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Wolbachia requirement for oogenesis: occurrence within the genus *Asobara* (Hymenoptera, Braconidae) and evidence for intraspecific variation in *A. tabida*

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Wolbachia are symbiotic bacteria that induce a diversity of phenotypes on their numerous invertebrate host species. In the wasp *Asobara tabida* (Braconidae), each individual harbours three *Wolbachia* strains: wAtab3, which is required for host oogenesis, and wAtab1 and wAtab2, that do not have this function but induce cytoplasmic incompatibility. In this study, we surveyed and identified *Wolbachia* strains in four additional *Asobara* species. We detected *Wolbachia* in one of these species, but both the identity (based on *wsp* gene) and prevalence of the *Wolbachia* detected in natural population indicate that this host species is not dependent on *Wolbachia* for oogenesis. We also compared *A. tabida* lines of different geographical origin for their dependence on *Wolbachia*. All individuals from 16 *A. tabida* lines proved to be infected by the three *Wolbachia* strains wAtab1, wAtab2 and wAtab3, but, interestingly, we found variation among lines in the degree to which females were dependent on *Wolbachia* to produce their oocytes. In three lines, aposymbiotic females (cured from the three *Wolbachia* strains by antibiotics) can produce some oocytes. However, these aposymbiotic females produce fewer and smaller oocytes than symbiotic ones, and the larvae they produce die before full development. Thus, depending on which nuclear genotype they have, *A. tabida* females depend on *Wolbachia* either because they fail to produce any oocyte or because the few oocytes they do produce generate unviable offspring. We discuss the implications of these findings for the understanding of the physiological and genetic deficiency of aposymbiotic females.
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Introduction

Wolbachia form a monophyletic group of alpha-proteobacteria that infect many arthropods and filarial nematodes. Living within the cells of their host, they are maternally transmitted from mother to offspring through the egg cytoplasm (Werren, 1997; Bandi *et al*, 1998; Stouthamer *et al*, 1999). *Wolbachia* have received much attention for three main reasons. First, several surveys suggest that about 20% of all insect species may be infected with *Wolbachia*, making these bacteria one of the most widespread parasites yet identified (Werren *et al*, 1995; West *et al*, 1998; Werren and Windsor, 2000). Second, *Wolbachia* have proved to be masters of modifying arthropod reproduction, including cytoplasmic incompatibility, parthenogenesis, male-killing or feminization. All these modifications allow the infection to spread and persist within host populations (Werren, 1997; Stouthamer *et al*, 1999). Third, in addition to these immediate reproductive modifications, *Wolbachia* infection may have a range of long-term effects on host taxa, such as providing a possible mechanism of rapid speciation (Bordenstein, 2003) or influencing the evolution of sex-determining mechanisms (Rigaud, 1997).

In the great majority of arthropods, *Wolbachia* are facultative for host physiology: individuals cured of the infection remain physiologically functional and able to feed and reproduce. In contrast to this rule, however, some species have become completely dependent on *Wolbachia* infection for reproduction (reviewed in Gottlieb and Zchori-Fein, 2001; Dedeine *et al*, 2003). One of these exceptions is the *Drosophila* parasitoid wasp *Asobara tabida* (Hymenoptera, Braconidae), in which females completely depend on *Wolbachia* infection for oogenesis. Aposymbiotic individuals (antibiotic-cured) of both sexes have an apparently normal morphology, physiology and behaviour, except that females have empty ovaries and fail to produce any oocyte (Dedeine *et al*, 2001). The possibility that the specific inhibition of oocyte production is caused directly by antibiotics, or indirectly through the release of endotoxins from decaying bacteria, has been rejected, thus strongly suggesting that oogenesis is totally dependent on infection itself (Dedeine *et al*, 2001). More recently, it has been demonstrated that, of the three genotypic *Wolbachia* strains co-infecting each *A. tabida* individual, only one strain, wAtab3, is required for oogenesis, whereas the two others, wAtab1 and wAtab2, do not have this ability, but induce cytoplasmic incompatibility (Dedeine *et al*, 2004).

