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DO VARIABLE COMPENSATORY MECHANISMS EXPLAIN THE POLYMORPHISM OF THE DEPENDENCE PHENOTYPE IN THE *ASOBARA TABIDA-WOLBACHIA* ASSOCIATION?

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Wolbachia **are symbiotic intracellular bacteria, which are classified as reproductive parasites. Although generally facultative,** ***Wolbachia*** **is necessary for** ***Asobara tabida*** **(Hymenoptera), because aposymbiotic females do not produce any offspring. Interestingly, the ovarian phenotype of aposymbiotic females is variable: some females do not produce any eggs, whereas others do produce some eggs, but these are aborted. Here, we show that the ovarian phenotype of aposymbiotic females is highly polymorphic within populations, although dependence remains complete in both cases. We also identified some lines in which aposymbiotic females were able to produce a very few viable offspring, further extending the range of variation observed. These results suggest that various factors actively maintain polymorphism. We demonstrated that** ***Wolbachia*** **is necessary to trigger oogenetic processes, but that the ovarian phenotype was determined by the host only. Phenotypic variation was also correlated with the differential expression of genes controlling iron homeostasis and oxidative stress, which are potentially involved in the evolution of dependence. This suggests that variation in the ovarian phenotype could reflect selection for different levels of compensatory mechanisms in response to** ***Wolbachia*** **infection, and that polymorphism is maintained through selection on different antagonist traits influenced by oxidative stress.**

KEY WORDS: **Apoptosis, oxidative stress, symbiosis, tolerance.**

Wolbachia (Anaplasmataceae) are intracellular bacteria that are best known for manipulating the reproduction of their numerous arthropod hosts (Werren et al. 2008). This reproductive parasite is maternally transmitted through the cytoplasm of eggs, and various different strategies allow it to spread and maintain itself within host populations. These strategies can increase the production of females versus males through male-killing, feminization of genetic males, or induction of thelytokous parthenogenesis. Another strategy is to decrease the number of offspring produced by uninfected females through cytoplasmic incompatibility. In the great majority of cases, *Wolbachia* are thus facultative for the survival and reproduction of their arthropod hosts. On the contrary, they are obligate mutualists in filarial nematodes (Fenn and Blaxter 2004). Because interactions between *Wolbachia* and their hosts encompass the whole parasitism–mutualism continuum, *Wolbachia* are considered to provide one of the best model systems for studying the evolution of symbiosis.

One surprising extended phenotype of *Wolbachia* occurs in *Asobara tabida* (Hymenoptera: Braconidae), a *Drosophila* parasitoid wasp. In this species, eliminating *Wolbachia* leads to

the inability of females to complete oogenesis (Dedeine et al. 2001). This is one of the rare instances of host dependence upon *Wolbachia* in Arthropods (Dedeine et al. 2003). This transition toward host dependence is even more surprising in that it must have evolved very recently, because no other species of the *Asobara* genus depends on *Wolbachia* for oogenesis (Dedeine et al. 2005; Kremer et al. 2009a). Cellular and molecular studies have begun to shed light on how dependence evolved. Cytological analysis of the ovarian phenotype has shown that eliminating *Wolbachia* triggers programmed cell death (PCD) in egg chambers within the ovaries, explaining why aposymbiotic females are unable to complete oogenesis, and suggesting that *A. tabida* has lost its ability to control PCD in the absence of *Wolbachia* (Pannebakker et al. 2007). At the molecular level, *Wolbachia* interferes with iron metabolism in *A. tabida*, and especially with the expression of ferritin, a protein involved in iron homeostasis and oxidative stress control (Kremer et al. 2009b). Cellular physiology has also been shown to be disrupted in other systems by *Wolbachia* infection, especially via the generation of oxidative stress (Brennan et al. 2008). Based on these findings, it has been proposed that *Wolbachia* infection could generally disturb the cellular physiology by increasing oxidative stress, and this could in turn interfere with PCD. In *A. tabida*, compensatory mechanisms could have been selected for to reduce the side effects of this perturbation, resulting in the evolutionary development of dependence (Kremer et al. 2009b).

Another characteristic of this association is that the ovarian phenotype of aposymbiotic females (i.e., the egg load after eliminating *Wolbachia*) displays a high level of intraspecific variation. Of 14 European laboratory strains of *A. tabida*, 13 did not produce eggs after *Wolbachia* had been eliminated (Dedeine et al. 2005). In contrast, aposymbiotic females of the 14th line, as well as from both of the two North American lines tested, did produce a few eggs. These eggs were successfully laid and hatched, but the larvae died at an early developmental stage (Dedeine et al. 2005). Despite this variation in the ovarian phenotype, dependence was complete in all cases, because no viable offspring are produced. This implies that ovarian phenotype of aposymbiotic females cannot be subject to direct selection, which raises questions about the nature, origin, and maintenance of variation in this particular phenotypic trait. Does it reflect drift of a neutral polymorphism resulting in the fixation of different phenotypes in different populations? Is this phenotypic trait indirectly subjected to selection by acting on another trait, which indirectly influences egg production in aposymbiotic females?

To understand the origin of this polymorphism, comparing populations that have diverged over a long period of evolution constitutes a major methodological issue because these populations have experienced distinct evolutionary histories, and therefore may have accumulated major genetic differences. For this reason, we analyzed the variation of the egg load in symbiotic and aposymbiotic females within and between various *A. tabida* populations collected in France. To access the natural variation within these populations, lines were analyzed three generations after collection, limiting the bottleneck effect of laboratory rearing. This allowed us to determine the extent to which egg load is polymorphic in natural populations, and to assess the link between the degree of dependence on *Wolbachia* for producing eggs (i.e., their ovarian phenotype), and the number of eggs produced by *Wolbachia*-infected females. We also conducted crossing experiments between lines to analyze the genetic basis of the variation observed. Finally, we used this variation to compare the expression of genes involved in iron metabolism and oxidative stress control in lines exhibiting different ovarian phenotypes. Such comparisons might make it possible to determine the extent to which the ovarian phenotype is linked to controlling oxidative stress.

Material and Methods

BIOLOGICAL SYSTEM

Asobara tabida is a solitary parasitoid wasp (Hymenoptera; Braconidae) that parasitizes different species of *Drosophila*. *Asobara tabida* females lay eggs into a first- or second-instar larva of *Drosophila* without killing it. As an endoparasite, the wasp larva consumes the hemolymph of the host larva to complete its growth. After *Drosophila* pupation, the parasitoid becomes an ectoparasite, and consumes its host before pupating and emerging. All individuals of *A. tabida* are naturally infected by three strains of *Wolbachia* (*w*Atab1, *w*Atab2, and *w*Atab3). *w*Atab1 and *w*Atab2 induce cytoplasmic incompatibility, whereas *w*Atab3 is necessary for oogenesis to be completed (Dedeine et al. 2001, 2004).

