The ISME Journal (2016) 10, 376–388

www.nature.com/ismej

ORIGINAL ARTICLE

A novel intracellular mutualistic bacterium in the invasive ant *Cardiocondyla obscurior*

Antonia Klein[1,8], Lukas Schrader[1,8], Rosario Gil[2], Alejandro Manzano-Marín[2], Laura Flórez[3], David Wheeler[5], John H Werren[6], Amparo Latorre[2,7], Jürgen Heinze[1], Martin Kaltenpoth[3,4], Andrés Moya[2] and Jan Oettler[1]

[1]*Institut für Zoologie, Universität Regensburg, Regensburg, Germany;* [2]*Institut Canvanilles de Biodiversitat i Biologia Evolutiva (ICBiBE), Parc Cientific de la Universitat de Valencia, Paterna (Valencia), Spain;* [3]*Max Planck Institute for Chemical Ecology, Jena, Germany;* [4]*Johannes Gutenberg University Mainz, Institute for Zoology, Department for Evolutionary Ecology, Mainz, Germany;* [5]*Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand;* [6]*Department of Biology, University Rochester, Rochester, NY, USA and* [7]*Área de Genómica y Salud de la Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana (FISABIO)–Salud Pública, Valencia, Spain*

The evolution of eukaryotic organisms is often strongly influenced by microbial symbionts that confer novel traits to their hosts. Here we describe the intracellular Enterobacteriaceae symbiont of the invasive ant *Cardiocondyla obscurior*, '*Candidatus* Westeberhardia cardiocondylae'. Upon metamorphosis, *Westeberhardia* is found in gut-associated bacteriomes that deteriorate following eclosion. Only queens maintain *Westeberhardia* in the ovarian nurse cells from where the symbionts are transmitted to late-stage oocytes during nurse cell depletion. Functional analyses of the streamlined genome of *Westeberhardia* (533 kb, 23.41% GC content) indicate that neither vitamins nor essential amino acids are provided for the host. However, the genome encodes for an almost complete shikimate pathway leading to 4-hydroxyphenylpyruvate, which could be converted into tyrosine by the host. Taken together with increasing titers of *Westeberhardia* during pupal stage, this suggests a contribution of *Westeberhardia* to cuticle formation. Despite a widespread occurrence of *Westeberhardia* across host populations, one ant lineage was found to be naturally symbiont-free, pointing to the loss of an otherwise prevalent endosymbiont. This study yields insights into a novel intracellular mutualist that could play a role in the invasive success of *C. obscurior*.

The ISME Journal (2016) **10,** 376–388; doi:10.1038/ismej.2015.119; published online 14 July 2015

Introduction

Interactions between organisms drive biological complexity (Maynard Smith and Szathmáry, 1997), shaping life as we know it. Symbioses with prokaryotes are considered to promote Eukaryote diversification (Brucker and Bordenstein, 2012), particularly in insects (Moya *et al*., 2008; Gil *et al*., 2010). Some bacterial symbionts provide novel ecological traits to their insect hosts, for example, defense against pathogens or parasitoids (Oliver *et al*., 2003; Kaltenpoth *et al*., 2005), enhanced stress tolerance (Russell and Moran, 2006) or nutrients (Douglas, 2009). Nutrient-providing symbionts are commonly found in hosts with restricted diets, for example, aphids feeding on phloem sap (Baumann, 2005), blood-feeding diptera (Wang *et al*., 2013) or grain weevils (Heddi *et al*., 1999). Symbionts can provide essential amino acids, vitamins or help in nitrogen recycling (Nakabachi *et al*., 2005; Feldhaar *et al*., 2007; Michalkova *et al*., 2014; Patino-Navarrete *et al*., 2014). Such bacteria are commonly harbored in bacteriocytes, specialized host cells that sometimes form special organ-like structures, the bacteriomes (Baumann, 2005) or are confined to the insect gut (Engel and Moran, 2013). Provisioning with nutrients can lead to increased fitness (Michalkova *et al*., 2014), which may enable invasive species to exploit novel habitats or food sources (Feldhaar, 2011).

Cardiocondyla obscurior (Wheeler, 1929) is an invasive ant that forms small multiqueen colonies in disturbed, arboreal habitats throughout the tropics. A peculiarity of the genus *Cardiocondyla* is the occurrence of wingless males that mate with closely related queens in their maternal nest (Oettler *et al*., 2010). New colonies are established via colony

Correspondence: J Oettler, Institut für Zoologie, Universität Regensburg, Universitätsstrasse 31, Regensburg 93053, Germany.
E-mail: joettler@gmail.com
[8]These authors contributed equally to this work.
Received 9 January 2015; revised 29 April 2015; accepted 9 June 2015; published online 14 July 2015

splitting (Heinze *et al.*, 2006). This unique life history with frequent genetic bottlenecks and high levels of inbreeding makes it an interesting model for the study of rapid adaptation to novel environments (Schrader *et al.*, 2014).

Here, we describe a so-far unknown intracellular symbiont of *C. obscurior*, for which we propose the name '*Candidatus* Westeberhardia cardiocondylae' strain obscurior (from here on referred to as *Westeberhardia*). We analyzed its distribution within and across host populations, and compared infection of individual ants depending on morph and age. Furthermore, we localized *Westeberhardia* in the host and scrutinized its genome focusing on its metabolic functions. *Westeberhardia* has lost many metabolic capabilities, but retained most of the shikimate pathway and the ability to synthesize the tyrosine precursor 4-hydroxyphenylpyruvate. We suggest that its localization in gut-associated bacteriomes of pupae, and the increased titers during pupal development point to a role of *Westeberhardia* in cuticle formation.

Material and methods

Ant colonies

We reared *C. obscurior* colonies from Brazil (BR), Japan (JP) and Spain (SP) in the laboratory. The BR colonies originated from two collection sites ~70 km apart, a cacao plantation in Ilhéus (2009 and 2013) and a citrus plantation near Una (2012) (Brazilian Ministry of Science and Technology, permits 20324-1/40101-1). JP colonies were collected from two coral trees 100 m apart (lineages 'OypB', 'OypC') in the Oonoyama park in Naha, Okinawa (2011) and from additional trees of the same park ('OypU', 2013). SP colonies were collected at a campsite in Los Realejos, Tenerife in 2012 and 2013. All colonies were housed in plaster nests under 12 h 28 °C light/12 h 23 °C dark cycles, with constant humidity and *ad libitum* provided honey and pieces of cockroaches. All animal treatment guidelines applicable to ants under international and German law have been followed.

Westeberhardia detection and phylogenomic analyses

During analyses of the *C. obscurior* genome (Schrader *et al.*, 2014), we identified prokaryotic scaffolds and candidates for horizontal gene transfers (HGTs) (Wheeler *et al.*, 2013). These were then further characterized by blasting (blastx) all annotated genes against a database of prokaryotic proteins. Besides *Wolbachia,* we identified six scaffolds of an unknown Enterobacteriaceae. Following *de novo* genome assembly and annotation (see below), we used translated CDS sequences for phylogenetic placement following Husník *et al.* (2011). Briefly, we performed Dayhoff6 recoding followed by a phylogenomic reconstruction with PhyloBayes v.3.3f (Lartillot *et al.*, 2009), based on 64 single-copy protein clusters (Supplementary Information).

