of Bacterial Endotoxins Action. In larval treatments, insects were exposed to antibiotics over their whole development, making possible a direct effect of released LPS molecules from the *Wolbachia* cell membrane at the time of oocyte formation. Adult treatment *per os* avoids this difficulty, because oogenesis can be checked in treated females and in their daughters (which had no direct contact with antibiotics). Oocyte load of treated adult females (fed with antibiotics mixed with honey) did not significantly differ from that of control-group females that were fed pure honey ($F_{2, 58} = 0.955$; $P = 0.391$). This result suggests that potential LPSs did not act on formed oocytes. However, significant differences were detected among their progeny. Daughters of rifampicin- or tetracycline-treated females had far lower oocyte loads than the control group ($F_{15,135} = 34.283$; $P < 0.0001$), and a proportion of these daughters had empty ovaries (Table 4). On the contrary, ciprofloxacin and gentamicin treatments had no effect (results not shown). Rifampicin and tetracycline ingestion by adult females thus strongly affected or inhibited oogenesis of their daughters. The effect did not differ from the larval treatment, except that it was less complete and less regular (owing either to variation in antibiotic ingestion by adult females or to differential antibiotic penetration within eggs at different stages of maturation).

Based on these results, it is very unlikely that *Wolbachia*–LPS is responsible for oogenesis inhibition in treated *A. tabida* females. First, the hypothesis has difficulty accounting for results

Table 2. Traits measured on treated and untreated *A. tabida* individuals for all antibiotics

Antibiotics	Mean (±SE) of sex-ratio offspring of males (no. tested)	Mean (±SE) of female locomotor activity rate (no. tested)	Mean (±SE) of female tibia length, mm (no. tested)
Untreated control	0.811 ± 0.015 (16)	0.561 ± 0.021 (30)	0.705 ± 0.004 (35)
Rifampicin	0.798 ± 0.009 (13)	0.538 ± 0.024 (29)	0.712 ± 0.006 (30)
Tetracycline	0.846 ± 0.008 (12)	0.504 ± 0.111 (27)	0.714 ± 0.005 (28)
Ciprofloxacin	0.811 ± 0.018 (11)	0.563 ± 0.081 (24)	0.716 ± 0.006 (28)
Gentamicin	0.808 ± 0.017 (12)	0.595 ± 0.123 (24)	0.707 ± 0.005 (32)
ANOVA	$F_{4,59} = 1.578$	$F_{4,129} = 2.271$	$F_{4,148} = 0.803$
	$P = 0.192$	$P = 0.065$	$P = 0.525$

Sex ratio is the proportion of females. Statistical analysis was performed by one-way ANOVA (after transformation for sex-ratio offspring of males and for female locomotor activity).

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Table 3. Infection by *Wolbachia* (percentage of females PCR positive) and oocyte load (numbers of mature oocytes in ovaries) in untreated and treated *A. tabida* and *A. citri* females for all antibiotics (larval treatment, concentration indicated)

Antibiotics	Dose, mg/g	*A. tabida*		*A. citri*
		Percentage of infected females (no. tested)	Mean (±SE) of oocyte load (no. tested)	Mean (±SE) of oocyte load (no. tested)
Untreated control	0	100% (32)	225 ± 6 (42)	196 ± 8 (24)
Rifampicin	2.00	0% (23)	0 (32)	202 ± 10 (17)
	1.00	0% (18)	0 (28)	197 ± 8 (12)
	0.13	0% (21)	0 (29)	201 ± 16 (8)
Tetracycline	2.00	0% (24)	0 (29)	199 ± 8 (16)
	1.00	0% (20)	0 (30)	190 ± 16 (10)
	0.13	0% (30)	0 (31)	197 ± 18 (8)
Ciprofloxacin	2.00	100% (16)	238 ± 8 (23)	199 ± 16 (15)
	1.00	100% (12)	233 ± 10 (16)	195 ± 12 (13)
Gentamicin	2.00	100% (4)	253 ± 32 (3)	199 ± 20 (4)
	1.00	100% (16)	243 ± 14 (27)	200 ± 8 (18)

Note for high concentration (2 mg/g): gentamicin enhances *Drosophila* larvae mortality, which explained the low *A. tabida* females tested for this treatment. Statistical analysis was performed by one-way ANOVA. ANOVA on all oocyte load values for *A. citri* and on values where oocytes are present in ovaries for *A. tabida* are not significant ($P > 0.05$).

of experiments in which antibiotics are applied to one generation, and oogenesis is checked in the following one, because it would imply LPS transmission across generations. Second, most studies dealing with insect–*Wolbachia* associations have used aposymbiotic strains obtained after antibiotic treatments (adults fed with antibiotic mixed with honey; refs. 6, 26, and 34), and no such case of oogenesis inhibition has been reported in any species. Moreover, oogenesis is ostensibly a conserved function in the course of evolution, and should therefore be equally susceptible to *Wolbachia*–LPS in other insect species. Third, it is difficult to explain how the expression of toxicity to the host of a vertically transmitted, strictly intracellular bacteria such as *Wolbachia* would be maintained through evolutionary time.