GENERAL PROCEDURES

We measured two main phenotypic traits: (1) the number of eggs produced by "*Wolbachia*-infected" or "symbiotic" females, a characteristic that we designate here the "egg load"; (2) the number of eggs produced by "antibiotically cured females" or "aposymbiotic" females, a characteristic that we designate here the "ovarian phenotype."

Antibiotic treatment

As described in Dedeine et al. 2001, rifampicin 2% (Hoechst, Germany) was added to the axenic nutritive medium to reach a final concentration of 2 mg/g of standard diet. Seventy *D. melanogaster* eggs were deposited in this medium, and allowed to be parasitized by three female wasps. The developing *Drosophila* thus transferred the antibiotic to each of the endoparasitoid wasp larvae, rendering them aposymbiotic. As a control, the same procedure was performed without the antibiotic treatment. Wasps

were maintained under controlled conditions (climate chambers at 21°C, 70% relative humidity and cycle LD 12:12).

Infection status

To test for the infection status, females were individually crushed (TissuLyser, Qiagen, Germany; 30 sec, 25 Hz) in 150 μL of 5% Chelex solution (Bio-Rad, CA), and kept at 56°C overnight. After 15 min at 95°C, the samples were centrifuged at 16,000 *g* for 4 min, and stored at −80°C. Multiplex PCR reactions (25 μL) were performed to detect both the bacterial *FtsZ* gene (F2/R2, Dedeine et al. 2001), and the ITS host nuclear region (ITS2U/ITS2L, Allemand et al. 2002) to confirm extraction efficiency. The reaction mix was composed of 1X Taq Buffer, 200 μM dNTP, 1.5 mM $MgCl_2$, 200 nM of each primer, 1 IU Taq DNA polymerase (Euroblue taq, Eurobio, France), and 2 μL of the previous extract. PCR was performed using the thermocycler Tetrad2 (Bio-Rad, CA). Thermal cycling conditions were 1 min at 95°C; a total of 35 cycles of 30 sec at 95°C, 1 min at 57°C, 1 min 30 sec at 72°C; and a final extension step of 10 min at 72°C. To confirm the effectiveness of the antibiotic treatment, the infection status of 10 hatching females/line was tested by PCR and revealed no amplification for *Wolbachia FtsZ* gene.

The diversity of *Wolbachia* strains infecting the symbiotic females (*w*Atab1, *w*Atab2, and *w*Atab3) was also characterized using the primers detailed in Mouton et al. 2004, and PCR products were directly sequenced for the *w*Atab3 strain on one individual per line/population (GenoScreen, France).

Egg load

Asobara tabida is a proovogenic wasp, which means that females emerge with a stock of eggs, almost all of which are mature. Females were isolated at emergence, and fed with honey for five days, to ensure that egg maturation was complete. The two ovaries were dissected in a Phosphate Buffer Saline solution (1X PBS pH 7.4, Eurobio, France), individually transferred onto a slide, and then gently crushed between a slide and coverslip to disperse the egg content. These preparations were observed under a microscope (AxioCam Imager Z.1, Zeiss, Germany), and the eggs in each ovary counted (ImageJ software, Abramoff et al. 2004). Egg production was calculated as the sum of eggs in the two ovaries.

Offspring production after antibiotic treatment

Some lines produced a few eggs. To determine whether these eggs were viable and able to develop up to adulthood, three aposymbiotic females were allowed to oviposit on ∼200 *D. melanogaster* eggs in an axenic nutritive medium (10 replicates/line). Pupae were examined to find out whether there was an abortive parasite inside (i.e., an "abnormal" pupae), and any adults that emerged were counted. These adults were allowed to reproduce. Then their infection status was checked by PCR (see above).

Table 1. Geographic origin of the *A. tabida* lines. Laboratory strains are written in italic.

Population	Number of lines	Coordinates
Montigné sur Moine (Mtg)	1	47.08; −1.13
Igé (Igé)	1	46.39; 4.74
Ville Sollier (Vsl)	1	46.14; 4.88
Sainte-Foy-lès-Lyon B (SFb)	3	45.73; 4.81
Sainte-Foy-lès-Lyon R (SFr)	3	45.73; 4.81
Saint Laurent d'Agny (SLA)	4	45.64; 4.68
Villette de Vienne (Vi)	2	45.58; 4.91
Les Roches de Condrieu (Co)	4	45.45; 4.77
Saanich (NA)	Laboratory strain	48.46; −123.33
Pierrefeu (Pi)	Laboratory strain	43.87; 7.08
Pierrefeu (Pi3)	Laboratory strain	43.87; 7.08
Sainte-Foy-lès-Lyon (SF)	Laboratory strain	45.73; 4.81

VARIATION OF THE EGG LOAD WITHIN AND AMONG NATURAL POPULATIONS OF *A. TABIDA*

We performed a survey of field populations in France, especially along the north–south axis of the Rhône valley (Table 1). Open traps baited with split bananas were placed in orchards. These traps were exposed to natural colonization for 10 days before being brought back to the laboratory. Newly emerged parasitoids were then collected from these traps. Because *A. tabida* females often do not parasitize *Drosophila* larvae when set up as isofemale lines, lines were created from three females that were allowed to oviposit on *Wolbachia*-free *D. melanogaster* eggs (originating from Sainte-Foy-lès-Lyon, France) reared on an axenic nutritive medium (David 1962). These lines were maintained and amplified for three generations (using three females each time) before experiments were conducted under controlled conditions (climate chambers at 21°C, 70% relative humidity and cycle LD 12:12).

Egg load was assessed for 10 females per experimental condition (combination of population, line and infection status; see protocol for general procedures). The variation in the egg load can be estimated between ovaries within female (residuals), between females within line (female factor), between line within the same population (line factor), or between population (population factor). For that purpose, we carried out a generalized linear nested mixed model because egg loads are count data and all of the three factors are random. We fitted this model thanks to the lmer function from the lme4 package (Bates et al. 2008) for the R software: lmer (eggs ∼ 1 + (1 | population) + (1 | line) + (1 | female)). Variance components were estimated at all levels of the nested

analysis except the last one (residuals), because this component does not actually exist given the way that the model is formulated in lmer. The developmental variability between the two ovaries of a given female was estimated as the absolute difference in the number of eggs between the right and left ovaries. Females with only one ovary and females producing no eggs were excluded from the analysis. Variances were compared between symbiotic and aposymbiotic females using a Fisher's test.