We detected one prokaryotic gene incorporated into the host genome. After manual correction of the HGT gene model, we used blastx analyses against NCBI's non-redundant database to identify homologs. RNA-seq data were used to verify expression in seven larval and seven adult queens by mapping reads against the *C. obscurior* genome (Schrader *et al.*, 2014). We generated count tables with htseq (Anders *et al.*, 2015) against *C. obscurior* gene annotations (including the manually corrected gene) and calculated untransformed, size factor-normalized read counts.

Genome assembly, annotation and functional analyses

Paired-end Illumina reads from Schrader *et al.* (2014) were used for *de novo* assembly of the *Westeberhardia* genome. We removed *Wolbachia* sequences based on their blastx result, and then assembled remaining prokaryotic reads using SOAPdenovo2 (Luo *et al.*, 2012). The resulting contigs were scaffolded using a custom-modified version of SSPACE v.2.0 (Boetzer *et al.*, 2011). Raw reads were then mapped back to the contigs using MIRA 4.0.1 (Chevreux *et al.*, 1999) and manually joined. Scaffold corroboration and visual inspection of contigs were performed in the Staden Package (Staden *et al.*, 2000). Inconsistencies were broken and manually reassembled. Base-calling correction was carried out using POLISHER (Lapidus *et al.*, 2008). No corrections were made to the consensus, which consisted of a single 532 684-bp contig (average coverage 204.5x).

The replication origin was predicted using originX (Worning *et al.*, 2006). A first round of open reading frame prediction was performed using Prodigal v.2.5 (Hyatt *et al.*, 2010) and the predicted open reading frames were annotated using the BASys server (Van Domselaar *et al.*, 2005). tRNAs were predicted using the 'COVE-only' algorithm of tRNAscan-SE v.1.3.1 (Lowe and Eddy, 1997), and checked with TFAM v.1.4 (Tåquist *et al.*, 2007). tmRNAs and their tag peptides were predicted using ARAGORN v.1.2.36 (Laslett and Canback, 2004). The genome was searched against Rfam v.11 (Burge *et al.*, 2013) using Infernal v.1.1 (Nawrocki and Eddy, 2013), and the resulting ncRNAs were manually integrated into the annotation following the INSDC conventions (http://www.insdc.org/files/feature_table.html). Ribosome-binding sites were predicted using RBSfinder (Suzek *et al.*, 2001) and signal peptides were predicted using SignalP v.4.1 (Petersen *et al.*, 2011). The resulting annotation was manually curated in Artemis (Rutherford *et al.*, 2000).

Metabolic functions were automatically predicted and analyzed using Pathway Tools v.17.5 (Karp *et al.*, 2010) against BioCyc and MetaCyc databases (Caspi *et al.*, 2012), following manual curation. Functional information was retrieved from the EcoCyc (Keseler *et al.*, 2013), KEEG (Ogata *et al.*, 1999) and BRENDA (Scheer *et al.*, 2011) databases.

Coverage analyses
For comparison of *Westeberhardia* infection between the sequenced reference colonies from BR (Ilhéus, 2009) and JP (OypB) (Schrader *et al.*, 2014), we mapped genomic reads from pools of 30 BR and 26 JP males (140 million reads each) against the *C. obscurior* and *Westeberhardia* genomes, and compared coverage between BR and JP with samtools' depth algorithm (Li, 2011) and custom bash/perl/R scripts as described in Schrader *et al.* (2014).

Analyses of intraspecific infection dynamics by PCR and real-time qPCR
To assess *Westeberhardia* presence across host populations, we screened 42 *C. obscurior* samples collected worldwide and the sister species *Cardiocondyla wroughtonii* (Forel, 1890) by performing a diagnostic PCR assay on a 204-bp fragment of the *nrdB* (*ribonucleoside-diphosphate reductase 1 subunit beta*) gene of *Westeberhardia* (*WEOB_403*) (*nrdB*-for: 5′-GGAAGGAGTCCTAATGTTGCG-3′; *nrdB*-rev: 5′-ACC AGAAATATCTTTTGCACGTT-3′), using the ant housekeeping gene *elongation factor 1-alpha 1* (*Cobs_01649*) (*EF1*-for: 5′-TCACTGGTACCTCGCAAGCCGA-3′; *EF1*-rev: 5′-AGCGTGCTCACGAGTTTGTCCG-3′, 104-bp fragment) as a control. We used DNA from a previous study (Oettler *et al.*, 2010), samples from laboratory colonies and stored tissues from which we extracted DNA using a chloroform-based method (Sambrook and Russell, 2001) (Table 1). To verify infection with the same *Westeberhardia* species, we sequenced a 917-bp fragment of the 16S rRNA gene of *Westeberhardia* (*WEOB_122*) (*WE16S*-for: 5′-CATTTGAATA TGTAGAATGGACC-3′; *WE16S*-rev: 5′-AACTTTTA CAAGATCGCTTCTC-3′) from one individual each of the BR, JP and SP populations and of *C. wroughtonii* (see Supplementary Information for PCR details).

We assessed inter- and intrapopulational *Westeberhardia* prevalence in laboratory colonies using PCR and quantitative PCR (qPCR) assays for workers and queens, respectively (Supplementary Information). For this purpose, we sampled 6–10 dealate queens and 9–10 workers from 6–8 colonies from three lineages from BR and JP, respectively, and from the SP population, resulting in a total of 538 analyzed workers and 517 queens. Worker and queen DNA was extracted using the hotshot method (Alasaad *et al.*, 2008) and the NucleoSpinTissue XS Kit (Machery-Nagel, Düren, Germany), respectively. To control for age effects on infection (see below), we selected freshly eclosed workers when possible.

We quantified *Westeberhardia* of single individuals by determining normalized *nrdB* copy numbers with qPCR (Supplementary Information) across developmental stages (larvae and prepupae of unknown sex and caste, young and old female pupae) for JP (OypB) and BR (Una, 2012) and across morphs (queens, workers, winged males, wingless males), ages (queens = 2, 14, 28 and 48 days; workers = 2, 14 and 28 days), and fertilization state of queens (virgin, mated) for only the BR population (Una, 2012).

