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The endosymbiont Wolbachia increases insulin/IGF-like signalling in Drosophila

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Insulin/IGF-like signalling (IIS) is an evolutionarily conserved pathway that has diverse functions in multi-cellular organisms. Mutations that reduce IIS can have pleiotropic effects on growth, development, metabolic homeostasis, fecundity, stress resistance and lifespan. IIS is also modified by extrinsic factors. For instance, in the fruitfly Drosophila melanogaster, both nutrition and stress can alter the activity of the pathway. Here, we test experimentally the hypothesis that a widespread endosymbiont of arthropods, Wolbachia pipientis, can alter the degree to which mutations in genes encoding IIS components affect IIS and its resultant phenotypes. Wolbachia infection, which is widespread in D. melanogaster in nature and has been estimated to infect 30 per cent of strains in the Bloomington stock centre, can affect broad aspects of insect physiology, particularly traits associated with reproduction. We measured a range of IIS-related phenotypes in flies ubiquitously mutant for IIS in the presence and absence of Wolbachia. We show that removal of Wolbachia further reduces IIS and hence enhances the mutant phenotypes, suggesting that Wolbachia normally acts to increase insulin signalling. This effect of Wolbachia infection on IIS could have an evolutionary explanation, and has some implications for studies of IIS in Drosophila and other organisms that harbour endosymbionts.

Keywords: Drosophila; insulin-like signalling; Wolbachia

1. INTRODUCTION

The insulin/IGF-like signalling (IIS) pathway is ubiquitous in multi-cellular animals and may have been involved in the evolution of multi-cellularity itself (Skorokhod et al. 1999). The pathway, via a conserved intracellular signalling cascade including phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB or AKT), negatively regulates the activity of several transcription factors of the forkhead family, by their phosphorylation and subsequent exclusion from the nucleus. Mutations that reduce the activity of the IIS signalling cascade have pleiotropic effects on many traits, including growth (Brogiolo et al. 2001; Butler & Le Roith 2001; Ikeya et al. 2002; Rulifson et al. 2002), development (Kimura et al. 1997), metabolic homeostasis (Saltiel & Kahn 2001), adult lifespan, resistance to stress (Lithgow et al. 1995; Clancy et al. 2001; Holzenberger et al. 2003) and fecundity (Partridge & Gems 2002; Liang et al. 2003; Nelson & Padgett 2003; Tatar et al. 2003; Broughton et al. 2005; Piper et al. 2008) in the nematode worm Caenorhabditis elegans, the fruitfly Drosophila melanogaster and the mouse Mus musculus. Natural variation in some ecologically significant traits has also been found to be associated with IIS. For example, allelic variants of the PI3K are implicated in the latitudinal variation of reproductive diapause in Drosophila melanogaster (Williams et al. 2006).

A primary function of IIS is to match energetically demanding activities such as growth and reproduction to nutrition and other informative environmental inputs such as various stresses, and to ensure metabolic homeostasis. Thus, consistent with its role in nutrient and stress sensing, a variety of extrinsic factors can be important regulators of insulin sensitivity in animals and humans. For example, the stress-responsive Jun-N-terminal kinase (JNK) pathway regulates IIS, linking responses to environmental stresses with metabolic homeostasis (see Karpc & Jasper 2009 for review). In both C. elegans and Drosophila, genetically upregulating JNK signalling leads to decreased IIS, increased resistance to oxidative stress and extension of lifespan (Oh et al. 2005; Wang et al. 2005). The evolutionarily conserved nutrient-sensing TOR pathway regulates protein synthesis and growth in response to amino acids and growth factors (Wullschleger et al. 2006), and interacts with IIS to control lifespan in worms and flies (Vellai et al. 2003; Jia et al. 2004; Kapahi et al. 2004). In humans, diet (Romao & Roth 2008), stress (Depke et al. 2008) and exercise (Colberg 2007) have all been shown to affect insulin sensitivity. Acute (transient) insensitivity can also be experienced following infections and injuries in humans (Ikezu et al. 1997; Del Aguila et al. 2000; Van den Berghe et al. 2001) and in Drosophila, infection by pathogenic Mycobacterium has been found to alter IIS (Dionne et al. 2006).