To permit comparison with previous findings (Dedeine et al. 2001, 2005), we also included in this study two other lines that had been maintained in the laboratory for ∼100 and ∼50 generations respectively: (1) Pi3 from Pierrefeu (France), which only harbored the *w*Atab3 strain (necessary for oogenesis completion), and produced no eggs after antibiotic treatment, (2) SF from Sainte-Foy-lès-Lyon (France), which produced some eggs after antibiotic treatment. All the lines are described in Table 1.

CROSSING EXPERIMENTS AND THE GENETIC BASIS OF EGG LOAD

For crossing experiments, we first used two laboratory lines (Pi and NA), which have been kept in the laboratory for eight years (around 100 generations), and are probably highly homozygous. Aposymbiotic females belonging to the Pi line (Pierrefeu, France) do not produce eggs, whereas those belonging to NA line (Saanich, Canada) still produce a few eggs, which develop abnormally and die (Dedeine et al. 2005). The egg load of 30 females was estimated for Pi × NA crosses. Pupae of both populations were isolated to ensure that the females were virgins. Direct crosses (♀Pi × ♂Pi and ♀NA × ♂NA) and reciprocal crosses (♀Pi × ♂NA and ♀NA × ♂Pi) were performed by mating three virgin females with males. After mating, these females were allowed to oviposit on 70 *D. melanogaster* eggs in the presence/absence of the antibiotic. Back crosses were also performed by crossing ♀Pi-NA × ♂Pi, ♀Pi-NA × ♂NA, ♀NA-Pi × ♂Pi and ♀NA-Pi × ♂NA, the first code indicating the genotype of the female's mother, and the second that of the female's father. Finally, the same procedure (eight generations of successive back crosses corresponding to 99.6% of genomic substitution) was performed to produce genetic introgressions, creating new nucleo-cytoplasmic associations (nuclear genotype from Pi with cytotype from NA, and nuclear genotype from NA with cytotype from Pi).

The same procedure was also performed with two extreme phenotypic lines trapped in Sainte-Foy-lès-Lyon (France), designated SFr2 (aposymbiotic females without eggs) and SFr3 (aposymbiotic females producing eggs), respectively, until the F1 generation (20 females/cross).

QUANTITATIVE EXPRESSION BY REAL TIME RT-PCR

Gene expression was measured by real-time quantitative RT-PCR from five biological replicates composed of 10 young females (0- to 2-day old) as described in Kremer et al. 2009b. Briefly, total RNA was extracted as described in Chomczynski and Sacchi 1987, and treated with DNAse (TurboDnase, Ambion, Applied Biosystems, Austin, TX). First-strand cDNA synthesis was performed from 750 ng of total RNA using oligodT primers (Superscript III, Invitrogen, France). Each biological sample was amplified in duplicate from 25 ng using Lightcycler 480 SYBR green I master (Roche, Paris, France). Quantitative RT-PCR reactions were performed as described in Kremer et al. 2009b, using the following primers: HCH-At-3F and HCH-At-2R (Ferritin heavy chain (FN395057), amplicon: 233 bp), LCH-At-F and LCH-At-R (Ferritin light chain (FN395057), amplicon: 218 bp), Tsf-At-F: ACC GCA ATA TAC GAC GAC AT and Tsf-At-R:TTG AGT GCT CAC CAA TGA GA (Transferrin (FN652903), amplicon: 232 bp), SOD-At-F: CTG GAC TCA TCC CCC TAT TT and SOD-At-R: CCA ACC ACC AAA ATT AGC AC (Superoxide Dismutase (FN652905), amplicon: 165 bp), GST-F: CCC AAT CGT CCT TTC CTA CA and GST-R: GGG AGC TGA GTT TGA GGA TG (Glutathione-S-Transferase (FN652904), amplicon: 176 bp). Standard curves were plotted using seven dilutions (10–10^7 copies) of a previously amplified PCR product purified using Nucleospin Extract II kit (Macherey-Nagel, Hoerdt, France). Expression data were estimated by calculating E^{-Cp}, where E corresponds to the efficiency of the PCR reaction, and Cp to the crossing point (Pfaffl 2001). For Pi and NA populations, ferritin gene expression was normalized by L6-ribosomal expression. Expression analysis of genes involved in iron homeostasis and oxidative stress was carried out in SFr2 and SFr3 lines. In these lines, the expression of LCH, HCH, transferrin, SOD, and GST was normalized by the geometric mean of the expression level of three housekeeping genes (Ribosomal L6, β-tubulin, and Elongation factor 1γ). Expression ratios were power-transformed (box.cox.powers function, car package, R software) for normalization before analysis (populations Pi/NA: $LCH^{-2.95}$, $HCH^{-2.03}$, populations SFr2/SFr3: $LCH^{-0.92}$, $HCH^{-0.99}$, $TSF^{-0.20}$, $SOD^{-3.23}$, $GST^{-1.48}$). Analysis of variance (ANOVA) residuals were checked for normality by Shapiro's test, and for homoscedasticity by Levene's test. Pairwise comparisons were performed using Tukey's HSD test (R software).

Results

EGG LOAD OF SYMBIOTIC FEMALES IN NATURAL FRENCH POPULATIONS

Wasps were sampled from eight French populations. After three generations of insect rearing, up to four lines per population had been stabilized. Diagnostic PCR revealed that in each line, individuals simultaneously harbor all three strains of *Wolbachia* (strains *w*Atab1, *w*Atab2, and *w*Atab3 necessary for oogenesis completion). All the *wsp* sequences of *w*Atab3 were the same as those

Table 2. Egg load of symbiotic and aposymbiotic lines. Egg load (mean±SE), number of abnormal pupae (pupae containing abortive parasite), and offspring production in lines of *A. tabida*. By way of comparison, the egg load was also measured in laboratory lines (italic). The egg loads of females from three populations were estimated again two generations later.