Fluorescence in situ hybridization
To localize *Westeberhardia*, we performed fluorescence *in situ* hybridization (FISH) as described previously (Kaltenpoth *et al.*, 2012, 2014 and Supplementary Information) on abdominal sections of queen, worker and wingless male pupae from BR (Ilhéus, 2009) and adult queens from BR (Ilhéus, 2009) and JP (OypB) with the general eubacterial probe *EUB338* (5′-GCTGCCTCCCGTAGGAGT-3′) (Amann *et al.*, 1990) and one of the *Westeberhardia*-specific probes *Wcard1* (5′-ATCAGTTTCGAACG CCATTC-3′) and *Wcard2* (5′-CGGAAGCCACAATT

Table 1 Prevalence of *Westeberhardia* across populations of *Cardiocondyla obscurior* and the closely related species *C. wroughtonii*[a]

Sampling site (year)	*Sample description*	*Morph*	*Westeberhardia (sample size)*
C. obscurior			
Brazil: Ilhéus (2004)	Laboratory colonies	W	Y (3)
Brazil: Ilhéus (2009)	Laboratory colonies	Q	Y (4)
Brazil: Una (2012)	Laboratory colonies	Q	Y (4)
Brazil: Ilhéus (2013)	Laboratory colonies	Q	Y (4)
Japan: Ishigaki (2002)	Oettler *et al.* (2010)	W	N (2)
Japan: Naha (2011) 'OypB'	Laboratory colonies	Q	N (4)
Japan: Naha (2011) 'OypC'	Laboratory colonies	Q	Y (4)
Japan: Naha (2013) 'OypU'	Laboratory colonies	Q	Y (4)
Spain: Tenerife (2012)	Laboratory colonies	Q	Y (4)
Egypt: Talkha (2003)	Oettler *et al.* (2010)	W	N (1)
Fiji (2007)	EtOH material	W	Y (1)
Malaysia: Ulu Gombak (2002)	Oettler *et al.* (2010)	W	Y (1)
Sri Lanka (2006)	Oettler *et al.* (2010)	W	N (1)
USA: Lake Alfred, Florida (2004)	Oettler *et al.* (2010)	W	Y (3)
C. cf. *obscurior* Singapore (2014)	EtOH material	W	Y (2)
C. wroughtonii			
Japan: Nakijin (2013)	Laboratory colonies	W	Y (2)

Abbreviations: EtOH, ethyl alcohol; Q, queen; N, no; W, worker; Y, yes.
[a]Based on a diagnostic PCR screen using the *nrdB* gene.

CAAGAT-3′), targeting the 16S rRNA gene. Probes were labeled with Cy3 or Cy5, and samples were counterstained with DAPI (4′,6-diamidino-2-phenylindole).

Test for reproductive manipulation and paternal inheritance

Several bacterial symbionts are known to be reproductive manipulators, with cytoplasmic incompatibility (CI) and parthenogenesis induction (PI) being the most common phenotypes (Cordaux *et al.*, 2011). Although CI results in the incompatibility of crosses between infected males and uninfected queens, PI causes parthenogenetic production of diploid female offspring in infected females. We crossed uninfected JP queens (OypB) with infected BR males (Ilhéus, 2009), by placing sexual pupae together with 20 workers into new nests ($n = 10$), which were observed twice a week for the presence of male and female brood.

To test for paternal inheritance of *Westeberhardia*, crosses of infected males and uninfected freshly eclosed virgin queens were initiated in a mating arena overnight. The following day, we dissected and macerated the spermathecae of queens ($n = 8$) in dH_2O and isolated DNA using the NucleoSpinTissue XS Kit (Machery-Nagel). We performed a diagnostic PCR assay with the *nrdB* gene and the housekeeping gene *EF1* as a positive control. We further analyzed infection status of two worker pupae each emerging from four of the above crosses using the *nrdB* PCR assay.

Results

Microbial associates of C. obscurior

Blastx analyses of the *C. obscurior* hologenome (Schrader *et al.*, 2014) retrieved 1.5 Mb of *Wolbachia* sequence and 543 172 bp in six scaffolds of an unknown γ-proteobacterium. A preliminary assembly of the *Wolbachia* sequences is hosted on antgenomes.org. The 16S sequence of the γ-proteobacterium showed 98.4% identity with an Enterobacteriaceae of a *C. obscurior* sample from Florida, USA (voucher RA0330, GenBank: GQ275143), detected during a survey of ant-associated bacteria (Russell *et al.*, 2009).

Blastx analyses further revealed a 360-bp intronless gene of putative prokaryotic origin encoding a xanthine-guanine phosphoribosyltransferase (EC: 2.4.2.22), which is incorporated into the host genome and has its closest ortholog in *Enterobacter cloacae* (WP_023478997). Xanthine-guanine phosphoribosyltransferase has a central role in the synthesis of purine nucleotides through the salvage pathways, converting xanthine and guanine to XMP and GMP, respectively. The gene is present in genomic reads of *C. obscurior* from BR (Ilhéus, 2009) and JP (OypB). We used published RNAseq data from adult queens and queen-destined larvae (Schrader *et al.*, 2014) to confirm *in vivo* transcription of the HGT and found a fivefold increased expression in larvae compared to adults ($median_{larvae} = 1140.1$, $median_{queens} = 223.2$, Mann–Whitney *U*-test: $W = 79$, $P < 0.001$).

Westeberhardia genome assembly, annotation and functional and phylogenomic analyses

De novo assembly of the *Westeberhardia* genome produced a single scaffold representing a circular chromosome of 532 684 bp (23.41% GC content) with 372 protein-coding genes and six pseudogenes (Figure 1). Sequences are accessible through the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/LN774881) under study number PRJEB8217, chromosome accession number LN774881 and are hosted on antgenomes.org. The phylogenomic analysis placed *Westeberhardia* within a group of Enterobacteriaceae symbionts comprising both facultative and obligate insect endosymbionts, including *Sodalis*, *Baumannia*, *Blochmannia* and *Wigglesworthia* (Supplementary Figure S1).

The genome codes for a simplified but functional informational machinery, with complete setups for DNA replication, transcription, translation and protein folding, but few genes involved in DNA repair (Figure 2). *Westeberhardia* has a limited metabolic repertoire but is capable of glycolysis, pentose phosphate pathway, fatty acid biosynthesis, nucleotide synthesis and ATP production through oxidative phosphorylation. The pathway for glycerophospholipid biosynthesis is impaired. Metabolite transport appears to be based on electrochemical potential-driven transporters and inorganic phosphate transporters, whereas ATP-binding cassette transporter as well as phosphotransferase system transporter genes are missing. *Westeberhardia* has lost the pathways for synthesis of all essential and most non-essential amino acids and cofactors, but has retained an incomplete shikimate pathway. Thus, it is able to synthesize chorismate, a central metabolite in the biosynthesis of many aromatic compounds (e.g. phenylalanine, tryptophan, *p*-hydroxybenzoate or enterobactin). However, it can only use chorismate for the biosynthesis of 4-hydroxyphenylpyruvate, a precursor of tyrosine (Hopkins and Kramer, 1992; Andersen, 2012). Although *Westeberhardia* cannot complete the last step in this pathway, the host genome codes for tyrosine aminotransferase (EC 2.6.1.5), converting 4-hydroxyphenylpyruvate to tyrosine (*Cobs_01567*). Further conversion of tyrosine to DOPA (3,4-dihyroxyphenylalanine), an important component of insect cuticles (Andersen, 2012), might occur through tyrosine 3-monooxygenase (EC 1.14.16.2) encoded in the host genome (*Cobs_14710*).

Intraspecific infection dynamics

Coverage analysis of genomic reads showed that, in contrast to males from a BR lineage (Ilhéus, 2009), males from a JP lineage (OypB) are devoid of *Westeberhardia* (coverage BR: 30x; JP: 0.21; Figure 3a).