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How oogenesis has become dependent on wAtab3 in the course of evolution of this insect lineage remains an open question. However, a previous result has suggested that this event occurred recently in the *Asobara* lineage (Dedeine *et al*, 2001): another species of the same genus, *A. citri*, does not harbour any *Wolbachia*, suggesting that the dependence on wAtab3 has evolved after the divergence of these two *Asobara* species. The aim of this study was to test whether the dependence on wAtab3 has emerged recently in *Asobara* lineage by studying the prevalence, identity and role of *Wolbachia* in oogenesis both in different species of the genus *Asobara* and within the species *A. tabida*. We confirmed preliminary results from *A. citri*, and surveyed the presence and identity of *Wolbachia* infections in three other *Asobara* species: *A. rufescens*, a very closely related species to *A. tabida* (Vet and Janse, 1984; Vet *et al*, 1984), and two more divergent species, *A. persimilis* from Australia, and *A.* sp, an undescribed species from North America. We also looked for intraspecific variation in the degree of wAtab3 dependence of oocyte production within *A. tabida*. To accomplish this, we compared *Wolbachia* infection and their role in oocyte production in 16 laboratory lines of *A. tabida* established from foundresses obtained from different areas through Europe and North America. In three lines, we found that aposymbiotic females were able to produce some oocytes. We then characterized these oocytes (number and size), assessed whether females could deposit them into *Drosophila* host larvae and tested whether aposymbiotic eggs are able to develop into fertile adults.

Materials and methods

Biological material

Wasps of the genus *Asobara* (Hymenoptera, Braconidae: Alysiinae) are solitary endoparasitoids of several *Drosophila* species and related genera. Females lay their eggs in fly larvae, within which parasitic larvae feed and develop (Carton *et al*, 1986). In this work, we studied five *Asobara* species from 25 localities of four continents (Table 1). Phylogenetic relationships between these species have not been established so far, but several morphological, genetic and ecological studies clearly indicate that *A. rufescens* is the closest related species to *A. tabida* (Vet and Janse, 1984; Vet *et al*, 1984). Except for *A. rufescens*, all the wasps were from established laboratory lines. Parasitoids were reared on a *Wolbachia*-free *D. melanogaster* strain originating from St Foy-lès-Lyon (Rhône, France). Rearing was conducted in vials containing standard *Drosophila* diet (David, 1962), at 20°C under a 12:12 light:dark cycle and 70% RH. *A. rufescens* wasps are known to develop on another host species, *Scaptomyza pallida*, which is more difficult to maintain in laboratory than *D. melanogaster*. For this reason, all *A. rufescens* individuals studied here were collected from the field.

Wolbachia detection and identification

To detect *Wolbachia* infection by PCR, we used a set of internal primers specific for the *Wolbachia ftsZ* gene (for primer sequences, see Dedeine *et al*, 2001). ITS 2 primers

Table 1 Presence and identity of *Wolbachia* in the *Asobara* wasps studied

Species	*Locality*	*Country*	*Individuals tested (no. males–females)*	*Proportion of infection*	*Wolbachia strains*[a]
A. tabida	Cordes	France	5–5	1	1, 2, 3
	Evans	France	5–7	1	1, 2, 3
	Lablachère	France	4–6	1	1, 2, 3
	Malaucène	France	5–5	1	1, 2, 3
	Pierrefeu	France	12–32	1	1, 2, 3[b]
	Plascassier	France	5–5	1	1, 2, 3
	Sospel	France	5–5	1	1, 2, 3
	St Foy-lès-Lyon	France	5–5	1	1, 2, 3
	St Laurent	France	5–5	1	1, 2, 3
	Villette	France	5–5	1	1, 2, 3
	Wervicq-sud	France	5–5	1	1, 2, 3[b]
	Hoge veluwe	The Netherlands	6–8	1	1, 2, 3
	Leiden	The Netherlands	8–9	1	1, 2, 3[b]
	Kos	Greece	5–5	1	1, 2, 3[b]
	Saanich	Canada	7–10	1	1, 2, 3[b]
	Seattle	United States	6–9	1	1, 2, 3[b]
A. rufescens	Leiden	The Netherlands	13–9	1	1[b]
	Wervicq-sud	France	10–10	0	—
A. citri	Brazzaville	Congo	7–10	0	—
	False Bay	South Africa	8–8	0	—
A. persimilis	Brisbane	Australia	6–7	0	—
	Sydney	Australia	5–6	0	—
	Adelaide	Australia	6–6	0	—
	Melbourne	Australia	6–6	0	—
A. sp	Concord	United States	10–12	0	—

[a] *Wolbachia* strains wAtab1, wAtab2 and wAtab3 are here named 1, 2 and 3, respectively.
[b] In these lines, PCR products of the wsp gene have been sequenced and compared with already available sequences (Vavre *et al*, 1999). For the three *Wolbachia* strains, wAtab1, wAtab2 and wAtab3, no variation has been observed (100% of homology).

were used to amplify wasp nuclear DNA as a positive control for template DNA quality (see primers in Allemand *et al*, 2002). All extracts tested negative for this ITS-specific PCR were excluded from the analysis. DNA extraction and PCR amplification were performed as described in Dedeine *et al* (2001).

