**Wolbachia** Interferes with Ferritin Expression and Iron Metabolism in Insects

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Abstract

*Wolbachia* is an intracellular bacterium generally described as being a facultative reproductive parasite. However, *Wolbachia* is necessary for oogenesis completion in the wasp *Asobara tabida*. This dependence has evolved recently as a result of interference with apoptosis during oogenesis. Through comparative transcriptomics between symbiotic and aposymbiotic individuals, we observed a differential expression of ferritin, which forms a complex involved in iron storage. Iron is an essential element that is in limited supply in the cell. However, it is also a highly toxic precursor of Reactive Oxygen Species (ROS). Ferritin has also been shown to play a key role in host-pathogen interactions. Measuring ferritin by quantitative RT-PCR, we confirmed that ferritin was upregulated in aposymbiotic compared to symbiotic individuals. Manipulating the iron content in the diet, we showed that iron overload markedly affected wasp development and induced apoptotic processes during oogenesis in *A. tabida*, suggesting that the regulation of iron homoeostasis may also be related to the obligate dependence of the wasp. Finally, we demonstrated that iron metabolism is influenced by the presence of *Wolbachia* not only in the obligate mutualism with *A. tabida*, but also in facultative parasitism involving *Drosophila simulans* and in *Aedes aegypti* cells. In these latter cases, the expression of *Wolbachia* bacterioferritin was also increased in the presence of iron, showing that *Wolbachia* responds to the concentration of iron. Our results indicate that *Wolbachia* may generally interfere with iron metabolism. The high affinity of *Wolbachia* for iron might be due to physiological requirement of the bacterium, but it could also be what allows the symbiont to persist in the organism by reducing the labile iron concentration, thus protecting the cell from oxidative stress and apoptosis. These findings also reinforce the idea that pathogenic, parasitic and mutualistic intracellular bacteria all use the same molecular mechanisms to survive and replicate within host cells. By impacting the general physiology of the host, the presence of a symbiont may select for host compensatory mechanisms, which extends the possible consequences of persistent endosymbiont on the evolution of their hosts.

Introduction

Symbiotic interactions, in which long-term interactions take place between two partners belonging to different species, are common in nature [1]. These associations form a continuum ranging from parasitism to mutualism with respect to the outcome of the association (*i.e.* the cost or benefit for the host), and can be either facultative or obligate for the host. It has usually been assumed that parasitic, commensal or mutualistic symbionts interact in fundamentally different ways with their host. However, many bacterial symbionts can exist either as a mutualist or as a parasite, depending on their host [2,3]. In addition, increasing reports in the literature indicate that the same molecular mechanisms are used by both parasitic and mutualistic symbionts to interact with their host [4]. If we focus on endocytobionts (*i.e.* symbionts living within the cells of their hosts), several mechanisms are known to be shared by parasites and mutualists, such as recognition and specific binding to the host cell, internalization within the cell, and finally intracellular survival and growth [5]. Common molecular mechanisms have been identified which are related to (i) communication processes, such as symbiosis/virulence factors that encode genes classically involved in secretion systems [6] (ii) survival and replication processes, including the expression of colonization factors [7], evading host immune systems [8], and regulating bacterial growth, and (iii) physiological processes involved in environmental adaptation, such as pH modification, induction of specific metabolic pathways, development of iron uptake strategies [9] and induction of stress proteins [10].

In this paper, we focus on *Wolbachia* (Rickettsiales), a well-known genus of bacteria that are reproductive parasites when associated with arthropod hosts [11] but mutualists when associated with nematodes [12]. Unlike other *Wolbachia* strains, which are generally facultative for their arthropod hosts, *Wolbachia* is necessary for oogenesis to be completed in the parasitoid wasp *Asobara tabida* (Hymenoptera, Braconidae) [13]. *Wolbachia* does not...
Author Summary

Wolbachia are intracellular bacteria that infect numerous invertebrate species, where they are generally facultative for their host. Surprisingly, the wasp Asobara tabida is dependent on Wolbachia for egg production: in uninfected females, the cells necessary for egg maturation die prematurely as a result of apoptosis. When we analyzed the genetic basis of this dependence, we found that ferritin, a protein involved in the regulation of iron homeostasis, was over-expressed in uninfected individuals. We also found that Wolbachia interferes with iron metabolism and ferritin expression in other host–Wolbachia associations. Furthermore, Wolbachia itself responds to changes in iron concentration by changing the expression of bacterioferritin. Iron is in short supply within the cell, and is necessary for both host and symbiont; our findings highlight the key role of iron in host–symbiont interactions, as had previously been shown for host–pathogen interactions. Furthermore, iron homeostasis is involved in the regulation of oxidative stress, which in turn is involved in inducing cell death. Wolbachia could also interfere with iron in a way that limits oxidative stress and cell death, thus promoting its persistence within host cells. In A. tabida, we show that iron induced cell death in the ovaries, suggesting that iron metabolism could also be linked to the evolution of dependence.

induce any cost or benefit affecting other life-history traits of A. tabida individuals, suggesting that the mutualistic interaction is exclusively due to the restoration of egg production in presence of Wolbachia. A. tabida is the only species within the genus Asobara to be dependent on Wolbachia for its oogenesis [14], which indicates this evolutionary transition towards obligatory dependence is recent, and makes it possible to investigate the molecular mechanisms underlying this dependence. A recent study has revealed that apoptosis is vastly greater in the nurse cells surrounding the oocytes in aposymbiotic ovaries than those in symbiotic ovaries [15]. Apoptotic processes are necessary for normal oogenesis to occur [16], but in the case of A. tabida, the absence of Wolbachia activates an early checkpoint during oogenesis [15]. This suggests that Wolbachia could be manipulating the host’s apoptotic machinery, either indirectly or directly, as microbial pathogens often do to evade the host immune system [17]. A strategy usually associated with parasitism could thus be what has led to the swift transition from parasitism to obligate mutualism observed in this species.