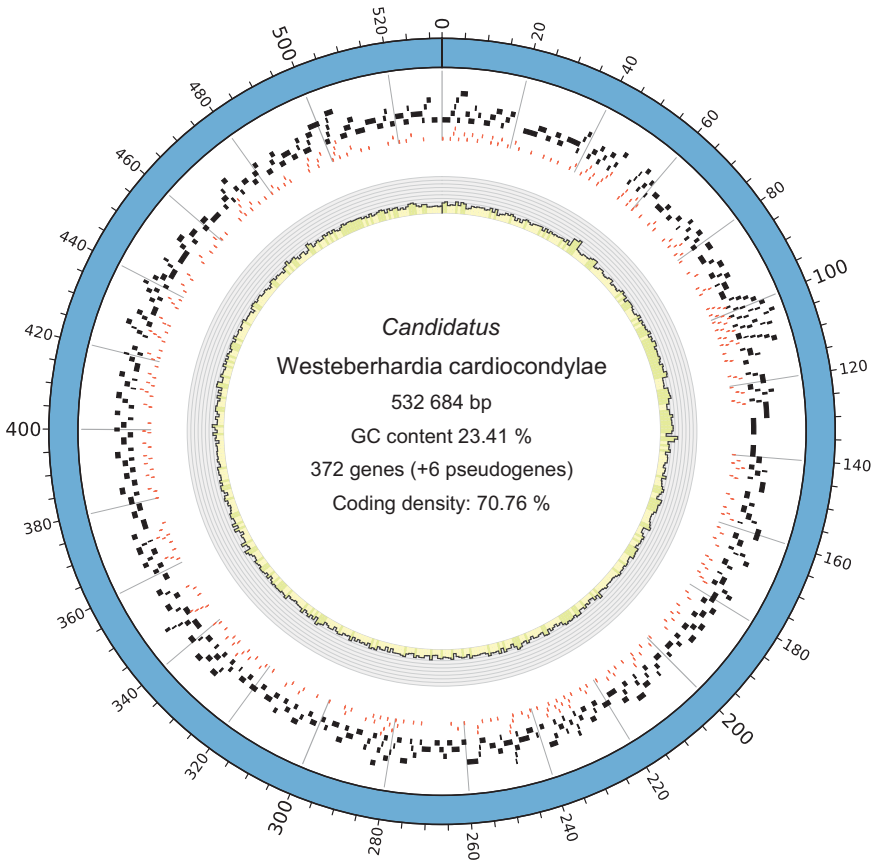

Figure 1 Genomic structure of *Westeberhardia* representational Circos plot (Krzywinski *et al*., 2009) illustrating genomic properties of *Westeberhardia*. Tile plots show the distribution of protein-coding genes (black bars) and ribosomal binding sites (red bars). The histogram in the inner circle shows GC content in percent for 1 kb windows.

qPCR of the *Westeberhardia*-specific *nrdB* gene in the female pupae, as well as in the larvae and prepupae of unknown sex and caste verified that *Westeberhardia* is completely absent in OypB (Figure 3b). Accordingly, *Westeberhardia* was not detected by FISH in sections of adult OypB queens.

Analyses of *C. obscurior* samples collected worldwide showed that *Westeberhardia* is present in 34 of 42 tested samples (81.0%), including all samples from BR, but absent in some JP populations and in material from Egypt and Sri Lanka (Table 1). The closely related species *C. wroughtonii* also contains *Westeberhardia*. A 917-bp 16S rDNA fragment of *Westeberhardia* is identical between the three *C. obscurior* populations from BR, JP and SP, and between *C. obscurior* and *C. wroughtonii*.

Westeberhardia infection of workers varies considerably within and among infected lineages, ranging from 42.5% to 96.3% (Figure 3d), whereas queen infection is almost fixed in all populations (88.6% to 100%; Figure 3c and Supplementary Table 5). However, in the OypB lineage, only one of 60 workers and two of 60 queens were infected at low levels (indicated by weak bands on the agarose gel or late C_q values in the qPCR, respectively). These values are not significantly different from zero (one-sample *t*-tests: workers: $t(59)=1$, $P=0.32$; queens: $t(59)=1.43$, $P=0.16$), and could possibly be caused by contamination. Intriguingly, individuals from colonies collected in a tree merely 100 m away (OypC) show infection rates of 96.3% (workers) and 100% (queens).

In the BR (Una, 2012) population, relative densities of *Westeberhardia* increase significantly during pupal development (worker and queen pupae combined) from white (early) to brown (late) pupae ($N_{\text{pupa white}}=9$, $N_{\text{pupa brown}}=8$; *t*-test: $t(14.7)=-4.3$, $P<0.001$), but is not different between larvae, prepupa and early pupae (Figure 3b and Supplementary Table 1). *Westeberhardia* titers are higher in queen compared with worker pupae (*t*-test: $t(13.3)=2.6$, $P=0.023$). A comparison of 2- to 14-day-old adults of each morph (queens, workers, winged and wingless males) shows that *Westeberhardia* titers differ significantly across castes (Kruskal–Wallis: $X^2=24.2$, d.f. = 3, $P<0.001$; Figure 3e), with queens having significantly more *Westeberhardia* compared with the other morphs, which are not different from each other (pairwise Mann–Whitney *U*-tests with Benjamini–Hochberg correction for multiple testing (Benjamini and Hochberg, 1995); Supplementary Table 2). We calculated generalized linear models (with a Gaussian distribution and identity link function to model age dependency of *Westeberhardia* in adult females (Figure 3f). In workers, infection decreases with age (generalized