Population	Line	Third generation				Fifth generation	
		Symbiotic	Aposymbiotic			Symbiotic	Aposymbiotic
		Eggs	Eggs	Replicates with "abnormal pupae"	Offspring production	Eggs	Eggs
Mtg	1	281±10.8	8.3±5.5	2/10	–	–	–
Igé	1	277.5±11.3	0.4±0.2	0/10	–	–	–
Vsl	1	284.1±12.7	2.1±1.5	0/10	–	–	–
SFb	1	302.2±6.2	18.6±15.9	0/10	–	293.3±13.2	3.2±2.4
SFb	2	270.1±15.5	84.8±19.9	2/10	–	261.0±17.8	10.2±3.9
SFb	3	317.2±12.1	88.1±27.9	5/10	–	–	–
SFr	1	261.8±21.6	109.9±26.1	9/10	–	–	–
SFr	2	274.5±20.1	21.1±21.1	3/10	–	272.7±17.0	0.0±0.0
SFr	3	301.6±14.4	165.5±30.4	8/10	7	315.8±6.3	270.3±17.0
SLA	1	286.2±25.2	16.7±6.4	4/10	1	–	–
SLA	2	293.9±7.6	17.1±8.6	4/10	–	–	–
SLA	3	297±6.3	8±5.6	2/10	–	–	–
SLA	4	289.6±8.2	48.6±19.4	5/10	–	–	–
Vi	1	296.1±18.1	165.7±18.1	10/10	–	–	–
Vi	2	296.2±19.0	55.1±20.6	5/10	–	–	–
Co	1	293.3±5.4	2.4±1.8	1/10	–	281.6±7.1	2.8±2.3
Co	2	284.3±7.4	105±23.2	6/10	3	326.5±6.7	111.9±32.1
Co	3	289.9±24.7	143±22.5	10/10	–	–	–
Co	4	325±9.4	3.8±2.6	0/10	–	–	–
-Pi3	*Laboratory strain*	*280.8±12.8*	*0.0±0.0*	*0/10*	–	–	–
-SF	*Laboratory strain*	*370.6±9.7*	*165.1±20.7*	*7/30*	*13*	–	–

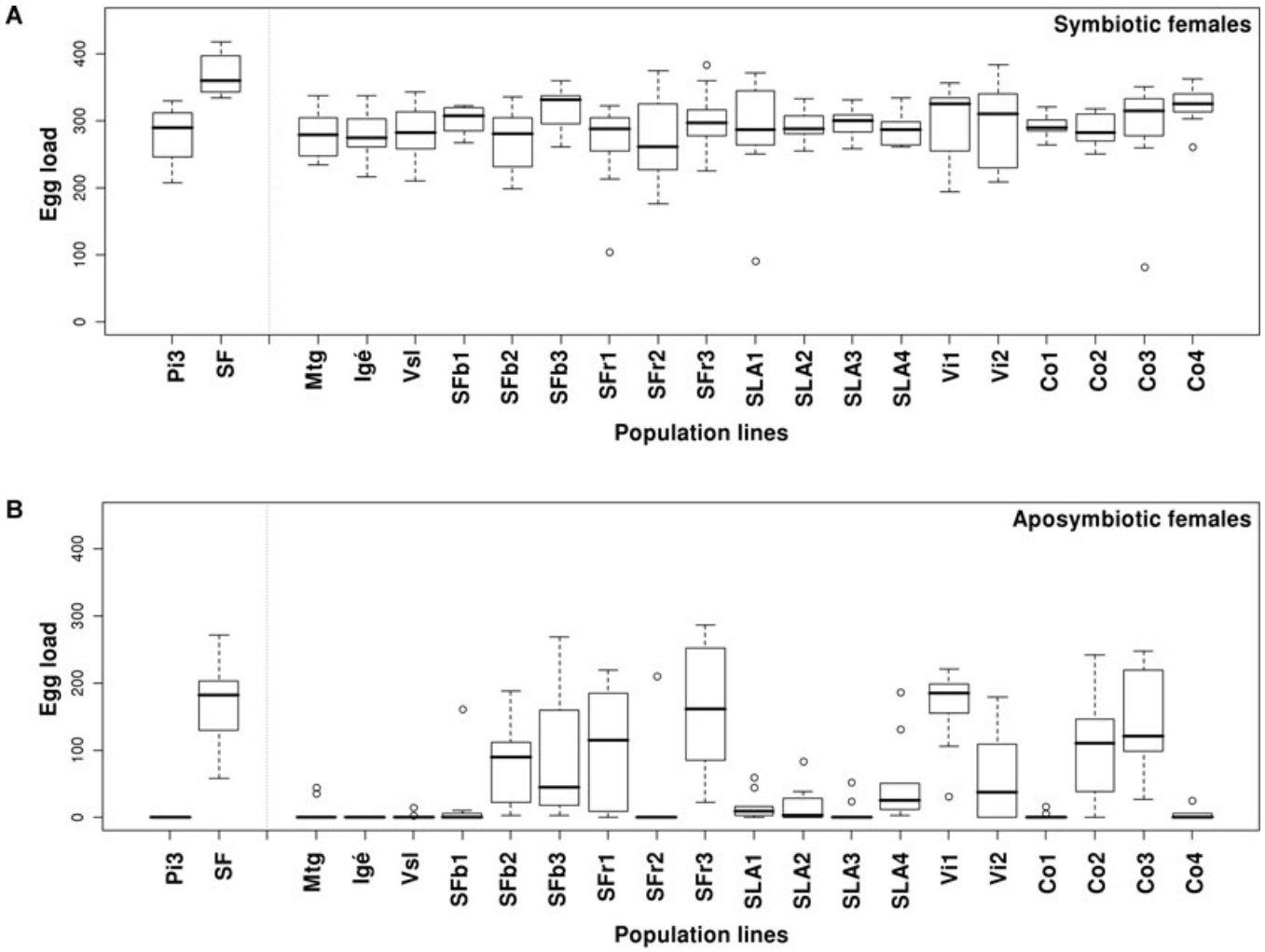
obtained previously in another strain of *A. tabida* (AF124859, positions: 135–560).

We quantified the number of eggs produced five days after hatching (i.e., the egg load) by each line (Table 2, Fig. 1A). Because *A. tabida* is a proovogenic wasp, the egg load of symbiotic females might provide a good estimate of their potential fecundity, and represents a key parameter of their fitness. Egg load did not differ between populations, or between lines within a given population (GLM summarized in Fig. 1C), indicating that genetic variation for egg load in symbiotic females is low at this geographic scale and under these experimental conditions.

VARIATION OF THE OVARIAN PHENOTYPE AFTER THE ELIMINATION OF *WOLBACHIA* IN FRENCH NATURAL POPULATIONS

We counted the eggs produced five days after hatching by aposymbiotic females of each line (i.e., their ovarian phenotype) (Fig. 1B). The ovarian phenotype is not directly related to the host fitness but rather measures the level of dependence of aposymbiotic females after manipulation of the biological system. Diagnostic PCR on cured females never amplified *Wolbachia* DNA, suggesting a complete efficiency of the antibiotic treatment. Considerable variations were observed: females in some lines were unable to produce any eggs when *Wolbachia* was removed, whereas others produced on average almost two-thirds as many eggs as symbiotic females. This variation was not only detected among lines (GLM, $P < 0.001$), but also within lines (GLM, $P < 0.001$), indicating a remarkable polymorphism for the ovarian phenotype in aposymbiotic females. Furthermore, this phenotype also displayed variation between the two ovaries of each female, to a greater degree than in symbiotic females ($F_{92,191} = 2.52$, $P = 8.9 \times 10^{-08}$), suggesting considerable instability during the development of ovaries in aposymbiotic females.