In order to investigate the molecular mechanisms underlying host dependence, we used a comparative transcriptomic approach (Suppression Subtractive Hybridization (SSH) to compare symbiotic and aposymbiotic ovaries of A. tabida females. The heavy (HCH) and light (LCH) chains of ferritin, which form complexes involved in iron storage and the oxidation of labile iron in the cell [18], were found to be over-expressed in aposymbiotic individuals. This focus on ferritin is very relevant, due to the known major role played by iron in bacterial growth and infection [19], and hence its potential involvement in host-bacteria interactions. Furthermore, the anti-oxidant properties of ferritin have a direct effect in potential involvement in host–bacteria interactions. Furthermore, iron homeostasis is played by iron in bacterial growth and infection [19], and hence its potential involvement in host–bacteria interactions. Furthermore, iron homeostasis is linked to the evolution of dependence.

shown that Wolbachia infection could have a beneficial effect on fecundity by buffering iron fluctuations [22].

Starting from the observed over-expression of ferritin in aposymbiotic individuals, we focused on the influence of Wolbachia on host iron homeostasis, and the role of iron overload in apoptotic processes during oogenesis in A. tabida. We then extended our analysis to the facultative parasitic association known to exist between Drosophila simulans and Wolbachia wRi and Aegypti cells lines stably infected by Wolbachia wMel. We demonstrated that the influence of Wolbachia on iron homeostasis was not limited to A. tabida, and in fact constitutes a common mechanism used by both mutualistic and parasitic Wolbachia.

Results

Both heavy and light chains of ferritin were over-expressed in aposymbiotic individuals of A. tabida under standard conditions

We performed a Suppression Subtractive Hybridization (SSH) to compare the distal part of symbiotic (S) and aposymbiotic (A) ovaries. Among all the potential candidates for involvement in apoptotic processes, we focused on ferritin, a protein complex involved in iron storage, and composed of HCH and LCH subunits. We first performed Real-Time quantitative PCR to confirm the over-expression of these genes, and showed that the heavy and light chains of ferritin were indeed both over-expressed in aposymbiotic A. tabida individuals (Fig. 1), suggesting that transcription of the two genes responded in similar ways to the absence of bacteria (see statistics in Fig. 1C). Ferritin over-expression was observed in the ovaries where the apoptotic phenotype was observed, in whole aposymbiotic females, but also slightly in males, even though no apoptotic phenotype was visible. These findings suggest that the over-expression of ferritin is a more global phenomenon controlled by the presence of Wolbachia, rather than being simply a response to the apoptotic phenotype observed in the ovaries.

Genomic loci of host and Wolbachia ferritin

As has already been shown for Drosophila melanogaster, Anopheles gambiae, Apis mellifera and Bombyx mori (review [23]), ferritin HCH and LCH appeared to be clustered head-to-head in A. tabida to form a 9194-bp superlocus (Fig. 2A). In Aedes aegypti, LCH and HCH regulation is regulated by cis-regulatory elements [24,25]. Coordinated regulation has been demonstrated in several species, including D. melanogaster [26] and A. aegypti [27]. The ferritin intron/exon structure is known to be highly variable among insect species. Five and four exons were found in the HCH and LCH forms of A. tabida ferritin, respectively, without any evidence suggesting transposable element insertion or a repeated sequence. Multiple polyadenylation sites are present at the 3’-end of each gene, and could affect the length and stability of transcripts [28,29]. HCH and LCH are 220-aa and 223-aa peptides, respectively, and possess a signal peptide for secretion with a cleavage site located between the 19th and 20th amino acid in the N-terminal region. A. tabida maintains all the ferroxidase residues of the ferritin HCH which are necessary for iron oxidation and iron absorption [30]. Finally, a canonical Iron Response Element (IRE), consisting of a hairpin hexaloop (5-CAGUGG-3) and a stalk disrupted by a bulge with an unpaired G nucleotide [31,32], was found in the HCH transcript within the uncoding region of the first exon (Fig. 2B). This IRE could be responsible for translational control by iron via Iron Response Proteins (IRPs) [33–35].
**Figure 1.** Ferritin expression in *A. tabida* under standard conditions. **A**–B. Expression of ferritin HCH (**A**) or LCH (**B**) relative to ribosomal L6 gene in aposymbiotic (white) and symbiotic (gray) ovaries, females and males. Asterisks indicate a statistically significant difference (t-test, ***: p < 0.001) of ferritin expression in response to infection status. Expression was measured on 5 replicates of 10 individuals (Mean ratio ± SE).