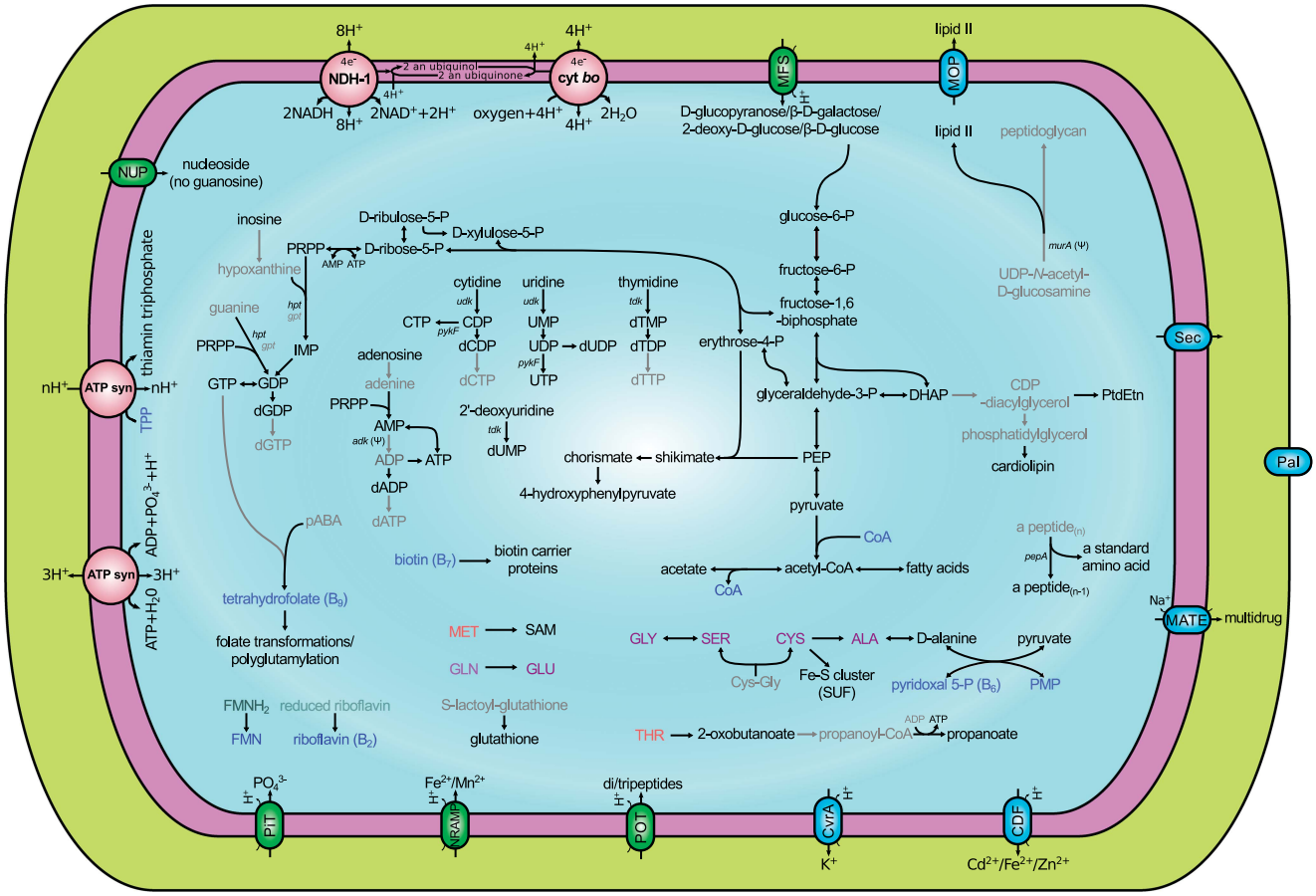

Figure 2 *Westeberhardia* metabolic reconstruction. Intact pathways are shown in black lines and unclear pathways (missing a specific gene or having it pseudogenized) are shown in gray lines. Exporters are represented using green ovals, whereas exporters/importers are represented using blue ovals with the name of the family/superfamily they belong to, otherwise the protein or complex name is used. ATP synthase is shown twice to represent an additional metabolic capability. Essential and non-essential amino acids are shown in red and purple lettering, respectively. Cofactors and vitamins are represented in blue. Blurred compounds represent those for which biosynthesis or import cannot be accounted for based on the genomic data, according to MetaCyc. Relevant genes involved in the biosynthesis of nucleotides and peptidoglycan are indicated. A single frameshift is found in *adk* and *mur*A; therefore, they might be young pseudogenes, or an RNA polymerase or ribosomal slippage would be required to produce a functional protein.

linear model: d.f. = 18, F = 12.7, $P = 0.002$; Supplementary Table 3) and is significantly more variable compared with that in queens (Fligner–Killeen test: $X^2 = 17.0$, d.f. = 1, $P < 0.001$). In queens, *Westeberhardia* significantly increase with age from day 2 after eclosion to day 48 (generalized linear model: d.f. = 38, F = 29.4, $P < 0.001$; Supplementary Table 4). Virgin and mated 28-day-old queens show no significant difference in infection (Wilcoxon's rank-sum test: W = 49, $P > 0.05$).

Localization of Westeberhardia

Westeberhardia is localized intracellularly in bacteriomes connected to the gut in queen, worker and wingless male pupae (Figures 4a–c). As in many other insect taxa with bacteriomes, bacteriocytes are densely packed with symbiont cells and exhibit enlarged host cell nuclei. In some sections, individual symbiont cells or symbiont-filled bacteriocytes were found in the gut lumen, suggesting beginning degradation of the bacteriome in later pupal stages. Concordantly, no bacteriomes were detected in adult queens (Figure 4d). However, both pupal and adult queens show high *Westeberhardia* abundances in the ovaries (Figures 4a, d–f). In particular, *Westeberhardia* is localized predominantly in the nurse cells. Several sections captured transgenerational infection events of the symbiont from the maternal nurse cells into late stages of the developing oocyte (Figure 4f).

Test for reproductive manipulation and paternal inheritance

Westeberhardia-free OypB queens mated to *Westeberhardia*-infected BR males produced viable diploid female and haploid male offspring. Furthermore, infected females generally also produce male offspring and unfertilized females exclusively produce males. In addition, *Westeberhardia* does not seem to be transmitted paternally, as revealed by the absence of the diagnostic *nrdB* gene in the spermatheca content of *Westeberhardia*-uninfected queens mated with infected males. Worker pupae emerging from the above crosses were also uninfected.

Description of 'Ca. W. cardiocondylae'

In accordance with the guidelines of the International Committee of Systematic Bacteriology, unculturable bacteria should be classified as *Candidatus* (Murray and Stackebrandt, 1995). We propose the name '*Ca.* Westeberhardia cardiocondylae' strain obscurior for this newly discovered γ3-proteobacterium. The genus name *Westeberhardia*