Diagnostic PCR assay was also used for specific detection of the three *Wolbachia* strains previously described in *A. tabida* (Vavre *et al*, 1999). Based on the *Wolbachia wsp* gene sequence of the three bacteria (Genbank accession numbers AF124856, AF124857, AF124859), specific primers were used for each of them (see Dedeine *et al* (2004) for sequences of primers). All three specific PCR assays were performed as separate reactions on DNA isolated from a single wasp. PCR conditions were as described in Vavre *et al* (2001), except that the annealing temperature used to detect wAtab3 was elevated to 55°C.

A subset of PCR amplification products (one or two extracts per insect line) were directly purified and sequenced. PCR amplicons were gel-purified using Qiagen gel extraction spin columns and used directly in standard fluorescent cycle-sequencing PCR reactions using the *Wolbachia* strain-specific set of *wsp* gene primers. Sequencing reactions were cleaned and run on an automated ABI Prism 310 sequencer. Sequences were aligned to the *Wolbachia* sequences already available in *A. tabida* (Vavre *et al*, 1999).

Obtention of aposymbiotic females and measure of oocyte production

Aposymbiotic females were obtained by antibiotic treatment as described in Dedeine *et al* (2001). The antibiotic (rifampicin, Hoechst) was applied to the wasps through the developing *Drosophila* host larva: 150 μl of a 2% rifampicin solution was added to 1.5 g of standard diet (2 mg/g). Treated *Drosophila* larvae transfer antibiotic to endoparasitic wasp larvae, which then develop into aposymbiotic adult wasps. To measure the number of oocytes produced, newly emerged females were fed honey for 5 days and then dissected in a phosphate-buffered saline solution (1 × PBS, pH 7.4). One ovary was transferred into a neutral red solution for 5 min and gently crushed between a slide and coverglass to disperse its contents. Oocytes were counted using a video system. Individual oocyte production was estimated as twice the number of oocytes in one ovary.

Measurement of oocytes

One ovary of females conditioned as described above was transferred on a slide in a phosphate-buffered saline solution (1 × PBS, pH 7.4). To avoid any deformation of oocytes, ovaries were neither covered with coverglass nor coloured. Preparations were observed under a light microscope (Zeiss, Germany), and the length (L) and width (W) of oocytes were measured using a digital video system. The volume of oocytes was estimated as the volume of a regular ellipsoid ($\pi/3LW^2$).

Developmental assay

Developmental success of parasitoids was measured as the total number of offspring produced by eight 5-day-old fertilized females provided for 48 h with about 200 *D. melanogaster* host larvae (24-h-old when parasitoids were introduced), in vials containing about 10 g of standard diet. Adult wasps normally emerged between 26 and 30 days after oviposition at 20°C, males emerging before females. When all emerging wasps were counted, the remaining full *Drosophila* puparia were dissected to detect and count parasitoid larvae that died during development. Three environmental factors that can potentially affect the developmental success of parasitoids were studied: (1) the *Drosophila* host species. Four species were tested: *D. melanogaster*, *D. subobscura*, *D. simulans* and *D. hydei*), (2) the temperature of development (15 and 20°C) and (3) the richness of *Drosophila* diet: two diets were tested: one in which the energetic content was normal, the other in which it was reduced to 25%.

Measurement of parasitoid larvae

At the time of host pupation, the parasitoid larva leaves the host body and stays in the puparium, thus becoming an ectoparasite (Carton *et al*, 1986). At this stage, *Asobara* larvae are easily visible through the *Drosophila* puparium. Batches were prepared with about 100 *Drosophila* eggs (0–8 h old) in vials containing standard diet. After 48 h incubation, eight 5-days-old fertilized parasitoid females were added to each vial and allowed to oviposit for 8 h into *Drosophila* hosts. At 1, 4 and 7 days (±3 h) after *Drosophila* pupation, samples of parasitized *Drosophila* were dissected and parasitoid larvae were collected and measured (L: length and W: width) using a micrometer. The volume of larvae was estimated as the volume of a regular ellipsoid ($\pi/3LW^2$).