To find out whether variation in the ovarian phenotype among lines was due to a stochastic developmental effect, or was genetically determined, the same measurement was repeated for the same lines two generations later (Table 2). Lines where females had produced eggs, still produced eggs, whereas those where females had produced almost no eggs still produced no eggs,



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Origin of total variance	Symbiotic females			Aposymbiotic females		
	Variance	Percentage	Statistics	Variance	Percentage	Statistics
Inter-populations	0	0	-	17	1	-
Intra-population, inter-lines	0	0	-	817	42	***
Intra-line, inter-females	525	76	***	748	39	***
Intra-female, inter-ovaries	163	23	n.d.	333	17	n.d.

Figure 1. **Variation of egg production in French natural populations. Total egg production in (A) symbiotic and (B) aposymbiotic females belonging to different natural populations (right side) or laboratory populations (left side). To confirm the effectiveness of the antibiotic treatment, we used the laboratory Pi3 line, in which aposymbiotic females do not produce any eggs. By way of comparison, we also used the established laboratory SF line, in which aposymbiotic females do produce some eggs. The box-and-whisker plot shows the extreme of the lower whisker, the lower hinge, the median, the upper hinge, and the extreme of the upper whisker for each group (n = 10 females per line) (C) Statistical analysis of variance between natural populations containing at least two lines. GLM, –: $P > 0.05$, ***: $P < 0.001$, n.d.: statistics could not be determined (no residuals at this level of variance).**

showing that the ovarian phenotype is heritable. However, this phenotype tended to display extreme values, which could have been due to a reduction in genetic diversity during these two additional generations of rearing in the laboratory.

Finally, the egg load of symbiotic females was not correlated to the ovarian phenotype of aposymbiotic female (Pearson, $R^2 = 0.0001$, $P = 0.96$). This suggests that the ovarian phenotype does not reflect any indirect selection acting on the egg load of infected females in these environmental conditions.

OFFSPRING EMERGED IN SOME APOSYMBIOTIC LINES

We examined the development of the eggs produced by aposymbiotic females belonging to these natural populations. Eggs were

laid, and embryos generally developed until the larval stage, but died within the pupae as described in Dedeine et al. 2005. Some of them, however, although very few (∼1 ‰), developed to adulthood (SFr3: 6♂ and 1♀, Co2: 2♂ and 1♀, SLA1: 1♀, Table 2), 8.0 ± 0.53 days later than eggs laid by symbiotic females. Even though they were all uninfected, as determined by diagnostic PCR, the emerging females were fertile but, like their mothers, produced numerous "abnormal pupae," making it impossible to maintain these lines for more than three generations. The production of offspring by aposymbiotic females indicates a kind of gradation in the severity of *Wolbachia*-dependence, ranging from no eggs to the production of abnormal and almost "normal" eggs.

GENETIC BASIS OF THE OVARIAN PHENOTYPE

We crossed laboratory lines Pi and NA, originating from Pierrefeu (France) and Saanich (Canada), respectively, because aposymbiotic females from these populations exhibit a clear and stable established ovarian phenotype. As described in Figure 2A, aposymbiotic females from Pi did not produce any eggs (mean ± SE: 0.0 ± 0.0), in contrast to those from NA, which produced 85.9 ± 8.0 eggs on average. The ovarian phenotype of hybrid females (either ♀NA × ♂Pi or ♀Pi × ♂NA, Fig. 2B) did not significantly differ (Mann–Whitney U-test, $P > 0.05$), suggesting a priori that dependence is mainly under nuclear control. Furthermore, the two types of hybrid females produced significantly fewer eggs than NA females (♀NA × ♂Pi: 14.1 ± 3.8, ♀Pi × ♂NA: 8.3 ± 2.9), indicating that the Pi genotype is dominant over the NA genotype. These results were confirmed by the four F2 backcrosses (Fig. 2C and 2D). Compared to F1 hybrids, a high proportion of females with 75% of Pi genome did not produce any eggs. Conversely, this proportion decreased in females with 75% of NA genome. Finally, we introgressed Pi cytoplasm (and thus Pi *Wolbachia*) into NA nuclear genome, and conversely NA cytoplasm (thus NA *Wolbachia*) into Pi nuclear genome (Fig. 3). Backcrossed females (symbiotic and aposymbiotic) had a similar egg load to parental lines that have the same nuclear genotype. Thus, the egg load of infected females as well as the ovarian phenotype of uninfected females were determined by the host genotype alone, and not influenced by any maternal effect (including *Wolbachia* and/or mitochondria).

Because Pi and NA lines have probably diverged over a long period of evolution, we also tested this genetic model by crossing individuals from two insect lines that were collected within the same population (SFr), which might limit the genetic divergence between lines (Fig. 4). First, the ovarian phenotype of hybrid females was much lower than that of the SFr3 line (SFr3 vs. ♀SFr2 × ♂SFr3, Wilcoxon, $W = 15$, $P = 9.4 \times 10^{-07}$; SFr3 vs. ♀SFr3 × ♂SFr2, Wilcoxon, $W = 20$, $P = 1.2 \times 10^{-06}$). Second, the two reciprocal crosses were not significantly different (*t*-test, $t = -0.68$, df = 37, $P = 0.50$), excluding any major maternal effect. These results suggest a similar genetic nuclear determinism with dominance of the "no egg" phenotype, even though the effect was less pronounced than that observed in Pi and NA crosses. This lower effect could be explained by the fact that the ovarian phenotype of SFr3 females was greater than that of NA females (270.3 ± 24.1 and 85.9 ± 8.0, respectively) or by the greater heterogeneity of ovarian phenotype between females in these natural populations than between Pi and NA lines.