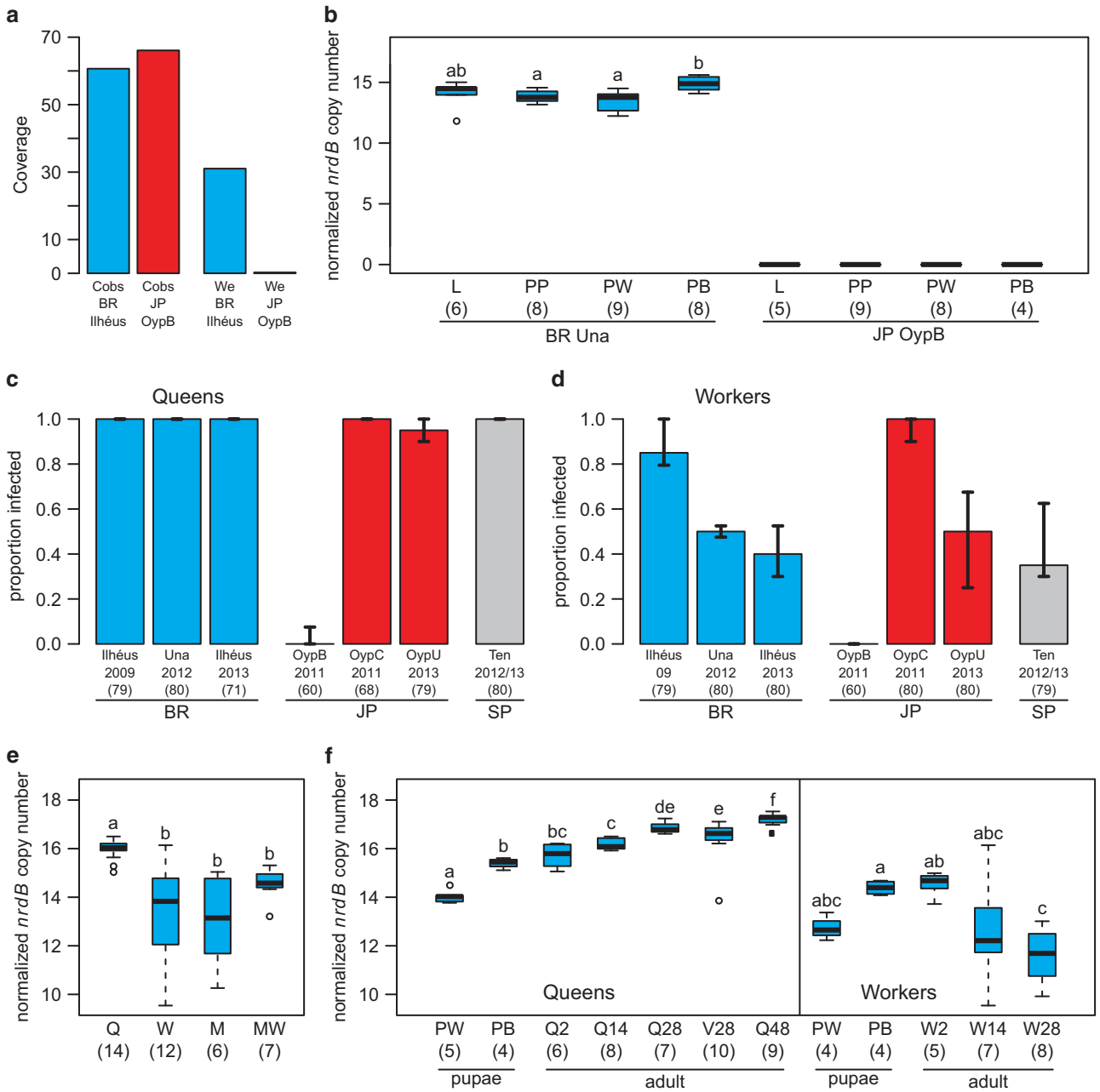

Figure 3 Intraspecific and temporal dynamics of *Westeberhardia* infection. (**a**) In genomic coverage data for pooled haploid males mapped against the *Westeberhardia* reference, *Westeberhardia* reads (We) were exclusively present in the Brazil, Ilhéus (2009) sample (BR, blue) and no reads could be detected in the OypB, Japan (JP, red) sample, whereas coverage of *C. obscurior* reads (Cobs) mapped against the *C. obscurior* reference is similar. (**b**) Real time-quantitative PCRs on DNA level for the *nrdB* gene confirm the absence of *Westeberhardia* in larvae (L), prepupae (PP) and female (queen and worker) pupae (PW = pupa white, PB = pupa brown) of the JP OypB population, whereas all those developmental stages are infected in the BR Una (2012) (letters indicate significances for within-population comparisons for BR). (**c** and **d**) Prevalence of *Westeberhardia* in queens (**c**) and workers (**d**) across different populations of *C. obscurior* from BR (blue), JP (red) and Tenerife, SP (gray), as revealed by qPCR (**c**) and diagnostic PCR (**d**), of the *nrdB* gene. For each lineage, 6–8 colonies and per colony 9–10 young workers and 6–10 queens were tested. Bars represent medians and whiskers denote quartiles. Note that while *Westeberhardia* infection status of workers varies between and within populations of *C. obscurior*, it is almost fixed in queens across all lineages except OypB. (**e** and **f**) Morph (**e**) and age (**f**) dependency of relative amounts of *Westeberhardia* in *C. obscurior* individuals from BR (Una, 2012) determined by real-time quantitative PCR. Normalized *nrdB* copy numbers are elevated in queens compared with all other morphs (Q = queens; W = workers; M = males winged; MW = males wingless) (**e**), increase with age in queens, but decrease with age in workers (numbers after Q/W show age in days, V = virgin queens, letters indicate significant differences for within-caste comparisons) (**f**). Sample sizes are given within parentheses.

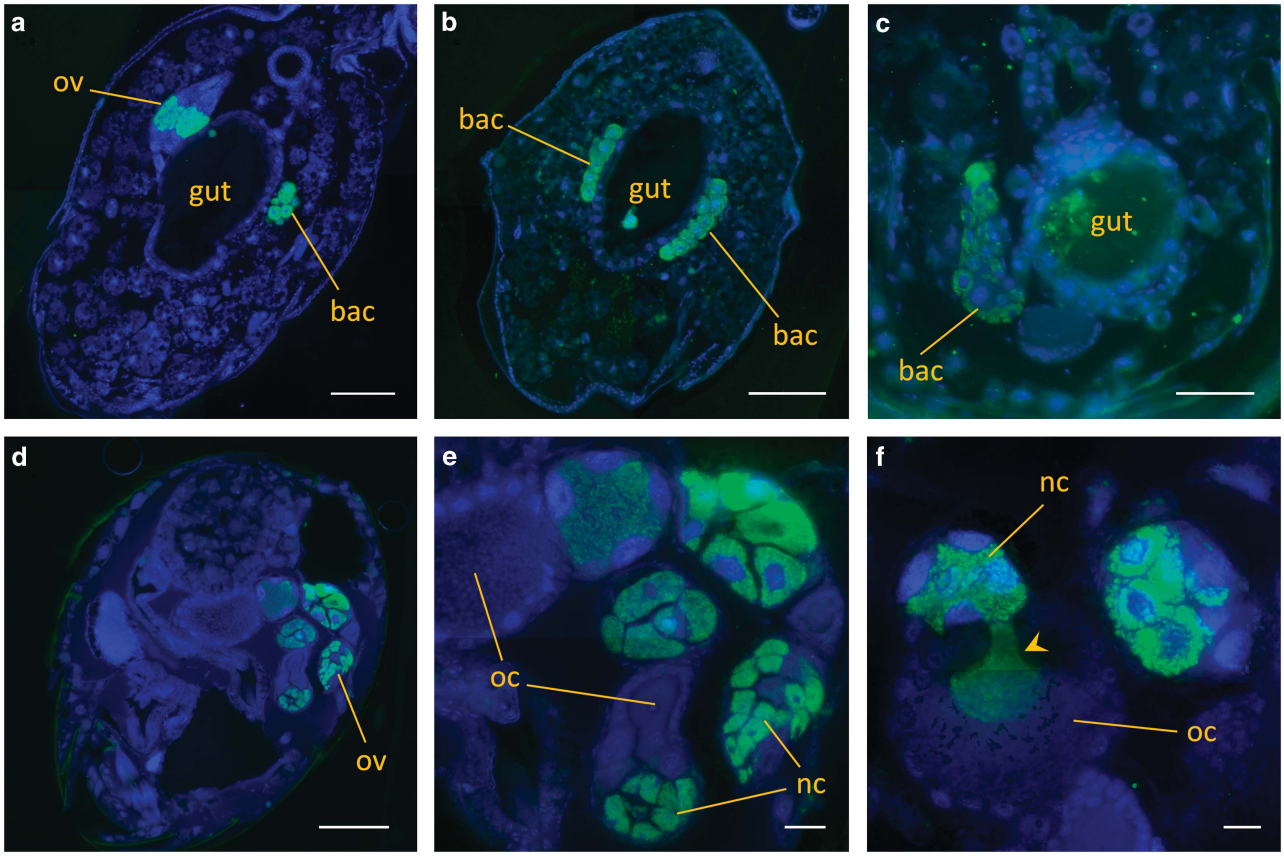

Figure 4 Localization of *Westeberhardia* in adults and pupae of *C. obscurior* (from Brazil, Ilhéus, 2009). Symbionts were stained in longitudinal sections through the abdomen with the *Westeberhardia*-specific probe Wcard2-Cy5 (green) and host cell nuclei were counterstained with DAPI (blue). (**a**–**c**) Localization of *Westeberhardia* in gut-associated bacteriomes (bac) in the pupae of a queen (**a**), a worker (**b**) and a male (**c**). Note the additional presence of symbionts in the queen ovaries (ov). (**d**) Section of the abdomen of an adult queen, with symbionts visible in the ovaries (ov). (**e** and **f**) Ovaries of an adult queen. Symbionts are mainly localized in the nurse cells (nc), but enter the developing oocyte (oc), probably during nurse cell depletion (arrowhead). Scale bars: 100 μm (**a**, **b** and **d**), 50 μm (**c**) and 20 μm (**e** and **f**).

refers to Mary Jane West-Eberhard, expressing our admiration for her far-reaching advances in evolutionary developmental biology. The specific epithet, cardiocondylae, indicates that it is an endosymbiont of *Cardiocondyla* ants.