Relationship Among Antibiotic Concentration, Oocyte Load, and the Presence of *Wolbachia*. To determine the relationship between oocyte production and the presence of *Wolbachia*, other treat-

ments were performed by using low rifampicin concentrations (larval treatment). These treatments produced females containing intermediate numbers of oocytes demonstrating the continuous (dose-dependent) nature of the antibiotic effect on oocyte production. Moreover, partial oocyte loads were associated with partial *Wolbachia* infection rates (Fig. 2). Fig. 2 shows that oocyte load decreases faster than the concentration of *Wolbachia* DNA necessary for PCR detection—inhibition occurs before bacteria are totally eradicated. Moreover, signal intensities of PCR products are lower for DNA extract from treated females than for untreated control-group females, suggesting a limit to the PCR-detection threshold (F.D., personal observation).

These results strongly suggest a direct relationship between *Wolbachia* density and female oocyte production, which was confirmed by confocal microscopy. The high concentration of *Wolbachia* in the posterior cytoplasm did not permit their precise

Table 4. Number of mature oocytes in *A. tabida* females fed or unfed with antibiotics and in their progeny

Parents			Progeny		
Adult treatment (food for 5 days after emergence)	Mean (±SE) of oocyte load after treatment	Females without oocyte/total tested, no.	Iso-female lines, name	Mean (±SE) of oocyte load after 5 days with pure honey	Females without oocyte/total tested, no.
Honey	228 ± 8	0/22	H1	230 ± 15	0/10
			H2	229 ± 19	0/10
			H3	227 ± 18	0/10
			H4	228 ± 14	0/10
Honey + rifampicin, 2%	232 ± 8	0/19	R1	197 ± 46	0/10
			R2	34 ± 43	5/10
			R3	20 ± 27	4/10
			R4	165 ± 45	0/10
			R5	193 ± 56	0/10
			R6	25 ± 24	3/10
Honey + tetracycline, 2%	237 ± 10	0/20	T1	19 ± 22	5/10
			T2	6 ± 5	4/10
			T3	7 ± 4	4/10
			T4	166 ± 62	0/10
			T5	7 ± 7	6/10

At the end of the parent treatment, lines were started with one female only (iso-female lines). Their progeny were kept individually.
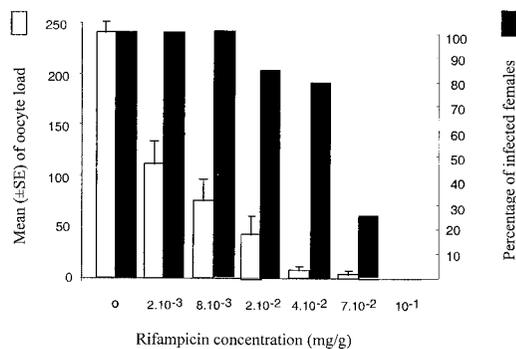
Fig. 2. Effect of rifampicin concentration (larval treatment, from 0 to 0.1 mg/g) on oocyte load and on the rate of infection by *Wolbachia* (percentage of females that were PCR positive). For each value, at least 20 females were used per rifampicin concentration.

Fig. 3. Comparison between ovaries and oocytes from treated females (larval treatment, rifampicin, 2.10^{-2} mg/g) with ovaries and oocytes from untreated control females. (*A* and *B*) Epifluorescent images of ovaries stained with 4',6-diamidino-2-phenylindole. Ovaries were collected from a control *A. tabida* female, which had 228 mature oocytes in ovaries (*A*), or a rifampicin-treated female, containing only 36 mature oocytes (*B*). The ovaries were spread to reveal the oocytes and were oriented with the germarium to the right. (*C–H*) Confocal images of oocytes stained with propidium iodide. Oocytes having been collected from a control female (*C–E*) or a rifampicin-treated female as in *B* (*F–H*). Accumulation of *Wolbachia* is seen at the posterior extremity of the control oocyte (*C*). *D* and *E* are magnified views of the nucleus and posterior regions from the same oocyte, respectively. In contrast, the oocyte in *F* contains very low levels of *Wolbachia* in the posterior region (*H*). [Bar in *A* = 500 μm (for *A* and *B*).]; [Bar in *C* = 50 μm (for *C* and *F*).]

quantification in the present study (Fig. 3 *C–E*). However, bacterial density was reduced dramatically in the posterior oocyte poles of treated females (Fig. 3 *F–H*).

Conclusions

Antibiotic treatments specifically inhibit oogenesis in the wasp *A. tabida*. By using several antibiotics and different doses of active molecules, we showed a clear relationship between the presence of *Wolbachia* in females and their oocyte production. The possibility that this effect could result from LPS release from killed bacteria is rather unlikely, because experiments demonstrated that the effect of antibiotics is not expressed in treated females but only in their untreated daughters. Moreover, it is hardly conceivable that LPS toxicity should be so specific such that oogenesis is the only detectable target. Thus, our results strongly suggest that *Wolbachia* themselves are necessary for *A. tabida* oogenesis, indicating that this is an obligate mutualism. It is already known that *Wolbachia* are able to manipulate chromosomal behavior or strongly influence sex determination. Our results add a new effect of symbiotic bacteria on host biology and fitness.