Statistical analysis

We conducted an ANOVA with one (infection status) or two (infection status, insect line) factors. The significance level α of ANOVAs, $P = 0.05$, was adjusted following the Bonferroni procedure, to correct for multiple analyses (Sokal and Rohlf, 1995). Statistical analysis was performed using JMP™ version 5.1.1 (Software).

Results

Wolbachia infection in the genus *Asobara*

In *A. tabida*, all individuals proved co-infected by the three *Wolbachia* strains wAtab1, wAtab2 and wAtab3, and no variation was observed in the *wsp* sequences of *Wolbachia* strains from different insect lines (Table 1). Among the four other *Asobara* species surveyed, three proved uninfected (*A. citri*, *A. persimilis* and *A. sp*). One population of *A. rufescens* (Wervicq-sud) was uninfected, while all individuals from the other population (Leiden) were infected (Table 1). Diagnostic PCR assay and sequencing analyses both showed that the infected *A. rufescens* population only harbours a single *Wolbachia* strain that is 100% homologous (based on 378 base pairs of *wsp* gene) to the strain wAtab1 infecting *A. tabida* (gene bank access: AF124856).

Oocyte production in symbiotic and aposymbiotic *A. tabida* females

The efficiency of antibiotic treatment was checked by testing at least 10 females per line for the presence of *Wolbachia* (217 females in total). All treated females were PCR-negative for the *fts-Z Wolbachia* gene and PCR-positive for the ITS 2 region of the insect DNA.

We compared oocyte production of symbiotic and aposymbiotic females from all *A. tabida* lines (Table 2). Interestingly, the statistical analysis performed only on symbiotic females shows that the oocyte production varies significantly between the *A. tabida* lines ($F=14.488$; df $=15$, 225; $P<0.0001$). Consistent with previous studies (Dedeine *et al*, 2001, 2004), ovaries of aposymbiotic females were found completely empty of any oocyte in 13 European lines, indicating their total dependence on *Wolbachia* for oogenesis. Conversely, aposymbiotic females from Saanich, Seattle and Leiden lines were able to mature oocytes (Table 2). Comparing these three insect lines (two-way ANOVA) shows that (1) aposymbiotic females produced far fewer oocytes than symbiotic females ($F=1068.6$; df $=1$, 110; $P<0.0001$) with 58.7% reduction in Saanich strain, 68.7% in Seattle and 80.4% in Leiden, and that (2) despite the lack of significance of the factor 'line' ($F=2.2$; df $=1$, 110; $P=0.114$), the interaction between the two factors (infection status × line) is significant ($F=21.4$; df $=1$, 110; $P<0.0001$), suggesting that the degree of dependence for oocyte production may be a continuous trait among *A. tabida* populations. Surprisingly, the three *A. tabida* lines able to produce a certain number of oocytes without *Wolbachia* are also the lines that produce on average the smaller number of oocytes when females are infected by *Wolbachia* (see Table 2).

Oocyte size

We compared the length and width of symbiotic and aposymbiotic oocytes (Table 3). In all cases, aposymbiotic oocytes are far smaller than symbiotic ones, both for width ($F=445.5$; df $=1$, 594; $P<0.0001$) and length ($F=2637.9$; df $=1$, 594; $P<0.0001$). The factor 'line' is also significant for width ($F=23.7$; df $=2$, 594; $P<0.0001$) and interacts significantly with the factor 'infection status' ($F=20.703$; df $=2$, 594; $P<0.0001$). Conversely, the factor 'line' is not significant for length ($F=1.6$; df $=2$, 594; $P=0.205$) and does not interact with the factor 'infection status' ($F=1.367$; df $=2$, 594; $P=0.255$). It is clear, however, that most of the variation is attributable to infection status. Accordingly, reduction of oocyte volume ranges from 49.6% in Saanich strain and 53.7% in Seattle to 61.0% in Leiden.