Another possible infectious extrinsic factor modifying IIS in Drosophila is Wolbachia pipientis, a micro-organism of the rickettsial family. Wolbachia is a widespread endosymbiont of arthropods and often causes infection-induced reproductive incompatibility in the host. This Wolbachia-induced trait has been found to occur in many Drosophila species, including D. melanogaster (Bourtzis et al. 1996; Veneti et al. 2003; McGraw & O’Neill 2004). Wolbachia infection can also affect other aspects of host reproduction, and feminization, male

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killing and parthenogenesis have all been reported (Stouthamer et al. 1999; Hurst et al. 2000). Although the relationship between Wolbachia and the host species is usually symbiotic, a virulent form of Wolbachia that shortens lifespan has been found in D. melanogaster (Min & Benzer 1997). In the Bloomington stock centre, 30 per cent of D. melanogaster strains have been estimated to be infected by endosymbionts (Clark et al. 2005). Removal of Wolbachia by tetracycline treatment showed that it was involved in the sterility of the Sex mutant allele (Starr & Cline 2002) and the lifespan-extension phenotype of an Indy mutant stock (Toivonen et al. 2007). The mechanisms involved in these effects of Wolbachia are not yet understood.

In the course of specifying and standardizing the Wolbachia infection status of our D. melanogaster laboratory stocks, we observed that the removal of Wolbachia by tetracycline appeared to exacerbate IIS mutant phenotypes. We therefore made a systematic investigation by measuring the effect of Wolbachia infection status on a range of IIS-related phenotypes in control and IIS mutant flies. In the presence of Wolbachia, ubiquitous expression of a dominant negative form of the insulin receptor (InRDN) resulted in moderate dwarfism, reduced fecundity and extension of female lifespan, all typical phenotypes of reduced IIS. In the absence of Wolbachia, the previously moderate effects of InRDN expression were accentuated, resulting in flies that showed severe IIS-related phenotypes including extreme dwarfism, sterility, increased fat levels and shortened lifespan. The absence of Wolbachia in control genotypes resulted in a decrease in fecundity and adult weight compared with infected control genotypes, but no effect on other IIS-related phenotypes. These data suggest that Wolbachia can increase IIS in infected flies.

2. MATERIAL AND METHODS

(a) Fly stocks and husbandry

yw;UAS-dInR^R14009K (chr II) was obtained from the Bloomington Drosophila Stock Centre (BDSC; ref. number. FBAL0156359). The UAS-InRDN transgene causes an amino acid substitution in the kinase domain (R14009A) of the Drosophila insulin receptor (dInR), resulting in its dominant negative activity (Wu et al. 2005). w^{1115}; Daughterless-GALA (chr II) (Fly Base ID FBti0013991) was obtained from the BDSC. UAS-PTEN was provided by Prof. Ernst Hafen (ETH, Switzerland) and w^{1115};S^{1115}106-GeneSwitch (chr III) (Fly Base ID FBti0013991) was obtained from the Stock Centre (BDSC; ref. number. BG0156359). w^{1115};S^{1115}106-GeneSwitch (chr III) (Fly Base ID FBti0013991) was obtained from the Stock Centre (BDSC; ref. number. BG0156359). w^{1115};S^{1115}106-GeneSwitch (chr III) was obtained from the Stock Centre (BDSC; ref. number. BG0156359).

(b) Generation of Wolbachia-infected and -uninfected flies

PCR to detect the top gene (Zhou et al. 1998) confirmed that, after backcrossing, all stocks were infected with Wolbachia (Wol+; data not shown). To create genetically identical Wolbachia-uninfected stocks (Wol−), the backcrossed Wol+ GALA, UAS and w^{Dab} strains were treated with 30 μg ml^{-1} of tetracycline contained in standard food for two generations and retested by PCR to confirm that Wolbachia had been removed (figure 1a). All stocks were then cultured under normal conditions for at least five generations to allow full recovery from the effects of tetracycline treatment and to restore intestinal flora (Holden et al. 1993; Toivonen et al. 2007) prior to the generation of Wolbachia-infected and -uninfected daGAL4/UAS-InRDN, daGAL4/+, UAS-InRDN/+, and w^{Dab} genotypes for further analysis.