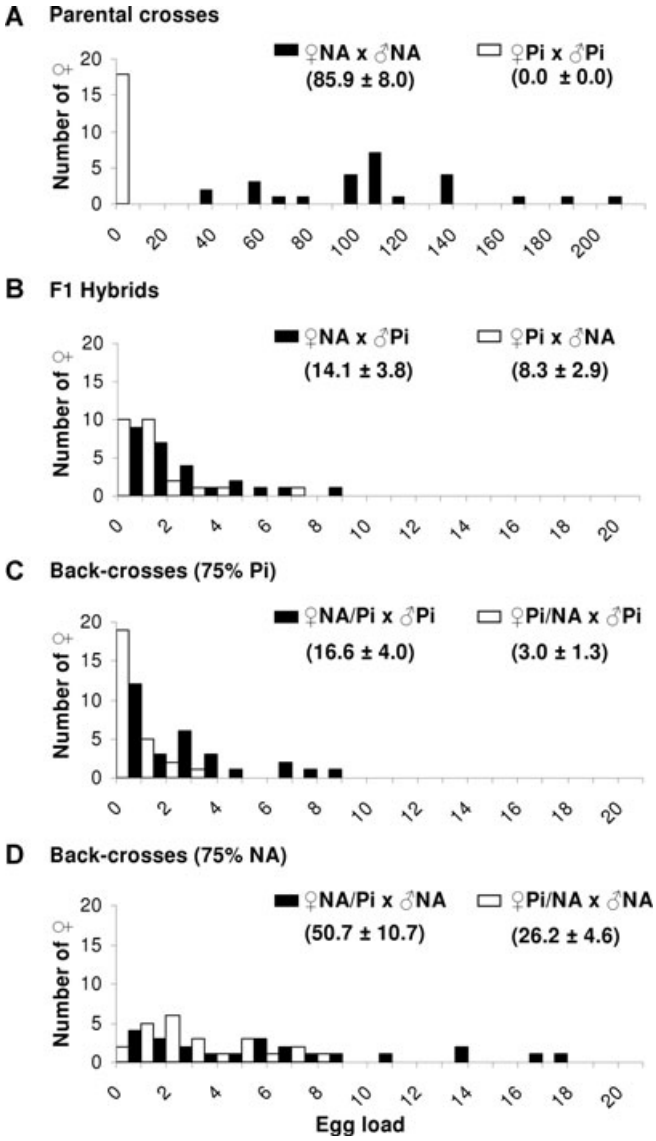

Figure 2. Egg load distribution of aposymbiotic offspring resulting from crosses between two laboratory lines (Saanich and Pierrefeu) with differing ovarian phenotypes. Egg load distribution of aposymbiotic offspring resulting from (A) parental crosses, (B) hybrid crosses, (C, D) back-crosses between two lines (NA: Saanich, Pi: Pierrefeu). The first code indicates the maternal genotype. Mean egg load ± SE are indicated under each cross. Note the 10-fold reduction scale between parental crosses and other crosses.

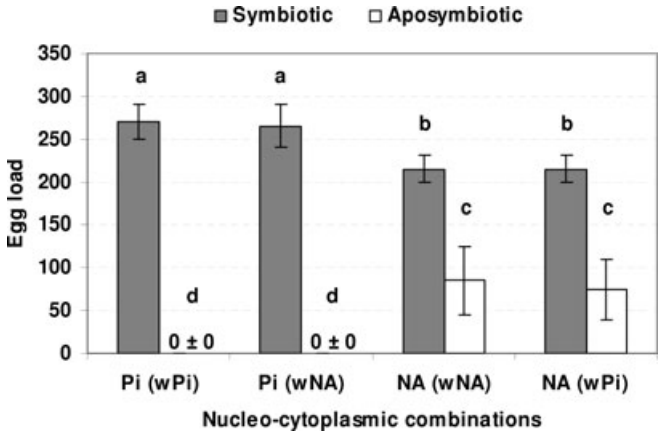

Figure 3. Egg loads of possible *Asobara/Wolbachia* combinations in the Saanich and Pierrefeu populations. Mean egg load ± SE of symbiotic (gray) and aposymbiotic (white) females is indicated for each nucleo-cytoplasmic combination. The first code indicates the nuclear genome (Pi: Pierrefeu, NA: Saanich). The second code indicates the cytoplasm (wPi: Pierrefeu cytoplasm, wNA: Saanich cytoplasm). Use of the same letters indicates that there is no statistical difference ($P > 0.05$), whereas different letters indicate a statistically significant difference ($P > 0.05$) between egg loads (Mann–Whitney U-test).

LINKS BETWEEN OVARIAN PHENOTYPE AND OXIDATIVE STRESS REGULATION

It has recently been shown that ferritin, a protein complex involved in iron storage and oxidative stress regulation (Arosio and Levi 2002), was differently expressed in symbiotic and aposymbiotic females of *A. tabida* in the Pi3 line (Kremer et al. 2009b).

Here, we first compared its expression in symbiotic and aposymbiotic females of populations with differing ovarian phenotypes (Pi and NA). Both the heavy- and light-chains of ferritin (HCH and LCH respectively) were overexpressed in aposymbiotic females exhibiting the "no egg" phenotype (Pi population; LCH: ratio aposymbiotic/symbiotic (i.e., A/S) = 1.94, $P = 2.23 \times 10^{-05}$; HCH: ratio A/S = 2.03, $P = 0.002$), whereas they were not overexpressed in aposymbiotic females producing some eggs (NA population; LCH: ratio A/S = 1.07, $P = 0.68$; HCH: ratio A/S = 1.15, $P = 0.288$). These results suggest that the regulation of ferritin expression differs in different lines, and is potentially linked to the ovarian phenotype of aposymbiotic females.

We then extended this analysis by studying the expression of the genes involved in oxidative stress control in lines exhibiting differing ovarian phenotypes, but originating from the same population (lines SFr2 "no eggs", and SFr3 "some eggs",