C. Ratio between mean expression in aposymbiotic (A) and symbiotic (S) individuals, and details of statistical analysis (t-test with Welch correction on log-transformed data).

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**Figure 2.** Genomic organization of Ferritin HCH and LCH. **A.** Genomic organization of ferritin in *A. tabida* (At) and in *D. melanogaster* (Dm) [48]. Introns are represented by lines, and exons by boxes to scale. Within exons, start and stop codons are indicated, delimiting the protein coding regions (shaded gray) and the untranslated regions (white). IRE is shown as dashed box. **B.** Sequence and secondary structure of the Iron Response Element (IRE) in *A. tabida* compared to the IRE consensus sequence [32] and the IRE of *D. melanogaster* [26].

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We also characterized the 474-pb bacterioferritin gene (bfr) of the Wolbachia strain necessary for A. tabida oogenesis (strain wAtab3). It contained all the residues involved in ferroxidase activity [36], suggesting that the bacterioferritin is likely to be functional. This gene is highly conserved in the Wolbachia strains of the A-clade that have been sequenced (wAtab 1 or 2 of A. tabida, wMel of Drosophila melanogaster [37], wRi of D. simulans [38]). Indeed, only eleven variable sites have been detected within the entire nucleotide sequence of these strains. Seven out of them are specific to wAtab3, but six are synonymous substitutions. The only non-synonymous substitution (His vs. Tyr, position 79) has already been identified in Wolbachia strains from other clades (wPip of Culex quinquefasciatus [39], wBm of Bungia malayi [20]), and does not correspond to an amino acid in the ferroxidase center [36].

Wolbachia limits parasite mortality after iron overload

In order to detect the potential effect of Wolbachia infection on host iron homeostasis, we artificially increased the iron load in the nutrient medium. Iron treatment was efficient since we measured a 5.24-fold increase of iron quantity in Drosophila larvae (t-test, t = 14.81, df = 4.13, p = 9.85e-05). The emergence of A. tabida adults slumped after a high iron load (Fig. 3, mixed LM, iron treatment: p < 10^-4), although iron overload had no effect on the development of D. melanogaster when they are not parasitized by A. tabida (mixed GLM with binomial error, iron treatment: p = 0.152). Furthermore, the decrease in wasp emergence was not attributable to a decrease in the infestation level (i.e. mean percentage of Drosophila hosts that have been parasitized±SE), which ranges from 84.1%±3.7 to 90.8%±3.9 for control treatment and from 86.2%±6.3 to 87.9%±5.0 for iron diet treatment, in symbiotic and aposymbiotic wasps, respectively. These findings suggest that the parasitoid development was affected by the iron increase, probably because parasitoids usually develop in the highly buffered environment of Drosophila larvae. Interestingly, while symbiotic individuals still suffer from higher iron load, the deleterious effect on wasp development was less pronounced than in aposymbiotic individuals (2.00-fold reduction; Mixed LM, iron treatment x infection, p = 0.007). This interaction between iron load and bacterial infection suggests that the presence of Wolbachia could act as a buffer in the wasp iron homeostasis after iron overload, and hence limit the deleterious effect of labile iron.

Little iron is absorbed by the wasps, and ferritin expression does not increase after iron treatment

In order to find out whether iron uptake differed between symbiotic and aposymbiotic wasps, we measured the iron absorbed by the wasp. We detected a marginally significant increase in iron content after the administration of an iron diet (ANOVA, F[1,14] = 3.70, p = 0.07), which ranged from 27.39±0.95 (mean±SE in nmol of iron/mg of protein) to 29.34±0.50 for aposymbiotic ones. However, we did not detect any effect of Wolbachia’s presence on the total iron absorption nor any interaction between Wolbachia and iron treatment (ANOVA, infection status, F[1,14] = 0.31, p = 0.58; iron treatment x infection, F[1,14] = 0.004, p = 0.95).

Ferritin expression was determined after iron treatment in both symbiotic and aposymbiotic females. We did not detect any over-expression of host ferritin or bacterioferritin transcripts in response to iron treatment (Table 1). This absence of a transcriptional response was not attributable to any change in bacterial density in response to iron load ([WSP]/[18S])x10^5 = 7.36±0.61 (mean±SE) for control treatment, and 7.83±1.13 for iron treatment; t-test on log-transformed data: t = -0.44, df = 3.49, p = 0.68). One interpretation is that emergence of parasitoids after iron overload occurred only in Drosophila larvae with efficient iron homeostasis and low iron content in their body, or in those larvae that were more efficient iron consumers. Alternatively, we could be observing a variation in the wasp’s tolerance to iron. However, this hypothesis is not well supported by the fact that the increase in iron content in the wasp was very limited after the iron treatment compared to that in the Drosophila larvae, and also that no transcriptomic response was detected.