Discussion

The 16S sequence of an unknown Enterobacteriaceae isolated from *C. obscurior* was previously published (Russell *et al.*, 2009), but the specificity and functionality of this association had not been addressed. Here, we describe it as '*Ca.* Westeberhardia cardiocondylae' strain obscurior and provide a first characterization of its relationship with *C. obscurior*. Phylogenomic analysis indicates that *Westeberhardia* is closely related to *Blochmannia*, the obligate endosymbiont of *Camponotus* ants (Feldhaar *et al.*, 2007). Nevertheless, its phylogenetic placement has to be considered with caution, because of long-branch attraction. As already observed by Husník *et al.* (2011), the monophyly of the cluster formed by *Sodalis*, *Baumannia*, *Blochmannia* and *Wigglesworthia*, in which *Westeberhardia* appears, needs to be further tested.

Transmission of Westeberhardia

Maternal transmission of *Westeberhardia* occurs through a different process than described for other endosymbionts (Koga *et al.*, 2012; Balmand *et al.*, 2013). In adult queens, *Westeberhardia* is localized in ovarial syncytial nurse cells, which originate from the same germline stem cell as the oocyte and are responsible for provisioning of the oocyte with metabolites. During the process of nurse cell depletion, when large amounts of cytoplasmic material are channeled into the oocyte (Mahajan-Miklos and Cooley, 1994), cytoplasmic *Westeberhardia* are swept into the developing oocyte, ensuring complete vertical transmission (Figure 4f).

CI is a widespread phenotype induced by some bacterial endosymbionts (Gotoh *et al.*, 2006; Werren *et al.*, 2008), but has to our knowledge not been shown for any social insect. In social Hymenoptera, males develop from haploid, unfertilized eggs and females from diploid, fertilized eggs; consequently, CI would only affect diploid offspring. *Westeberhardia*

does not appear to induce strong CI, if any, as uninfected queens mated to infected males produced diploid F1 females. Another common phenotype caused by reproductive manipulators, the induction of thelytokous PI, can also be excluded. This would lead to exclusive female offspring in infected queens, as *C. obscurior* does not show diploid male production (Schrempf *et al*., 2006). The regular occurrence of male offspring and the exclusive production of males by unfertilized females argue against PI. However, *Wolbachia*, also present in *C. obscurior*, could exert effects on host reproduction as well. In contrast to some other intracellular symbionts (Moran and Dunbar, 2006; Damiani *et al*., 2008; Watanabe *et al*., 2014), paternal transmission of *Westeberhardia* is unlikely as we did not detect *Westeberhardia* DNA in transferred sperm and/or seminal fluids stored in the spermatheca of uninfected queens mated to infected males.

Westeberhardia as a possible source of a HGT event

The bacterial gene *gpt* encoding the xanthine-guanine phosphoribosyltransferase (EC: 2.4.2.22), which was horizontally transferred into the host genome has its closest ortholog in *Enterobacter*, an Enterobacteriaceae. This indicates that *Westeberhardia* could be the origin of the HGT event. However, it could also be a relict of a former symbiont no longer present in *C. obscurior* (Husník *et al*., 2013). Homologs of *gpt* have been identified in most bacterial endosymbionts, including *Buchnera*, *Moranella*, *Blochmannia*, *Sodalis* and *Wigglesworthia.* The presence of the HGT in the OypB lineage suggests either an ancestral association between *C. obscurior* and *Westeberhardia* and a secondary loss of the symbiont in OypB, or the origin of the HGT from an unknown bacterium in the ancestor of both lineages. *Westeberhardia* is not capable of *de novo* synthesis of purines, but it is capable of producing all purine nucleotides from recovered bases and nucleosides. A functional genome annotation revealed the presence of *hpt,* a gene with similar function to *gpt*, in the *Westeberhardia* genome. However, RNAseq data show that infected hosts transcribe *gpt*. Therefore, *Westeberhardia* might be reliant on an effective salvage using the host-encoded *gpt* in some conditions (O'Reilly *et al*., 1984). Interestingly, *gpt* expression is higher in larval compared with adult queens, indicating that it is not correlated with *Westeberhardia* titers. Inhibiting *gpt* expression in *Westeberhardia*-infected and -uninfected individuals will help elucidate a putative effect of this gene on host and bacteria fitness.

Dependency of Westeberhardia on host-provided metabolites

With a genome size reduction to 533 kb and a GC content of 23.41%, the *Westeberhardia* genome exhibits features of degenerative genome evolution following the transition to obligate symbiosis (Moya *et al*., 2008). In addition to reduced effective population sizes in host-associated bacteria compared with free-living relatives, small effective population size of *C. obscurior* (Schrader *et al*., 2014) and social insects in general (Romiguier *et al*., 2014) could lead to even faster genome degeneration. With a coding density of 70.76%, the genome is surprisingly loosely packed, compared with other endosymbionts with similar-length genomes (88% coding density on average) (McCutcheon and Moran, 2012). Furthermore, the occurrence of six pseudogenes indicates that genome erosion in *Westeberhardia* is still incomplete. It was previously shown that even in advanced mutualistic relationships, endosymbiont genome reduction continues (Gil *et al*., 2002). Nevertheless, despite the substantial genome reduction, *Westeberhardia* appears capable of DNA replication, transcription, translation and protein folding, suggesting that it is close to a minimal cell status (Gil *et al*., 2004). On the other hand, the lack of *dnaA* and any other alternative mechanism for replication initiation might be an indication that bacterial replication could fall under host control, as suggested for *Wigglesworthia* and *Blochmannia* (Akman *et al*., 2002; Gil *et al*., 2003), although no potential mechanism has been identified yet. It has also been suggested that, in the absence of *recA*, the maintenance of *recBCD* might indicate that this complex has a role in DNA replication initiation instead of recombination (Wu *et al*., 2006). The gene count with only 372 coding genes and the impairment of essential pathways such as cofactor and essential amino-acid biosynthesis indicate a metabolic dependency on extrinsic resources. In particular, a highly simplified cell envelope and the absence of most transporter genes point towards dependency on the host machinery. In this, *Westeberhardia* resembles *Buchnera aphidicola* BCc, which also lacks the ability to synthesize cell surface components (Pérez-Brocal *et al*., 2006). Intracellularity allows the host to control endosymbiont populations (Vigneron *et al*., 2012), which together with the lack of *dnaA* suggests that *Westeberhardia* populations are controlled by *C. obscurior*.