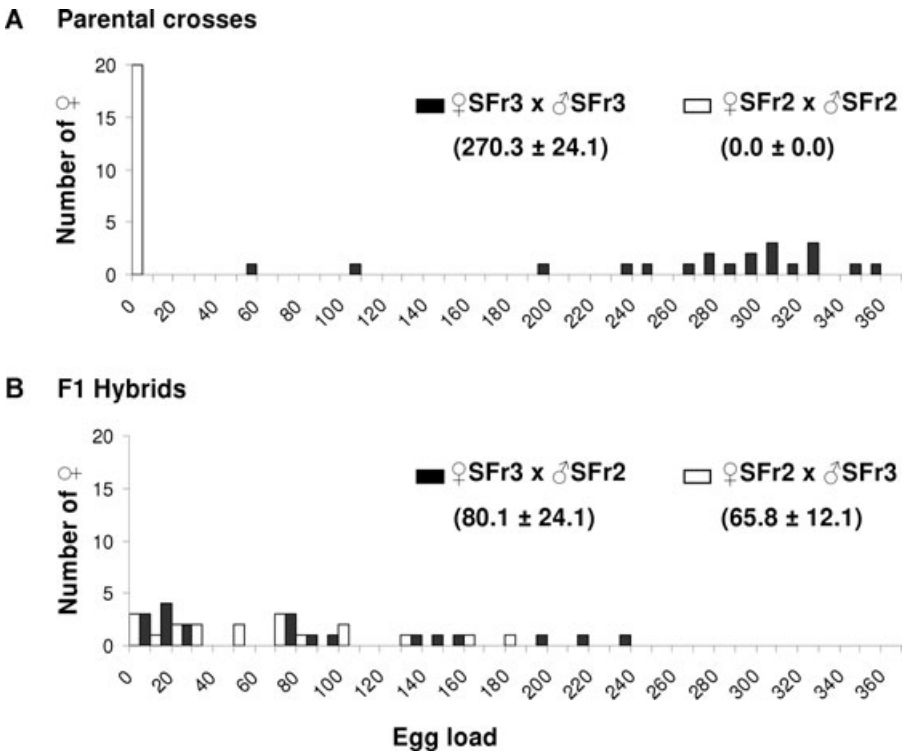

Figure 4. Egg load distribution of aposymbiotic offspring resulting from crosses between two lines collected within the same population, but with differing ovarian phenotypes. Egg load distribution of aposymbiotic offspring resulting (A) from parental crosses and (B) from hybrid crosses between two extreme lines (SFr2 and SFr3) belonging to the same natural population (SFr). Mean egg load ± SE are indicated under each cross.

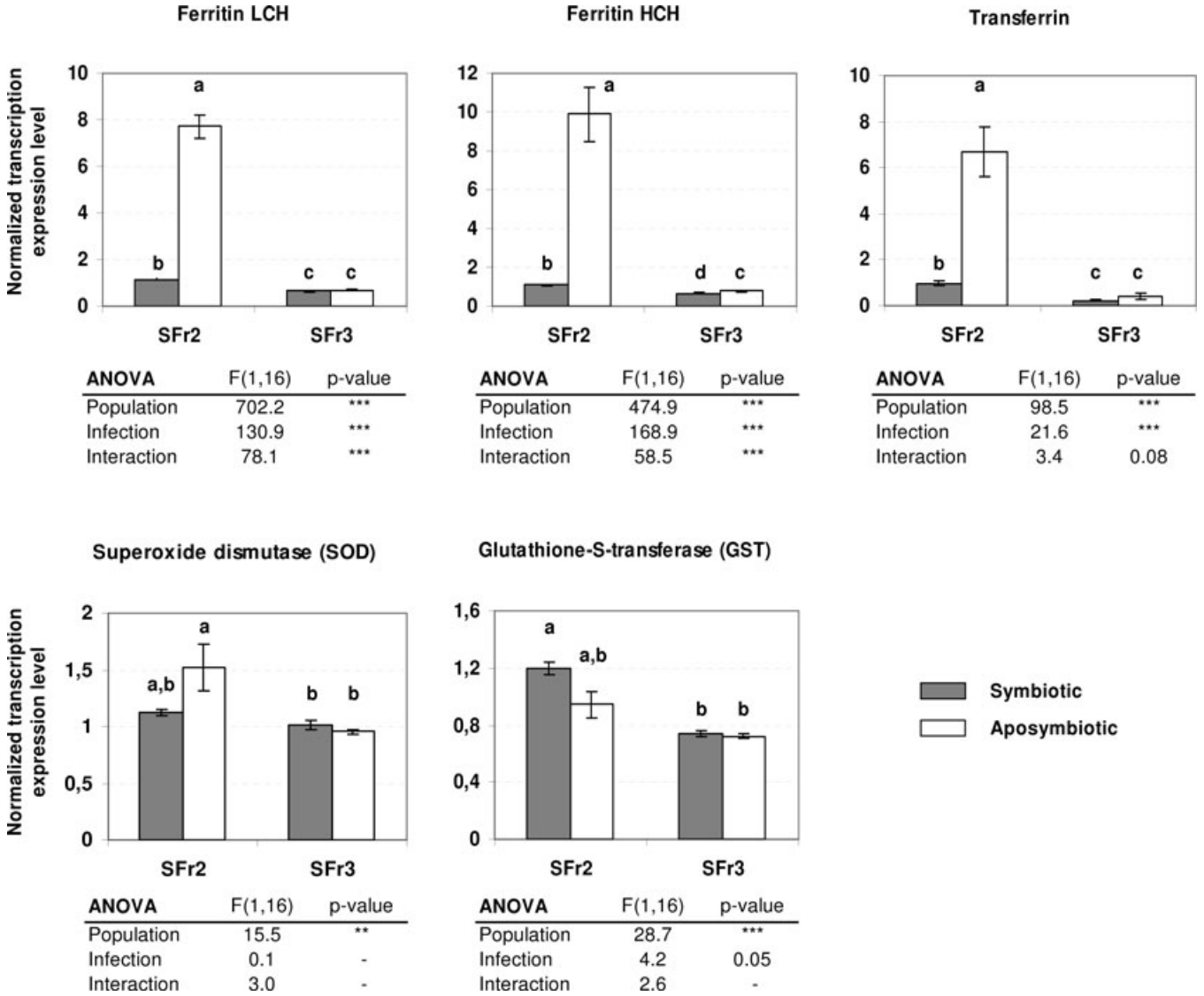

Figure 5. **Antioxidant gene expression in response to infection in two lines collected within the same population but with differing ovarian phenotypes. Gene expression is measured in symbiotic (gray) and aposymbiotic (white) females (normalization by three housekeeping genes, mean ± SE, five replicates each). Aposymbiotic females from the SFr2 populations did not produce any eggs, whereas those from SFr3 populations did produce some eggs. Statistical analysis of variance (ANOVA) values are indicated below each graph (–: $P > 0.05$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). Use of the same letters indicates that there is no statistically significant difference ($P > 0.05$), whereas different letters indicate that there is a statistically significant difference ($P < 0.05$) between the normalized gene expression levels (Tukey's HSD test).**

Fig. 5). Marked overexpression of both ferritin chains was observed in SFr2 aposymbiotic females compared to symbiotic ones, whereas no (or only slight) overexpression was detected in SFr3 aposymbiotic females (statistics in Fig. 5). This result reinforces the possibility of a link between the ferritin response to the elimination of *Wolbachia* and the phenotype expressed in the ovaries. In addition, the same expression pattern was highlighted with transferrin, suggesting the existence of correlation between the ovarian phenotype and the control of oxidative stress relative to iron homeostasis.