Figure 3. Influence of iron on wasp development. A. Emergence (mean±SE) of aposymbiotic (white) and symbiotic wasps (gray) following development on standard diet (control) or on iron-supplemented diet (iron). The results from two independent experiments (with 7 and 8 replicates for each condition, respectively) are pooled. B. The number of wasps emerged was analysed using mixed Linear Model (LM) with binomial error. The experiment was treated as a random factor, whereas iron treatment and infection status were treated as fixed factors. doi:10.1371/journal.ppat.1000630.g003

Apoptosis is induced in the ovaries of A. tabida after iron overload

In Drosophila, stress induces several different apoptotic checkpoints that control egg production [16]. In A. tabida, there was a marginally significant reduction in oocyte load after iron treatment (Wilcoxon, W = 1484, p = 0.07, Fig. 4C), with some ovaries being totally devoid of eggs after iron overload. These empty ovaries were similar to those observed in ovaries of aposymbiotic females where apoptosis was detected early during the oogenetic process [15]. In response to iron treatment, TUNEL staining was enhanced in ovaries from symbiotic females (Fig. 4), indicating an increase in apoptosis (Wilcoxon, W = 241, p = 0.01). This increase in TUNEL staining was particularly marked in egg chambers close to the germarium, and was detected either in all the nurse cells of an entire egg chamber, or in only some of them (erratic points). Thus, a toxic effect of iron on oogenesis was
**Table 1. Ferritin expression after iron treatment in A. tabida.**

| A | Experimental condition | LCH mean | LCH SE | HCH mean | HCH SE | Bacterioferritin mean | Bacterioferritin SE |
|---|------------------------|----------|--------|----------|--------|-----------------------|--------------------|
| Symbiotic/control | 1.05 | 0.05 | 0.24 | 0.01 | 11.31 e-04 | 1.66 e-05 |
| Symbiotic/iron | 1.13 | 0.05 | 0.25 | 0.01 | 11.07 e-04 | 1.83 e-05 |
| Aposymbiotic/control | 2.89 | 0.15 | 0.64 | 0.05 | - | - |
| Aposymbiotic/iron | 2.73 | 0.14 | 0.62 | 0.03 | - | - |

**B.** ANOVA on log-transformed data

| Experimental condition | LCH | HCH | Bacterioferritin |
|------------------------|-----|-----|------------------|
| Iron treatment | F(1,16) | 8.68 e-06 | 0.99 | 0.01 | 0.92 |
| Infection | 352.00 | 8.48 e-13 | 311.60 | 2.27 e-12 | - |
| Infection × treatment | NS | - | NS | - |

A. Relative expression of Ferritin HCH, LCH and of bacterioferritin in response to iron treatment and infection. Expression was measured on 5 replicates of 10 females. B. ANOVA and t-test on log-transformed data.

We subsequently looked at ferritin expression in response to iron treatment and infection (Fig. 6, statistics in Fig. 6D). As for iron absorption, *Wolbachia* had no effect on ferritin expression under control conditions. In response to iron treatment, HCH expression increased 1.67 times, but there was no interaction with infection. LCH expression also increased after iron treatment, but this increase was lower in infected (1.63-fold increase) than in uninfected females (2.13-fold increase). This could be explained by the transcriptional response of bacterioferritin, the expression of which doubled after iron treatment (Fig. 6C, statistics in Fig. 6D). This increase in bacterioferritin expression cannot be attributable to bacterial density as we did not observe any change in response to iron treatment ([WSP]/[RP49] = 1.07 ± 0.09 (mean ± SE) for control treatment, and 0.93 ± 0.11 for iron diet treatment; t-test on log-transformed data : t = 0.76, df = 18.68, p = 0.45).

Finally, we did not detect any influence of *Wolbachia’s* presence on morphometric traits (wing size) or offspring production under control or iron diet treatment conditions (data not shown). This suggests that *D. simulans* can easily adapt its metabolism to the change in iron homeostasis observed after iron overload and *Wolbachia* infection. The longevity of the infected flies was greater than that of the uninfected ones under both control and iron diet treatment conditions (Fig. S1), suggesting that *Wolbachia* infection confers some benefit. However, the effect of iron treatment was not significant, and there was no interaction between infection and iron diet (Cox model, infection status: p = 0.01, iron treatment: p = 0.14, infection × iron treatment: p = 0.50).

**Discussion**

**Role of iron metabolism in host-parasite interactions**

Iron is essential for survival in all organisms, since it is used as a cofactor or catalyst in electron transport. Bacteria also need iron to grow and to maximize the infection process [40,41]. Hence, competition for iron can emerge between partners in host-bacteria associations. Many proteins scavenge iron, including transferrin,
lactoferrin, ferritin or siderophores in eukaryotes, and bacterioferritin or siderophores in prokaryotes.

Although iron is essential for most organisms, an excess of iron in the cell is harmful. Indeed, labile iron catalyses Reactive Oxygen Species (ROS) via the Fenton reaction in the presence of H$_2$O$_2$, and activates the immune response and apoptosis [42]. Ferritin is an iron storage protein, which can complex labile iron and buffer the intracellular labile iron pool. Because its expression is regulated by iron [43] and by oxidant residues via an Antioxidant Responsive Element (ARE) [44,45], ferritin contributes to iron homeostasis and oxidative stress reduction [18].