(c) Lifespan experiments

Procedures for lifespan studies were as described in Clancy et al. (2001) and Mair et al. (2003). The experimental flies were allowed to mate for 48 h before collection and sorting. Lifespans were measured in flies kept at 10 per vial on standard food medium and transferred to new food three times a week. Deaths were scored five to six times in every 7 days.

(d) Fecundity and body weight

Females for measuring body weight were prepared as for lifespan experiments and weighed individually when 7 days old. For fecundity, the number of eggs laid per vial of 10 females per day in lifespan experiments was counted.

(e) Triacylglycerol assay

Triacylglyceride (TAG) content quantification was performed as described in Grönke et al. (2003), except that data were normalized to whole fly weight.

(f) Oil red O staining

Lipid droplets were stained with Oil Red O (Sigma; Guiterrez et al. 2007). Seven-day-old (adult) female abdomens were attached to a glass slide using double-sided tape and dissected to expose the adult fat body attached to the dorsal abdomen. Samples were embedded in Vectashield with DAPI (Vector). Images were taken using an ORKA-ER CCD camera (Hamamatsu Photonics) and an Axioskop 2 microscope (Zeiss), and subsequently analysed using ImageJ.

(g) AKT and FOXO protein detection

Anti-phospho-Drosophila Akt (no. 4054) and Akt (no. 9272) antibodies were purchased from New England BioLab (UK distributor for The Cell Signalling). Anti-dFOXO is described in Giannakou et al. (2007). Five flies were homogenized with 200 μl of 1× sample buffer containing proteinase inhibitor and phosphatase inhibitor cocktail I (Sigma). SDS–PAGE analysis was performed using a BioRad mini gel system and the proteins were transferred to a membrane by semidry blotter. Primary AKT antibodies were used at 1:1000 dilution and the dFOXO primary antibody at a dilution of 1:500. A secondary antibody conjugated with HRP was used at 1:10 000, and the signal developed using an ECL detection kit (GE Healthcare).

(h) Statistical analyses

Statistical analyses were performed using JMP (v. 7) software (SAS Institute). Lifespan data were subjected to log-rank tests and are presented as survival curves. Weight, fecundity, TAG levels and fat-cell number data were tested for normality using the Shapiro–Wilk W-test on studentized residuals (Sokal & Rohlf 1998). Two-way (genotype, Wolbachia status) analyses of variance (ANOVA) were performed and planned comparisons of means were made using the
The body weight of 7-day-old adult females was examined in Wolbachia and insulin-like signalling mutants. DNA obtained from tetracycline-untreated and their controls in parallel. IIS mutant flies were characterized typical IIS-related phenotypes in Wolbachia-infected (Wol+) and -uninfected (Wol−) flies for each genotype were performed using the Tukey HSD.

Tukey HSD test or Student’s *t*, as appropriate. Data are presented as means of raw values ± s.e.m., and asterisk denotes significant difference from controls.

### 3. RESULTS

**(a) Removal of Wolbachia enhances IGF-like signalling mutant phenotypes**

To determine how Wolbachia infection status affects IIS, we characterized typical IIS-related phenotypes in Wolbachia-infected and -uninfected IIS mutant flies and their controls in parallel. IIS mutant flies were created by expression of a dominant negative form of the insulin receptor (UAS-InRDN) under the control of the ubiquitous and constitutive daughterless-GAL4 (daGAL4) driver.

Reduced growth and fecundity are typical phenotypes of lowered IIS and, as expected, daGAL4/InRDN females weighed significantly less and laid fewer eggs than their daGAL+/+, InRDN/+ and w control genotypes, irrespective of Wolbachia status (figure 1b,c) and electronic supplementary material, figure 1a–d). However, removal of Wolbachia greatly accentuated the reduction in weight and fecundity of the daGAL4/InRDN genotype and moderately reduced these phenotypes in control genotypes (figure 1b,c; and table 1 for statistical analysis by Wolbachia status).