Developmental success

To compare the developmental success of aposymbiotic and symbiotic individuals, we allowed aposymbiotic and symbiotic females to parasitize *Drosophila* larvae. Offspring emerged from each vial were counted 30 days after oviposition. While symbiotic females produced a substantial number of adult offspring, aposymbiotic females did not produce any. In vials where aposymbiotic females had oviposited, we could observe two clear morphological abnormalities of *Drosophila* puparia: (1) dark orange coloration instead of the yellow characterizing puparia containing *Wolbachia*-infected parasitoids, and (2) presence of an internal gas bubble. We then counted these easily distinguishable puparia in all vials (Table 4; in total $n=659$). At dissection ($n=106$), all puparia contained a single living parasitoid *A. tabida* larva, a gas bubble taking approximately one-third of the *puparium* volume and a liquefied mass of white host tissues. Finally, after 21 extra days, nothing emerged from the remaining 553 puparia, and dissection showed that the wasp larvae had died.

Table 2 Number of oocytes (mean ± SD) produced by symbiotic and aposymbiotic females of the 16 *A. tabida* lines

A. tabida line	*Symbiotic*	*Aposymbiotic*
Villette	294.6 ± 20.4 (13)	0 (13)
Kos	291.4 ± 15.3 (14)	0 (14)
Gazeran	288.7 ± 23.7 (14)	0 (14)
St Foy-lès-Lyon	286.0 ± 24.2 (15)	0 (15)
Malaucène	283.4 ± 21.1 (16)	0 (16)
Villeneuve d'Ascq	283.3 ± 20.9 (15)	0 (15)
Vénérieu	280.5 ± 25.8 (13)	0 (13)
St Laurent	279.5 ± 31.4 (15)	0 (15)
Cordès	276.3 ± 30.9 (14)	0 (14)
Lablachère	275.4 ± 29.5 (15)	0 (14)
Wervicq-sud	271.0 ± 24.3 (14)	0 (14)
Hoge veluwe	267.5 ± 24.3 (14)	0 (12)
Pierrefeu	267.3 ± 23.2 (20)	0 (20)
Leiden	248.0 ± 31.9 (17)	48.7 ± 18.6 (20)
Seattle	242.1 ± 21.9 (15)	75.8 ± 20.5 (20)
Saanich	208.2 ± 14.6 (20)	85.9 ± 39.4 (24)

Number of dissected females in brackets.

Table 3 Length, width and estimated volume of symbiotic (S) and aposymbiotic (A) oocytes from Saanich, Seattle and Leiden *A. tabida* females

Traits	*Infection status*	*Saanich*	*Seattle*	*Leiden*
Length (mm)	S	0.238 ± 0.006	0.235 ± 0.009	0.237 ± 0.008
	A	0.222 ± 0.010	0.222 ± 0.010	0.221 ± 0.009
Width (mm)	S	0.037 ± 0.003	0.036 ± 0.003	0.037 ± 0.002
	A	0.027 ± 0.003	0.025 ± 0.003	0.024 ± 0.002
Volume (mm^3)	S	0.344 ± 0.004	0.325 ± 0.004	0.341 ± 0.004
	A	0.174 ± 0.004	0.151 ± 0.004	0.132 ± 0.004

Each mean (± SD) was calculated from the measure of 100 oocytes.

Table 4 Number of offspring and abnormal puparia (see text) produced (mean ± SD) by symbiotic (S) or aposymbiotic (A) females

Infection status	*Offspring produced*					
	Saanich		*Seattle*		*Leiden*	
	Adult	*Abnormal puparia*	*Adult*	*Abnormal puparia*	*Adult*	*Abnormal puparia*
S	86.8 ± 14.7 (15)	0.0 ± 0.0 (15)	82.7 ± 16.4 (12)	0.0 ± 0.0 (12)	72.1 ± 21.5 (14)	0.0 ± 0.0 (14)
A	0.0 ± 0.0 (24)	14.5 ± 11.1 (24)	0.0 ± 0.0 (20)	8.1 ± 8.0 (20)	0.0 ± 0.0 (22)	6.8 ± 9.3 (22)

Number of vials studied in brackets.

Since the developmental success of parasitoids may change according to complex interactions between the host species, temperature and feeding condition of host larvae (Vinson and Iwantsch, 1980; Carton *et al*, 1986; Wajnberg *et al*, 1990), we tested whether changing developmental conditions would allow aposymbiotic larvae to develop. In a series of similar experiments, we thus compared the developmental success of aposymbiotic and symbiotic *A. tabida* larvae under different rearing conditions (ie, we tested four different *Drosophila* species as developing host, two temperatures and two *Drosophila* diets), and clearly demonstrated that all aposymbiotic larvae failed to develop, although the presence of parasitoid larvae within abnormal host puparia (described above) demonstrated that wasps did lay eggs into *Drosophila* larvae (results not shown).