The expression of the Superoxide Dismutase (SOD) gene was greater in SFr2 aposymbiotic females than in SFr3 ones, whereas that of the Glutathione-S-Transferase (GST) gene was not. This suggests that some, but not all, antioxidant enzymes could respond to limit oxidative stress in aposymbiotic females, depending on the population line and its associated ovarian phenotype.

Finally, we showed that the genes involved in oxidative stress control were also differentially expressed in symbiotic females of lines exhibiting different ovarian phenotypes, with expression

being higher in the SFr2 than the SFr3 line (except for SOD). This suggests that expression of these genes varies within populations, which could reflect a potential response to selection.

Discussion

Because *A. tabida* is a proovogenic wasp, the egg load might provide a good estimate of the potential fecundity of symbiotic females. As *Wolbachia* is maternally transmitted through the eggs, this trait is a key factor for the fitness of both the host and *Wolbachia*. Crossing experiments indicated that egg load of symbiotic females is exclusively determined by the host's nuclear genome, and not by that of *Wolbachia*. Hence, *Wolbachia* interacts with oogenesis only by triggering its activation, and the bacterium apparently does not control the number of eggs produced by symbiotic females. These results contrast with a recent study on *D. simulans*, where a *Wolbachia* strain rapidly evolved from parasitism to mutualism, thus giving some host genotypes a fecundity advantage (Weeks et al. 2007).

Our results reveal considerable variation in the ovarian phenotype of aposymbiotic females, ranging from no egg production to the production of a certain number of eggs. In addition, some uninfected eggs reached adulthood, and uninfected lines created from these individuals were maintained for three generations in the laboratory. These results further extend the range of variation observed in previous studies (Dedeine et al. 2001, 2005). Furthermore, this new phenotype might shed some light on the mechanisms by which *Wolbachia* interferes with oogenesis. It has been hypothesized that the *Wolbachia*-dependence of host oogenesis could be due either to the induction of a poison/antidote system (Charlat and Mercot 2001), analogous with cytoplasmic incompatibility (CI), or to a functional interaction between *Wolbachia* and oogenesis, potentially linked to PCD (Pannebakker et al. 2007). In CI, a modification is activated by *Wolbachia* during spermatogenesis, and rescued by the presence of the same strain in the developing egg (Werren 1997). In *A. tabida*, dependence would rather correspond to a transgenerational CI, referred to as the Sterilization of Aposymbiotic Sisters hypothesis (SAS hypothesis, Charlat and Mercot 2001). Indeed, the poison would be synthesized by *Wolbachia* in the mother, and then would specifically inhibit oogenesis in any daughters that lack *Wolbachia*. In infected daughters, *Wolbachia* would synthesize the antidote, and therefore would allow egg production. This strategy could be selected for if sterilization of aposymbiotic females leads to an increase in the fitness of symbiotic females. However, it assumes that there is competition for hosts, and that the females laying eggs in a given patch are closely related, which might not be the case in natural populations of *A. tabida* (F. Vavre, pers. obs.). Furthermore, for transgenerational CI to be expressed, the poison must have a longer half-life than the antidote. The results obtained here, where aposymbiotic lines were maintained for three generations without a normal phenotype's being restored, seems to be incompatible with this hypothesis. It would indeed require the poison to have a half-life that extends over three generations, and to be able to act at extremely low concentrations, because of the dilution of the poison that will inevitably occur as the organism develops. It appears, therefore, that the ovarian phenotype is most probably due to a functional dependence rather than to an SAS phenotype.

Our results also show that variations in the ovarian phenotype of aposymbiotic females exist within populations. The stability of the ovarian phenotype across generations, together with the demonstration that the variation observed in the ovarian phenotype has a nuclear genetic basis, show that the phenotypic expression of dependence is heritable. It is puzzling that polymorphism of a trait that is not under direct selection should be maintained. It seems very unlikely that the polymorphism observed in French populations is transient, because almost all populations exhibit some variation between lines. Neutral polymorphism is also unlikely, because founder effects would be expected to lead to the homogenization of the phenotypic trait, at least at a reduced geographical scale (i.e., within the same population), unless the effective size of populations is very large, or there is migration between populations. Alternatively, this trait could be subject to indirect selection if it is correlated with other selected traits. Our results revealed that such a trait, if it exists, is unlikely to be the egg load of symbiotic females, because under our experimental conditions no correlation was observed with the ovarian phenotype of uninfected females. It has been hypothesized that dependence upon *Wolbachia* in *A. tabida* has been selected for to enable the host to tolerate the harmful effects of the symbiont on its physiology (Aanen and Hoekstra 2007). Indeed, a mid-oogenesis apoptotic checkpoint, that checks egg quality and adjusts egg production in response to environmental stresses (McCall 2004), is induced in aposymbiotic females (Pannebakker et al. 2007). It is also known that intracellular bacteria can sometimes act directly on apoptotic processes to sustain their cellular environment, but also indirectly by inducing oxidative stress, as has been shown for *Wolbachia* (Bazzocchi et al. 2007; Brennan et al. 2008). This means that host mutations that compensate for the harmful effect of *Wolbachia* on apoptosis can be selected for, even if they would be deleterious in the absence of *Wolbachia.* In the case of *A. tabida*, it is thus tempting to hypothesize that apoptotic compensation could lead to oogenetic dysfunction in the absence of *Wolbachia* and then to dependence (Vavre et al. 2008). Depending on the extent to which this apoptotic checkpoint is activated in aposymbiotic individuals, more or fewer eggs could be produced. Furthermore, deregulation of this apoptotic checkpoint could also result in the tolerance of vitellogenesis in some abnormal eggs, leading to the early death of the developing egg/larvae. In this context, we found that

the transcriptional response to the elimination of *Wolbachia* by genes involved in iron metabolism and oxidative stress reduction correlates with the ovarian phenotype of aposymbiotic females. These variations could reflect variability of the compensatory mechanisms leading to the polymorphism demonstrated in these French populations. Interestingly, we found that females from lines with different ovarian phenotypes not only differ in their response to the elimination of *Wolbachia*, but also exhibit differing expressions of antioxidant genes in *Wolbachia*-infected females, making direct selection a possibility. Because apoptosis and oxidative stresses are general processes that affect numerous traits in the organism, it is reasonable to suggest that various levels of tolerance may optimize different traits, leading to trade-offs, and making it possible to maintain a high level of polymorphism. The availability of various lines with contrasting ovarian phenotypes, and originating from geographically close areas, offers an opportunity to investigate this hypothesis by analyzing various traits, in males as well as in females, the expression of which could be correlated with ovarian phenotype. In addition, understanding the basis of this polymorphism might help elucidating the precise mechanisms underlying and regulating host-*Wolbachia* interactions.

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