Increases in TAG levels and alterations in adult fat tissue morphology are often associated with reduced IIS (Bohni et al. 1999; Tatar et al. 2001). Wol− daGAL4/InRDN females had normal TAG levels (figure 2a) and adult fat body morphology (figure 2b) compared to Wol+ controls. In contrast, Wol− daGAL4/InRDN females had significantly higher TAG levels (figure 2a; Tukey HSD, p < 0.05) and alterations in fat body morphology (figure 2b) compared to all other genotypes. Unlike the morphologically normal abdominal fat tissue of Wolbachia-infected daGAL4/InRDN and control flies (figure 2b(i–iii)), this tissue was misshapen and shrunken in Wol− daGAL4/InRDN flies (figure 2b(vi)), similar to that seen under starvation conditions (Scott et al. 2004). For these IIS-related phenotypes, Wolbachia status had no effect on control genotypes (figure 2a,b).

Lifespan is another trait that responds to changes in IIS and the effect of reducing the activity of the pathway can be either positive or negative. Some mutations or genetic manipulations of IIS, such as strong, ubiquitous downregulations, are often detrimental to lifespan (Clancy et al. 2001; Tatar et al. 2001; Taguchi et al. 2007; Selman et al. 2008), but moderate or tissue-specific reductions in IIS can extend it (Clancy et al. 2001; Tatar et al. 2001; Giannakou et al. 2004; Hwangbo et al. 2004; Broughton et al. 2005; Selman et al. 2008). It appears that alterations of specific IIS components at particular times or in specific tissues may be important, or that lifespan may peak at an intermediate level of IIS activity. Interestingly, Wol+ daGAL4/UAS-InRDN once-mated females showed a large extension of median and maximum lifespan (figure 2c; table 2 for statistical analysis;
This detrimental effect of InRDN expression to male found (electronic supplementary material, figure 2 measured, and a slight reduction in median lifespan was mental to survival. The effect of over-expression of expression, resulting in a reduction in IIS to levels detri-

Wolbachia (figure 2 supplementary material, figure 2 difference from all groups, [44x54]¼ 

TAG level by genotype/wolb ( ug)/weight(mg) were subjected to two-way ANOVA, with genotype (2009) and Wolbachia backgrounds are shown. The upper two bands of each panel are phospho-AKT (pAKT) and the bottom 2 and Wol–infected and -uninfected daGAL4/InRDN once-mated females and controls. Median lifespans and statistical ana-

Figure 2. The effect of Wolbachia infection status on fat, survival and AKT phosphorylation. (a) TAG concentration of individual whole virgin females (n = 12). Wol+ wolb = 15.15 µg mg⁻¹ of fly weight; Wol- wolb = 18.15 µg mg⁻¹; Wol+ InRDN/+ = 14.38 µg mg⁻¹; Wol- InRDN/+ = 15.74 µg mg⁻¹; Wol+ daGAL4/dInRDN = 18.29 µg mg⁻¹; Wol– daGAL4/dmRDN = 40.07 µg mg⁻¹; Wol+ daGAL4/+ = 12.25 µg mg⁻¹; and Wol– daGAL4/+ = 14.37 µg mg⁻¹. Data were subjected to two-way ANOVA, with genotype (p < 0.0001) and Wolbachia status (p < 0.0001) having significant effects. There was a significant interaction effect of genotype and Wolbachia status (p < 0.0001). Planned comparisons of the mean TAG level by genotype/Wolbachia status were made using the Tukey HSD. Error bars are s.e.m. and asterisk indicates significant difference from all groups, p < 0.05. Black bars, Wolbachia-infected; grey bars, -uninfected. (b) Adult abdominal fat body mor-