Size and growth of parasitic larvae

On days 1, 4 and 7 after *Drosophila* pupation, we compared the sizes of symbiotic and aposymbiotic wasp larvae after extraction from host puparia (Table 5). At 1 day after host pupation, aposymbiotic larvae are far smaller than symbiotic ones both in length ($F = 324.3$; df = 1, 32; $P < 0.0001$) and width ($F = 96.0$; df = 1, 32; $P < 0.0001$). Consequently, their volume is correspondingly reduced, showing their poor early development. Moreover, it is clear that symbiotic larvae go on growing till they have ingested all host *Drosophila* tissues (volumes increase by 3.5 between days 1 and 3, and by 1.2 between days 3 and 7; analysis of length: $F = 70.732$; df = 2, 46; $P < 0.0001$; analysis of width: $F = 155.957$; df = 2, 46; $P < 0.0001$). Conversely, aposymbiotic larvae do not grow further and remain unchanged over time both for length ($F = 0.02$; df = 2, 440; $P = 0.98$) and width ($F = 0.12$; df = 2, 44; $P = 0.89$).

Discussion

Many insect species depend on symbiotic microorganisms for essential physiological functions such as nutrition, reproduction or defense against predators or pathogens (Buchner, 1965; Margulis and Fester, 1991; Douglas, 1994). However, the evolutionary mechanisms by which such host functions have become dependent on the symbiont remain poorly understood. In this issue, the association between the parasitoid wasp *A. tabida* and *Wolbachia* may be an interesting model. Previous studies have shown that among the three *Wolbachia* strains infecting each *A. tabida* host individual, one strain, wAtab3, is required for host oogenesis, whereas the two other strains, wAtab1 and wAtab2, do not have this function, but induce cytoplasmic incompatibility (Dedeine *et al*, 2001, 2004). This unique insect–bacteria association may document an example of evolutionary transition, where a parasite has become obligate for a single essential function of its host (Dedeine *et al*, 2001, 2003, 2004). In the present study, we report inter- and intraspecific variations in the presence and identity of *Wolbachia*, especially wAtab3, and on the role of infection on oocyte production by the host.

No *Wolbachia* infection has been detected in *A. citri*, *A. persimilis*, *A.* sp (North America) and in one population of *A. rufescens*, showing that these insects do not need *Wolbachia* to reproduce. Moreover, individuals of the infected *A. rufescens* population do not harbour wAtab3, as expected if they shared the same dependence on *Wolbachia* as *A. tabida*. In contrast, infected *A. rufescens* individuals harbour a *Wolbachia* strain, wAtab1, which also infects *A. tabida*, where it induces cytoplasmic incompatibility (Dedeine *et al*, 2004). These results suggest that the dependence on wAtab3 for oogenesis has evolved after the divergence of *A. tabida* from the other species studied here. Furthermore, the close relatedness of *A. rufescens* and *A. tabida* (Vet and Janse, 1984; Vet *et al*, 1984) suggests that the dependence on wAtab3 for oogenesis may be specific to *A. tabida*.

Within *A. tabida* species, individuals from all 16 geographical *A. tabida* lines tested here harbour the three *Wolbachia* strains, wAtab1, wAtab2 and wAtab3. Moreover, no variation was found in the *wsp* sequence within each *Wolbachia* strain between the different insect lines. Nevertheless, we found intraspecific variation in the degree of dependence of females on *Wolbachia* to produce oocytes: while aposymbiotic females cannot produce any oocyte in 13 European lines, aposymbiotic females from Saanich, Seattle and Leiden did produce a certain number of oocytes. However, these aposymbiotic females produced fewer and smaller oocytes than those produced by symbiotic females. Further, even though embryos are apparently able to hatch, the larvae produced die early during development. Thus in all cases, the dependence on *Wolbachia* infection for reproduction is complete in *A. tabida*, but, depending on their nuclear genotype, females lacking wAtab3 either fail to produce oocytes or produce few small oocytes that do not develop.