Finally, iron is involved in the modulation of innate immunity mechanisms against pathogenic bacteria in mammals, such as the production of ROS and RNI (Reactive Nitrogen Intermediates), and limiting bacterial access to iron can also lower their infectivity [46]. In addition, HCH ferritin expression is under the control of the NF-κB signaling pathway in mammals. This pathway plays a major role in the immune response, inducing the expression of immune-related genes, and limiting ROS-induced apoptosis [47]. In D. melanogaster, ferritin genes possess two NF-κB binding domains [48], and ferritin up-regulation has also been described after immune challenge in starfish [49], the horseshoe crab [50] and Amphioxus [51].

Figure 4. Ovarian phenotype in response to iron stress. A. Distribution of TUNEL staining (whole Egg Chamber (EC) or Erratic Points (EP)) in the Germarium (G), Close to the Germarium (CG), Close to the Vitellarium (CV), or in the Vitellarium (V). B. TUNEL index of ovaries from females reared under standard (control, n = 17) or iron-supplemented diets (iron, n = 19). C. Oocyte load of females developed under standard (control, n = 60) or iron-supplemented diets (iron, n = 60). 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. D–G. TUNEL (green) and DAPI (blue) staining of ovaries. (D, E) PCD occurred only in the germarium (Ge) and during the dumping near the vitellarium (Vi) under control conditions. (F, G) PCD is induced in egg chambers (Ec) close to the germarium after iron-supplementation. Scale bar: 100 μm.

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**Wolbachia** influences iron metabolism in various biological systems

Our results strongly suggest that iron plays a pivotal role in the interaction between **Wolbachia** and its hosts. We detected a global influence of **Wolbachia** on ferritin expression in *A. tabida* under standard conditions, but also in *D. simulans* and *A. aegypti* cells after iron supplementation. The fact that similar results have been obtained using different systems suggests that the effect observed in *A. tabida* is not due to a direct action of the antibiotic treatment, but rather to an effect of the absence of **Wolbachia**. In *D. simulans* and the *Aedes* cell lines, we also observed a transcriptional response by the **Wolbachia** itself, with bacterioferritin expression increasing in response to an iron-supplemented diet. Possible interactions between **Wolbachia** and iron metabolism have already been suggested, since almost all the genes necessary for heme biosynthesis and also bacterioferritin, are retained by the **Wolbachia** infecting the nematode *Brugia malayi* [20], *Drosophila melanogaster* [37] and *Culex quinquefasciatus* [39]. Different lines of evidence suggest that this pathway could contribute to the **Wolbachia**-nematode mutualism [20]. In particular, several nematodes, among which *B. malayi*, have lost the machinery for heme production and depend on external sources [32]. **Wolbachia** infection in filarial nematodes could allow for this provision and explain why **Wolbachia** is a mutualist in these organisms while it is a parasite in others. However, this does not preclude any interaction between iron metabolism and **Wolbachia** in other systems. For example, **Wolbachia** participation in iron homeostasis has been shown to be beneficial in *D. melanogaster* under conditions of nutritional stress [22]. Our data provide clear evidence that **Wolbachia** does indeed interact with iron metabolism at the molecular level in both the host and the bacterium. This means that the intracellular life-style of **Wolbachia** may rely on similar pathways in mutualistic or parasitic associations, which confirms that iron plays a major role in host-symbiont interactions [46].

Since the primary function of ferritin in insects is to capture, store and transport iron [53,54], the decrease in host ferritin mediated by **Wolbachia** infection could be explained by two distinct mechanisms: (i) **Wolbachia** could repress the expression of host ferritin, in order to have more easily access to the iron within the cell, or (ii) **Wolbachia** could scavenge iron in the cell, thus reducing the concentration of labile iron, and leading to reduced expression of ferritin. This last mechanism is supported by the results obtained for the association between *D. simulans* and **Wolbachia** arRi, where the iron uptake following an iron overload was greater in infected females than in uninfected ones. This difference in iron uptake could be due to the iron scavenged by **Wolbachia**, as suggested by the increase in bacterioferritin expression after iron-supplementation. Similar results were also observed in *A. aegypti* cells, where the absence of transcriptional response of the light chain in infected cells could be offset by an increase in the expression of bacterioferritin.

**Influence of endosymbionts on host functions: from cellular physiology to immune system evasion?**

Intracellular infection by bacteria may alter the cellular environment. This alteration can be a side effect of infection, a defense reaction of the host, or a manipulation induced by the symbiont to allow it to survive within the host cell. For example, the presence of an intracellular bacterium in an organism is generally detected by the host, which can in turn induce stress in the infected cell (production of toxins, ROS ...), activate the immune system, or enter into apoptosis [55]. In order to allow them to persist within the host cells, micro-organisms have developed various strategies to counter host defense strategies, including producing anti-oxidant bacterial molecules, manipulating the expression of host genes, blocking immune effectors, and activating/inhibiting apoptosis [56,57].

It was shown recently that **Wolbachia** induces stress (ROS production) when present in *Aa23 Aedes albopictus* cell cultures [58]. 2D-PAGE analysis has shown that **Wolbachia** infection up-regulates various host anti-oxidant proteins in cell cultures, and that **Wolbachia** bacterioferritin and Fe superoxide dismutase were highly expressed [58]. Our results confirm the expression of bacterioferritin in *Aedes* cell cultures, and also in whole insects. In addition, we have also demonstrated a transcriptional response of this gene to environmental conditions in *D. simulans* and *Aedes* cell lines. In both biological systems, there was no increase in bacterial densities after iron overload, suggesting that iron is not a limiting factor for bacterial growth. This could indicate that overexpression of bacterioferritin is related to ROS limitation rather than being a metabolic requirement. This could also explain why, in these two systems, an effect of bacterial infection was only detected in iron
supplemented medium. Together with the results reported by [58], this suggests that host and symbiont share the task of limiting the cytotoxic effects resulting from infection and from environmental conditions.