phology for each genotype: (i) Wol+ InRDN/+; (ii) Wol+ daGAL4/+; (iii) Wol+ daGAL4/InRDN; (iv) Wol– InRDN/+; (v) Wol– daGAL4/+; (vi) Wol– daGAL4/InRDN. Arrow indicates area of missing tissue and scale bar is 20 µm. (c) Survival of Wolbachia-infected and -uninfected daGAL4/InRDN once-mated females and controls. Median lifespans and statistical anal-

yses are given in table 2. Red solid line, daGAL4/InRDN Wol+; red dashed line, daGAL4/InRDN Wol–; dark blue solid line, daGAL4 Wol+; dark blue dashed line, daGAL4 Wol–; green solid line, InRDN Wol+; green dashed line, InRDN Wol–; light blue solid line, wolf Wol+; light blue dashed line, wolf Wol–. (d) Levels of AKT and dFOXO phosphorylation were detected by Western blotting. For AKT phosphorylation, representative blots of two independent experiments each for Wol+ and Wol– backgrounds are shown. The upper two bands of each panel are phospho-AKT (pAKT) and the bottom two bands are tAKT. The presence of two bands is due to expression of two isoforms of AKT. pAKT/tAKT relative ratio was estimated from the signal intensity of the lower bands of both pAKT and AKT, which was averaged by ROI analysis in ImageJ. For dFOXO phosphorylation in the Wol– background, a single experiment was performed. The ratio of phosphoryl-

ated to unphosphorylated dFOXO (dFOXOppp/dFOXO ratio) was estimated from the intensity of the upper (dFOXOppp) to the lower (dFOXO) band in ImageJ.

Wol+ daGAL4/InRDN females were very short-lived (figure 2c; table 2 for statistical analysis; electronic supplemen-
tary material, figure 2b). For control genotypes, Wolbachia infection status had no effect on lifespan (figure 2c; table 2 for statistical analysis; electronic supplementary material, figure 2a–c). This effect of Wolbachia removal to drastically shorten the lifespan of daGAL4/UAS-InRDN females is consistent with an accentuation of the inhibitory effect of InRDN expression, resulting in a reduction in IIS to levels detri-

mental to survival. The effect of over-expression of InRDN on lifespan of males in the Wol+ strain was also measured, and a slight reduction in median lifespan was found (electronic supplementary material, figure 2d). This detrimental effect of InRDN expression to male lifespan appeared to be accentuated in the absence of Wolbachia as only very few Wol– daGAL4/UAS-InRDN males enclosed, and those that did died within a few days. This sex difference in the effect on survival is typical for IIS mutants in Drosophila (Clancy et al. 2001; Tatar et al. 2001; Giannakou et al. 2004; Broughton et al. 2005) and in mice (Holzenberger et al. 2003; Selman et al. 2008).

The previous data suggested that Wolbachia acts to enhance IIS. As a further indicator of a change in the magnitude of IIS downregulation in daGAL4/UAS-InRDN flies owing to Wolbachia infection, the activation of two key downstream effectors of IIS—AKT and the fly forkhead box O transcription factor, dFOXO—were measured. The Ser505 of AKT is a target of the TOR/ RICTOR complex, the phosphorylation of which is
Table 2. Median and maximum lifespans and statistical comparisons of survivorship data from figure 2c. Each pairwise comparison of survivorship data by genotype and Wolbachia infection status was made using the log-rank test, and $\chi^2$ $p$-values are given.