Our study would seem to be the first demonstration of an obligate association between *Wolbachia* and their arthropod hosts. The few cases where *Wolbachia* benefit their arthropod hosts have proven to be facultative (26, 27). *Wolbachia* thus display an astonishing diversity of effects, resulting in a range of relationships from parasitism to obligate mutualism. The respective contribution of host and bacterial genotypes in this diversity is a matter of discussion (44, 45). *A. tabida* harbor three types of *Wolbachia*, one of which is specific to this insect and which is isolated on the phylogenetic *wsp* tree (29), suggesting the possible phylogenetic uniqueness of this mutualism. Future attempts to obtain derived *A. tabida* strains with different combinations of bacterial variants will allow a more rigorous test of this hypothesis. Symmetrical investigations on *A. tabida* strains originating from other parts of the world are necessary to test whether the effect is specific to the European strains studied here or general to the whole species.

Mutualistic endosymbiosis with bacteria is common in insects (14–18), but the particularity identified in the present study is the restriction of the interaction to oocyte production. Male reproduction and other physiological functions in females are unaffected by *Wolbachia* removal. Thus, contrary to most insect–bacteria associations, the host does not obtain nutritional benefits, although it is unknown which physiological pathway or pathways are involved. We observed that ovaries of aposymbiotic females do indeed contain aborted egg chambers and there is no indication of vitellogenesis, suggesting that *Wolbachia* are some-
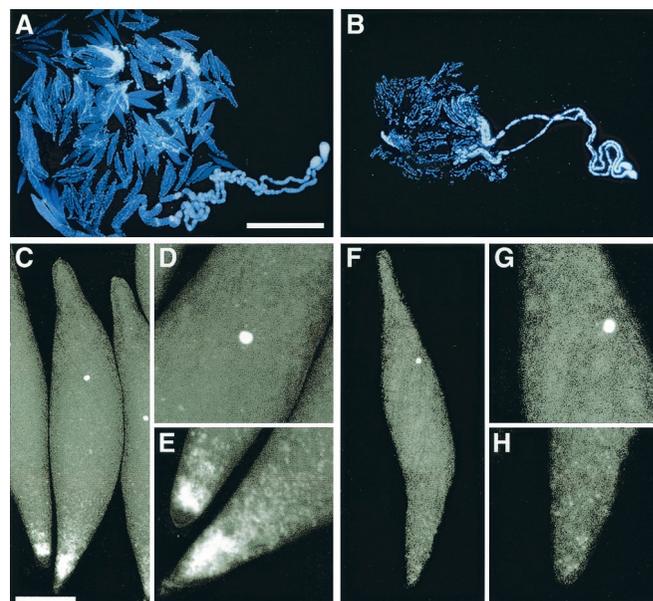
how involved in oocyte differentiation and yolk production and/or transportation rather than in the division of germ cells. Another particularity of the interaction investigated here is that the localization of *Wolbachia* is not restricted to the cytoplasm of one cell type as generally observed in other obligate endosymbioses (17, 18). That closely related species of *A. tabida* do not require *Wolbachia* to complete their oogenesis suggests that this association is rather recent.

From an evolutionary perspective, the transition from a facultative to an obligatory association for the host suggests that the wasp or its ancestor would have become associated with a *Wolbachia* encoding for a necessary oogenesis factor that preexisted the association. Such functional redundancy of host and symbiont genes has been evoked as an intermediate step in the evolution of endosymbiosis, but it is usually thought to precede the loss of function by the symbiont (46–48). Sterility of aposymbiotic *A. tabida* females demonstrates that the host itself would have lost the capacity to produce this now costly factor on its own, thus becoming totally dependent on bacteria for reproduction. A similar substitution of function mechanism may have occurred in endosymbiotic association between the intracellular prokaryote x-bacteria and the unicellular eukaryote *Amoeba proteus*. In this case, the interaction between the bacterium initially harmful to the host evolved to a beneficial state after ≈200 generations in culture. Moreover, experiments demonstrated that the host nucleus had become dependent on the infective organisms for its own functioning (49–51). These studies suggest that evolution from cell parasitism to obligate mutualism can occur rapidly, and that

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preexisting functions are either purged from the host genome or not expressed, because of the costliness of being redundant in the face of the symbiont bacteria. In insect–*Wolbachia* associations, another loss of function may have occurred in the parthenogenetic wasp *Encarsia formosa*, where curing the females of their usual *Wolbachia* reverts asexual reproduction to sexual, but where the male offspring of aposymbiotic females are sterile, thus making *Wolbachia*-induced thelytoky the only possible mode of reproduction in this species (34). However, in this system, the dependence on *Wolbachia* is caused by an indirect effect of the *Wolbachia* rather than a direct one on host physiology.

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