A striking finding is that the presence of \textit{Wolbachia} in host cells reduces ferritin expression, while it has generally been shown to be up-regulated by infection, even in close relatives of \textit{Wolbachia}, such as \textit{Anaplasma phagocytophilum} in human neutrophils [59] or \textit{Rickettsia montanensis} in ticks [60]. This finding might be related to the fact that the only Rickettsiales in which bacterioferritin has been found is \textit{Wolbachia}. These bacteria could rely on other systems to limit oxidative stress and its consequences, such as apoptosis, as has been demonstrated for the human pathogens \textit{Rickettsia rickettsii} (\textit{Rickettsiaceae}) and \textit{Anaplasma phagocytophilum} (\textit{Anaplasmataceae}) [61,62].

Is iron homeostasis involved in the evolution of dependence in \textit{A. tabida}?

Following iron overload, we did not detect any effect of infection status on fly development and life-history traits (size, longevity and oogenesis) in the facultative association between \textit{D. simulans} and \textit{wRi}. This contrasts with the fecundity benefits observed in \textit{D. melanogaster} [22], and could be due to differences in bacterial density or host tissue localization between the \textit{wRi} and \textit{wMel} strains [63]. We also found that \textit{Wolbachia} infection had an overall protective effect on developmental success in \textit{A. tabida}, which could be due to the ability of \textit{Wolbachia} to scavenge and thus to buffer the iron present within the cell. The high developmental mortality in iron-fed wasps suggests that \textit{A. tabida} has a very limited plasticity to changes in iron concentration. This difference between \textit{A. tabida} and \textit{Drosophila} species is probably related to their differing lifestyles: development in a highly buffered environment, consisting of \textit{Drosophila} larvae for the former, vs. heterogeneous substrates for the latter.

We also found that the toxicity of iron in infected \textit{A. tabida} females led to an increase in apoptosis in the ovaries comparable to the phenotype observed in aposymbiotic females in the most extreme cases observed. This result reinforces the idea that \textit{A. tabida} is very susceptible to iron, since this effect is observed despite...
a moderate increase of iron concentration in the wasps. This also raises the possibility that iron metabolism may be linked to the evolution of dependence. Indeed, egg production is controlled in Drosophila by the induction of two main apoptotic checkpoints in reaction to deleterious external stimuli, such as nutrient deprivation, cytotoxic chemicals and abnormal development [16]. If Wolbachia contributes to limiting ROS in A. tabida, then removing the bacteria or rearing the wasps on iron supplemented-medium could generate the accumulation of ROS during oogenesis, and the further activation of checkpoints. According to this hypothesis, removing Wolbachia would lead to ROS accumulation and the over-expression of host anti-oxidant genes, including ferritin. However, the low plasticity of A. tabida could lead to a response that is insufficient to counteract the apoptotic process in aposymbiotic ovaries. To comfort the link between iron metabolism and wasp dependence on Wolbachia, developing wasps on low-iron diet would be required to determine whether this affects oxidative stress and apoptotic processes. Unfortunately, all our attempts to chelate iron by adding BPS (Bathophenanthroline disulfonic acid) in the Drosophila food did not reduce the iron content in the wasp and it was thus not possible to limit iron in this Drosophila/parasitoid system.

Taken together, these data suggest that the presence of Wolbachia could dramatically affect the cellular physiology of its hosts. Consequences of endosymbiont infection may thus extend far beyond their effect on reproduction, which widen their possible impact on the evolution of their hosts. Compensation or tolerance may easily evolve for highly prevalent symbionts such as Wolbachia and contribute in some extreme cases to the emergence of dependence in an initially parasitic association.

Materials and Methods

Biological systems

Asobara tabida. Asobara tabida (Hymenoptera: Braconidae) is a solitary parasitoid species, and is naturally infected by three strains of the intracellular bacterium Wolbachia (wAtab1, wAtab2 and wAtab3) [64]. wAtab1 and wAtab2 induce cytoplasmic incompatibility, and only wAtab3 is required for oogenesis completion [65]. A. tabida females lay eggs into the first or second instar larvae of Drosophila. After Drosophila pupation, the parasitoid becomes an ectoparasite, and consumes its host before pupating and emerging. Wasps were maintained under controlled rearing conditions (20°C, 12 light/dark (LD) cycle) on axenic medium.

Aposymbiotic females are sterile, which makes it impossible to establish and maintain aposymbiotic lines, and so antibiotic treatments were administered just before the experiment in order to obtain aposymbiotic wasps [13].

Drosophila simulans. Drosophila simulans (Diptera: Drosophilidae) was trapped in Antibes (France), and was naturally infected by the wRi strain. Aposymbiotic flies were produced by two successive antibiotic treatments [13], followed by 12 generations without antibiotic treatment. Flies were maintained under controlled rearing conditions (20°C, 12 LD cycle) on axenic medium.