<table>
<thead>
<tr>
<th>genotype/ Wolbachia status</th>
<th>daGAL/ InRDN$^+$</th>
<th>daGAL/ InRDN$^-$</th>
<th>daGAL$^+$</th>
<th>InRDN$^-$</th>
<th>daGAL$^-$</th>
<th>InRDN$^+$</th>
<th>$w^-$</th>
<th>$w^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>median lifespan (days)</td>
<td>70.5</td>
<td>21.4</td>
<td>54.0</td>
<td>54.0</td>
<td>49.0</td>
<td>45.5</td>
<td>54.0</td>
<td>54.0</td>
</tr>
<tr>
<td>maximum lifespan (days)</td>
<td>82</td>
<td>36.0</td>
<td>66.0</td>
<td>65.0</td>
<td>63.0</td>
<td>66.0</td>
<td>69.0</td>
<td>66.0</td>
</tr>
<tr>
<td>daGAL/ InRDN$^+$</td>
<td>$2.197 \times 10^{-36}$</td>
<td>$1.632 \times 10^{-16}$</td>
<td>$3.861 \times 10^{-17}$</td>
<td>$1.853 \times 10^{-19}$</td>
<td>$3.707 \times 10^{-18}$</td>
<td>$2.923 \times 10^{-13}$</td>
<td>$1.488 \times 10^{-13}$</td>
<td></td>
</tr>
<tr>
<td>daGAL/ InRDN$^-$</td>
<td>$3.695 \times 10^{-35}$</td>
<td>$5.675 \times 10^{-29}$</td>
<td>$2.102 \times 10^{-32}$</td>
<td>$2.073 \times 10^{-31}$</td>
<td>$6.635 \times 10^{-27}$</td>
<td>$1.417 \times 10^{-25}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>daGAL$^+$</td>
<td>0.2081</td>
<td>0.00976</td>
<td>0.08486</td>
<td>0.46632</td>
<td>0.75395</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>InRDN$^-$</td>
<td>0.20105</td>
<td>0.52295</td>
<td>0.78930</td>
<td>0.11548</td>
<td>0.03060</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>daGAL$^-$</td>
<td>0.06816</td>
<td>0.19695</td>
<td>0.67115</td>
<td></td>
<td></td>
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</tbody>
</table>

Taken together, these data suggest that the removal of the endosymbiont Wolbachia from daGAL4/InRDN females caused a greater inhibitory effect of InRDN expression on IIS, resulting in enhanced IIS-related phenotypes. The similar but less pronounced reduction in weight and fecundity seen upon removal of Wolbachia from control genotypes suggests that these two phenotypes may be the most sensitive to changes in IIS and demonstrates that Wolbachia’s effect is not specific to the daGAL4/InRDN genotype.

(b) Removal of Wolbachia enhances the effect of expression of InRDN in adult fat body

The quantitatively different effects of InRDN expression on IIS-related phenotypes in the presence and absence of Wolbachia, described above, suggested a dose dependency of IIS downregulation. To begin to determine if these effects are general, we used the RU486 GeneSwitch-inducible GAL/UAS system (Osterwalder et al. 2001; Roman et al. 2001) to express InRDN in adult fat body. Manipulation of IIS by over-expression of the key IIS effector forhead transcription factor, dFOXO, in this tissue using the inducible S1106-GS driver has previously been shown to be sufficient to extend lifespan (Giannakou et al. 2004). This driver was initially reported as expressed in adult fat body (Roman et al. 2001) and has since been shown also to express in the digestive system and testes (Poirier et al. 2008).

Expression of InRDN using the S1106-GS driver under the same induction conditions as in Giannakou et al. (2004) had no effect on lifespan in the presence of Wolbachia (figure 3a). However, removal of Wolbachia from S1106-GS/UAS-InRDN female flies resulted in extension of lifespan and revealed a dose-dependent effect of expression of InRDN (figure 3b). At 200 $\mu$M, RU486 inducer Wol$^-$ S1106-GS/UAS-InRDN females showed a 6.5 per cent increase in median lifespan ($\chi^2 p = 1.16 \times 10^{-7}$) and at 300 $\mu$M RU486 a 13 per cent increase ($\chi^2 p = 3.65 \times 10^{-15}$) compared with the same
genitalia of these kinds of effects and exactly how Wolbachia modifies IIS-related phenotypes.