One question then is whether the two observed phenotypes (ie, no oocyte production and unviable larval offspring) have independent physiological bases, or whether they proceed from a unique deficiency of aposymbiotic females. Our results support the second hypothesis for two main reasons. First, aposymbiotic females produce far fewer oocytes than symbiotic ones (in all cases <50%), suggesting that, in addition to producing unviable offspring, they suffer strong inhibition of oogenesis. Second, during the curing process, triply infected females oviposit in *Drosophila* larvae which have been feeding on antibiotics. Thus, the *A. tabida* eggs were immersed in host body fluids that contain antibiotics once they had been injected into the host. It is likely that *Wolbachia* are eliminated at a very early developmental stage. However, the early removal

Table 5 Length, width and estimated volume of symbiotic (S) and aposymbiotic (A) *A. tabida* larvae (line of Saanich) at three developmental stages: 1, 3 and 7 days (±3 h) after host pupation

Traits	*Infection status*	*+1 day*	*+3 days*	*+7 days*
Length (mm)	S	1.738±0.112	2.015±0.178	2.211±0.096
	A	0.842±0.170	0.854±0.165	0.848±0.182
Width (mm)	S	0.555±0.018	0.959±0.103	1.006±0.096
	A	0.298±0.106	0.304±0.115	0.318±0.117
Volume (mm^3)	S	0.560±0.072	1.967±0.075	2.367±0.075
	A	0.098±0.075	0.105±0.075	0.113±0.078

Each mean (±SD) was calculated from at least 15 larvae.

of *Wolbachia* does not disturb further development of larvae, since we always obtain adult wasps of both sexes that look normal in all respects except for oocyte production (Dedeine *et al*, 2001). Thus, the two phenotypes observed are probably resulting from the same failure of aposymbiotic females to transmit a factor to their oocytes that is necessary for differentiation and development. Differences among *A. tabida* lines could simply result from some dosage effect.

One interesting result concerns the apparent relationship between the number of oocytes produced by infected females (their potential fecundity) and the number of oocytes produced by uninfected females (their degree of dependence on *Wolbachia*). Indeed, only females of the lines Saanich, Seattle and Leiden can produce some oocytes when uninfected. Unexpectedly, these lines also produce the fewest oocytes when infected. This suggests a genetic trade-off between the ability of females to produce some oocytes without *Wolbachia*, and their potential fecundity when infected, although additional quantitative genetic analyses are needed to better understand this relationship.

Another question is to understand how variation can be maintained within *A. tabida* species. Two main hypotheses have been proposed to explain how *A. tabida* became dependent on wAtab3 (reviewed in Dedeine *et al*, 2004). The hypothesis of the Sterility of Aposymbiotic Daughters (SAD) phenotype suggests that wAtab3 specifically sterilizes daughters that did not inherit wAtab3 from their infected mothers. As for cytoplasmic incompatibility, SAD-inducing *Wolbachia* would produce two molecules: a toxin produced in mothers but specifically inhibiting oogenesis of daughters, and a toxin-specific antidote produced in infected daughters only. According to the SAD hypothesis, differences between *A. tabida* lines that either completely or partly depend on wAtab3 to produce oocytes may be due either to differences in the efficacy of the toxic molecule produced by *Wolbachia* or to some resistance that insects have evolved against it.

Another possible hypothesis is that the high prevalence of wAtab3 within *A. tabida* populations protects nuclear genes that control oogenesis from selection, and could have allowed deleterious genetic changes (mutations and/or fixation of new allelic forms) to accumulate. According to this hypothesis, differences between *A. tabida* lines may be due to qualitative or quantitative differences in the genetic modifications that have accumulated. Lines from Saanich, Seattle and Leiden may have accumulated less genetic modification, or with less deleterious consequences, than the other lines. This outcome could be explained if these populations are in an evolutionary transitory phase and currently evolving towards complete dependence, or if some local specific selective pressures have partly purged them from deleterious genetic changes, either directly through selection or indirectly through pleiotropic effects. It is possible that the variation reported in this study may serve to distinguish the two hypotheses in future investigations.

In conclusion, this study raises many new questions, especially regarding the genetic basis of the poor performance of aposymbiotic females, the pathway of bacterial restoration and the way wAtab3 *Wolbachia* acquired this capacity. For all these questions, the reported intraspecific variation in the degree to which *A. tabida* females are dependent on wAtab3 to produce their oocytes will certainly provide a useful foundation for future genetic investigations.

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