Aedes aegypti RML12 cell line. The Aedes aegypti RML12 cell line derived from larvae [66] and infected by Wolbachia strain...
were plotted using 7 dilutions (10 to 10^7 copies) of a previously diluted cDNA (corresponding to 25 ng of cDNA). Standard curves in accordance with the manufacturer’s instructions. The genomic region was sequenced (EuroBlue Taq, Eurobio, France) to check the clustering of Light- and Heavy-chains at the same genomic locus, and the potential presence of Iron Response Elements (IRE) in introns. Signal peptides were detected using SignalP-3.0 software [68]. Genomic ferritin sequence has been deposited in the EMBL database under accession number FN395057.

Characterization of the ferritin genes

**Insect ferritin.** *A. tabida* ferritin ESTs obtained by Suppression Subtractive Hybridization were full-length sequenced using the GeneRacer Kit (Invitrogen, France) in accordance with the manufacturer’s instructions. The genomic region was sequenced (EuroBlue Taq, Eurobio, France) to check the clustering of Light- and Heavy-chains at the same genomic locus, and the potential presence of Iron Response Elements (IRE) in introns. Signal peptides were detected using SignalP-3.0 software [68]. Genomic ferritin sequence has been deposited in the EMBL database under accession number FN395057.

**Bacterioferritin (Bfr).** Whole bacterioferritin CDS was amplified using consensus primers (Bfr-F1: ATG AAT GAA GAG ATA GTA and Bfr-R1: ATT TGT TGT TCT TAA ATA) in *A. tabida* (ωAtab3 and one of the two other strains ωAtab1 or ωAtab2) and in *D. simulans* (ωRi). The bacterioferritin sequences have been deposited in the EMBL database under accession numbers FN395058 to FN395060.

**Quantitative expression by Real-Time RT-PCR**

**A. tabida.** We used a Real-Time quantitative RT-PCR technique to measure ferritin expression. Young individuals (0–2 days old) were isolated from symbiotic and aposymbiotic lines (5 replicates of 10 females, 10 males, or 10 ovaries (only the distal part which does not contain eggs was dissected in a drop of A-) and sterile, 30 µg of digested DNA per reaction). A total of 30 cycles of amplification was performed: 95°C for 10 s, 57°C for 20 s, and 72°C for 20 s. The reaction mixture consisted of 0.5 µM of each primer, 1 µl of diluted cDNA (corresponding to 25 ng of cDNA). Standard curves were plotted using 7 dilutions (10 to 10^7 copies) of a previously amplified PCR product purified using Nucleospin Extract II kit (Machery-Nagel, France). The number of gene copies was calculated as described in [70]. The L6-ribosomal gene (RibL6-At-F: CAC CGA TGA TGA GCT TTG CT and RibL6-At-R: CCC CGG GAT CCT AAC GAT GA, amplicon: 154 bp) was used as housekeeping gene for quantitative analyses. This gene was not differentially expressed in response to infection and iron treatment. In addition, similar results were obtained when Elongation factor 1γ and β-tubulin were used to normalize expression (data not shown).

**D. simulans.** The same procedure was used to quantify the ferritin in *D. simulans* (4 replicates from 5 whole females), except that the total RNA of the *D. simulans* females was extracted using RNaseasy kit (Invitrogen, France). The following primers were used for quantitative PCR: HCh-Ds-F: GAA TTT TGC TGT GCT CTT TT and HCH-Ds-R: CCG TGT AGG GTG GTG ATG AT (amplicon: 148 bp); LCH-Ds-F: CGT CTA CCT GTG CGA GT and LCH-Ds-R: GTG CTC TTA ATA ATG CT (amplicon: 190 bp). The RP49 gene [71] was used as housekeeping gene for quantitative analyses.

**A. aegypti cell line.** For total mRNA extraction, three biological replications were done using 5×10^7 cells of each line. Cells were homogenized in trizol solution, and total RNA was extracted using chloroform/trizol method as recommended. The total RNA contained in the aqueous phase was precipitated by 2 M LiCl, overnight at −20°C. After centrifuging at 16,000 g for 20 min, pelleted RNA was washed twice with cold ethanol (70%), then dried by vacuum centrifugation, and dissolved in 30 µl of DNase/RNase-free water. The extracted RNA was treated with DNase (TURBO DNA-free, Ambion, Applied Biosystems, TX, USA), in accordance with the manufacturer’s recommendations. RNA was reverse transcribed using SuperScript III and random primers (Invitrogen, France), in accordance with manufacturer’s instructions. For quantitative PCR, cDNAs were diluted to 10 ng/µl, and the following primers were used: cell ferritin (LCh2e-F: CTC AAA GGC GGA GTT ATT GG and LCH3eR-1: ATC GGA ACT CCT CCT CGA GA; amplicon: 233 bp) and bacterioferritin (see above); host ribosomal gene 7S RNA [72]. Quantitative PCR was performed using LightCycler LC480 system (Roche, France) as follows: 10 min at 95°C, 35 times [15 sec at 95°C, 10 s at 57°C (or 54°C for Bfr), 20 sec at 72°C], 20 sec at 70°C. The reaction mixture consisted of 0.4 µM of each primer, 10 µl of Fast SYBR-Green Master Mix (Roche, France), and 2 µl of diluted cDNA (corresponding to 20 ng of cDNA) in a total reaction volume of 20 µl.