Evolutionary considerations suggest that the mechanisms by which Wolbachia interacts with IIS may have its origin in the way that these endosymbionts are transmitted. Their habitat is inside the cytoplasm of cells, and they are transmitted maternally, via infection of eggs (Stouthamer et al. 1999). Wolbachia are thought to persist in host populations by providing females with a reproductive advantage via mechanisms that can include male killing, feminization of genetic males and cytoplasmic incompatibility (see Stouthamer et al. 1999 for review). In D. melanogaster, an effect of Wolbachia infection is to confer reproductive advantage through cytoplasmic incompatibility (Hoffmann et al. 1998). Wolbachia-infected females can successfully mate with infected or uninfected males, but uninfected females are incompatible with infected males. Thus, Wolbachia infection can spread and persist in the host population, although other mechanisms must also be involved (Olsen et al. 2001; Fry et al. 2004; Weeks et al. 2007; Brownlie et al. 2008).

In evolutionary terms, the interests of the female host and of the symbiont in host egg production are identical. Both host and symbiont are selected to maximize host fitness through this route. In populations of flies that are infected with Wolbachia, part of the response to selection for an optimal regime of host egg production through the life history may thus be attributable to evolution of the effects of the symbiont on host reproduction. However, this alone would not explain why Wolbachia would act as seen in this study, solely to promote high host fecundity. A possible explanation could come from events during adaptation to life in the laboratory. The life history of Drosophila evolves so as to optimize fitness under the prevailing culture regime (Sgro & Partridge 2000; Spencer & Promislow 2002). The usual regime consists of the transfer of young adults to fresh culture vials, with consequent selection for rapid maturation and copious early reproduction. This produces evolutionary increases in the speed of pre-adult development and in female fecundity relative both to the levels seen in wild flies and in laboratory stocks maintained with overlapping generations. These traits that are favoured in laboratory culture are also promoted by increased IIS, although it has not yet been determined whether laboratory adaptation is achieved in whole or in part by increased activity of this pathway. In stocks that are infected with Wolbachia, part of the adaptation to laboratory conditions may be achieved by the evolution of the effects of the symbiont on host reproduction. The tetracycline treatment used for the removal of Wolbachia from Wol+ normal-lived S106-GS/InRDN females to increase the inhibitory effect of InRDN in this tissue to a threshold sufficient for lifespan extension. The data further suggest that ubiquitous expression of InRDN by daGal4 may have affected fecundity, growth and fat phenotypes via expression during development and/or in other tissues.

5. DISCUSSION

The data presented here demonstrate that mutant IIS and its consequent phenotypes in Drosophila can be enhanced by removal of Wolbachia, suggesting that one of the normal consequences of Wolbachia infection is enhanced IIS. The data are consistent with a general effect on IIS, and not a specific interaction with mutants such as the daGal4/InRDN genotype, because removal of Wolbachia from control strains had similar, although weaker, effects. More work will be needed both to determine the
(Ballard & Melvin 2007). However, this study measured the effects only two generations after treatment and, because the effect is based on damage to the mitochondria, it is expected to decline rapidly. The parental strains used in the current study were reared on normal food for at least five generations following tetracycline treatment to restore flora in their intestine prior to the generation of experimental flies for analysis. That such a regime does not produce ill-effects as a result of the tetracycline treatment itself is supported by the finding that tetracycline treatment of already-Wolbachia-free flies had no effect on lifespan when tested several generations after treatment (Toivonen et al. 2007).

The variability of the phenotypes owing to IIS reduction in the fly is probably due to the complex nature of the pathway itself, as well as the developmental stage and tissue affected by the genetic manipulation in question. However, the dose dependency of IIS downregulation identified here supports the idea that some of the variability could be related to differing strengths of IIS downregulation. Over-expression of the FOXO transcription factor in the fly eye imaginal disks induced cell death (Junger et al. 2003), whereas the same gene expressed in the adult fat body extended lifespan (Giannakou et al. 2004). It is not known what intracellular differences exist between the adult fat body and imaginal disks (Giannakou et al. 2004; Hwangbo et al. 2004). It is important to note that separate FOXO’s longevity function from its apoptotic effect has implications for metabolic provisioning by a common invertebrate endosymbiont, Wolbachia pipientis, during periods of nutritional stress. Proc. Natl Acad. Sci. USA 102, 3105–3110. (doi:10.1073/pnas.0405775102)