**Exposure to iron and life-history traits**

**Iron treatment.** Seventy *D. melanogaster* eggs were placed on standard diet (Control), or on standard diet supplemented by FAC (Ferric Ammonium Citrate, Aldrich, MO, USA) 20 mM (Iron). The diet was kept unchanged all along larval development. Three *A. tabida* females were allowed to lay eggs on the larvae hatched from the eggs. As parasitoid larvae feed on the hemolymph of their *Drosophila* host, iron absorbed by the fly is available for the developing wasp.

For *D. simulans*, seventy eggs were placed on standard diet (Control) or on standard diet supplemented by FAC 20 mM (Iron).

In all the cell cultures, the medium was replaced by a fresh medium without fetal bovine serum when 80% confluence had been reached, and the cultures were then incubated overnight (~18 hours) [73]. After the incubation period, fresh medium supplemented with FAC 100 µM was added, and incubation was continued for a further 18 hours. For control experiments, the infected and uninfected cell lines were treated as above, but the iron was omitted. Cells were collected by centrifuging and Trypan Blue staining was used to estimate the proportions of live/dead cells. In all experiments, including the controls, cell viability was around 96% (mean) and ranges from 92% to 99%. Pelleted cells
were quickly frozen in liquid nitrogen, and stored at −80°C until use.

**Development of A. tabida.** Seventy Drosophila eggs were introduced in each vial, and three A. tabida females were introduced for parasitization. Four different treatments were performed: development on standard medium, on standard medium plus antibiotic, on iron-supplemented medium and on iron-supplemented medium plus antibiotic. The experiment was repeated two times with 7 and 8 replicates for each treatment, respectively. As a control, the same protocol was performed without adding the wasps (n = 8 replicates per condition). The Drosophila and A. tabida that emerged were counted.

**Total iron absorption.** Iron was measured using a Ferrozine assay adapted from [74] with 15 A. tabida females, 10 Drosophila females or 10 Drosophila larvae (stage L3), except that (i) Drosophila heads were removed in order to avoid any interference due to their red eye coloration and (ii) purple coloration was measured at 562 nm, and the background at 750 nm was deduced (Xenius spectrophotometer, Safas, Monaco).

**Bacterial density.** DNA from 6 females was individually extracted using DNeasy kit (Qiagen, France) in accordance with manufacturer’s instructions. Quantitative PCR was performed as described below, using 81-F: TGG TCG AAT AAG TGA AGA AAC/691-R: AAA AAT TAA ACG CTA CTC CA primers (Tm: 52°C) to quantify total Wolbachia density, and 18Slo1/ N58+2 [75] or RP49 F/R [71] as a normalizer for A. tabida and D. simulans, respectively.

**Ovarian phenotype.** Ovaries of A. tabida females reared under control or iron-supplemented diets were dissected in PBS, and individually transferred onto a slide using Roti-Liquid Barrier (Roth, France). Ovaries were either mounted in PBS to count the eggs (n = 60 per treatment), or stained as follows for the TUNEL assay (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labeling, n = 17 and 19 for control and iron, respectively). Ovaries were fixed in paraformamide 4%−PBST 0.1% for 30 min, washed with PBS, made permeable with 20 µg/mL Proteinase K (Eurobio, France) for three hours, and then washed with PBS and mounted in 25 µl of Vectashield Hardset containing 1.5 µg/mL DAPI (4′,6-Diamidino-2-phenylindole, Vector Laboratories, CA, USA). Positive and negative controls were treated by 50 U/Diamidino-2-phenylindole, Vector Laboratories, CA, USA). (Fermentas, France) for 5 min before TUNEL staining, with or without the wasps (n = 8 replicates per condition). The Drosophila and A. tabida that emerged were counted.

**Statistical analyses**

When possible, parametric tests were used directly or after transformation of data. Expression data and iron absorption measurements were log-transformed and 1/x-transformed, respectively, before analysis. ANOVA residuals were checked for normality by Shapiro’s test, and for homoscedasticity by Levene’s test. Pairwise comparisons were then performed using Tukey’s HSD test. To take into account all the experiments that were realized (2 blocks), number of wasps emerged and percentage of successful development of Drosophila were analysed using mixed Linear Model (LM) and mixed General Linear Model (GLM) with binomial error, respectively. In both cases, blocks were treated as random factor, whereas iron treatment and infection status were treated as fixed factors. All statistical analyses were performed using R 2.8.0 software.

**Supporting Information**

**Figure S1** Influence of Wolbachia infection and iron treatment on D. simulans survival. Survival curves of symbiotic and aposymbiotic females reared on standard diet (control) or iron-supplemented diet (n = 15 per treatment). Diet was renewed every 2 days and females were allowed to lay eggs. Found at: doi:10.1371/journal.ppat.1000630.s001 (0.01 MB PDF)

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**Author Contributions**

Conceived and performed the experiments: NK PM BM FV. Performed the experiments: NK DV. Analyzed the data: NK DC. Wrote the paper: NK DV PM BM FV.

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