Potential for mutualism: Shikimate pathway

Westeberhardia has retained almost the complete shikimate pathway, which produces chorismate, the precursor for tryptophan, tyrosine and phenylalanine, but lacks the downstream enzymes necessary for the synthesis of these aromatic amino acids. However, it can produce 4-hydroxyphenylpyruvate, which can then be converted to tyrosine by the host. Tyrosine is a precursor for DOPA and thereby essential for cuticle formation in insects (Hopkins and Kramer, 1992; Andersen, 2012). Insects cannot synthesize aromatic amino acids and acquisition from diet and/or provisioning by endosymbionts is a common phenomenon. For example, *B. aphidicola* has evolved overproduction of phenylalanine and

tryptophan (Lai *et al*., 1994; Jiménez *et al*., 2000). Similarly, *B. floridanus* can synthesize tyrosine, and increased tyrosine biosynthesis during the host's pupal stage (Zientz *et al*., 2006) coincides with elevated *Blochmannia* titers (Stoll *et al*., 2010; Ratzka *et al*., 2013). Accordingly, we found high densities of *Westeberhardia* in late *C. obscurior* pupae and young adults. Taken together with the detection of gut-associated bacteriomes in pupae, this suggests a role of *Westeberhardia* in cuticle synthesis during metamorphosis. Although the precise metabolites provided to the host are unclear at this stage, we hypothesize that the retention of the shikimate pathway is key to the mutualistic relationship between *Westeberhardia* and *Cardiocondyla*.

After eclosion, *Westeberhardia* declines slowly in workers but proliferates in queens with age. Although virgin queens exhibit significantly reduced egg laying rates compared with mated queens (Schrempf *et al*., 2005), we did not find an increase of *Westeberhardia* infection with reproductive output. Instead, it appears as if the mere availability of reproductive tissue allows proliferation of *Westeberhardia*. As a consequence of reproductive division of labor in a eusocial host, the major proportion of *Westeberhardia* encounters a dead end. *Cardiocondyla* workers completely lack ovaries (Heinze *et al*., 2006), thus likely impeding *Westeberhardia* proliferation in the absence of the appropriate microhabitat. In *Camponotus floridanus* ants, mid-gut connected bacteriomes populated by *Blochmannia* during the pupal stage become symbiont-free in adult queens and workers, whereas queens retain *Blochmannia* in their ovarioles (Sauer *et al*., 2000; Wolschin *et al*., 2004). Similarly, symbionts localized in gut-associated bacteriomes of cereal weevils are actively eliminated by the initiation of apoptosis after cuticle formation is finished, but ovary-associated symbionts are retained for vertical transmission (Vigneron *et al*., 2014). Probably due to slow degeneration of bacteriomes, *Westeberhardia* was still present in young adult males and workers. Bacteria detected in the gut lumen (from degrading bacteriomes) may be the source of continued bacterial infections found in adult workers and males. We have not ruled out that the symbiont continues to have a role in adult workers, although its general decline suggests that the role is not vital. It remains to be investigated if active degradation of bacterial populations in workers benefits individuals and, on a higher level, colony performance (Wenseleers *et al*., 2002).

Population differences cast doubts about the symbiosis status

We found a naturally occurring uninfected host lineage that continues to thrive in the laboratory, questioning the essentiality of *Westeberhardia*, at least under conditions including *ad libitum* protein provisioning. We verified the absence of *Westeberhardia* in freshly collected field colonies and established laboratory colonies with different methods and across different developmental stages. It remains elusive why *Westeberhardia* prevalence is so substantially different between colonies of two lineages (OypB, OypC) separated by such short distances (<100 m) in the field. This could indicate a facultative status of *Westeberhardia* as seen for many insect–microbe symbioses (e.g., Moran *et al*., 2005). However, *Westeberhardia* lacks the main characteristics shared by facultative symbionts, even those of a facultative symbiont in the transition to becoming obligate (i.e., large genomes with low coding density and abundance of pseudogenes, presence of repetitive sequences and transposable elements, high GC content (Manzano-Marín and Latorre, 2014)). The occurrence of *Westeberhardia* in *C. wroughtonii* and in different populations of *C. obscurior* indicates an ancestral infection and secondary loss of *Westeberhardia* in OypB. As the impact of facultative symbionts depends on the particular environmental conditions (Dale and Welburn, 2001; Hansen *et al*., 2007), a shift in diet or different gut microbiota could explain symbiont loss by natural selection. Owing to proximity of host lineages, an alternative, more parsimonious explanation is mutational loss of *Westeberhardia* and subsequent fixation through drift (Reuter *et al*., 2005). Future comparisons between infected and uninfected hosts with the same or different genetic backgrounds under varying environmental conditions will help to reveal potential effects of *Westeberhardia* on host fitness.

Conclusion

Our study describes a novel intracellular bacterium that maintains a mutualistic relationship with its host but can be lost in some conditions. Its genomic organization, metabolic capabilities, localization and prevalence during host development indicate a role of *Westeberhardia* in host cuticle formation, possibly facilitating an invasive lifestyle in nutrient poor arboreal environments. The putative monophyly with other insect endosymbionts, possibly facilitating cuticle buildup during development (Zientz *et al*., 2006; Vigneron *et al*., 2014), suggests a single origin of metamorphosis-based symbiosis.

Owing to novel traits emerging through host–symbiont associations, it is indispensable to evaluate possible fitness effects of symbionts on hosts, which are used as model organisms for broad biological questions. Although symbionts are situated along the boundary between biotic environmental factors and genomic composition of the host, it becomes obvious that selection pressures acting on the holobiont must be considered when studying adaptation: 'Contrary to common belief, environmentally initiated novelties may have greater evolutionary potential than mutationally induced ones' (West-Eberhard, 2005).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We are most grateful to Masaki Suefuji, Tina Wanke and Jacques HC Delabie for collecting the JP, SP and BR colonies. We thank Helena Lowack for help in the laboratory, the 'Molecular Ecology' class of 2014 (University Regensburg) and Franziska Scherl for help with the population screens. We also thank Benjamin Weiss for help with the sectioning of ant specimens for FISH; Yannick Wurm for hosting Wobs and WEobs on antgenomes.org; and Horacio Frydman, Abdelaziz Heddi, Filip Husník, Eva Schultner and Mary Jane West-Eberhard for helpful discussions. This study was funded by a grant from the German Science Foundation (DFG He1623/31) (to JO and JH). AL received Grant BFU2012-39816-C02-01 from the Spanish Ministry of Economy and Competitivity cofinanced by FEDER funds, and AM was funded by grant PrometeoII/2014/065 from Valencian Community (Spain). AMM was supported by a Marie Curie Grant (FP7-PEOPLE-2010_ITN SYMBIOMICS 264774) and a fellowship from the Mexican Federal Government (CONACYT 327211/381508). MK and LF were funded by the Max Planck Society, and an additional grant from the German Science Foundation (to MK) (DFG KA2846/2-1). DW and JW were supported by US NSF DEB0821936; JW was further supported by US NSF DEB1257053; and DW was further funded by a start-up grant from Massey University.

Author contributions

Designed the study: AK, LS, JO; wrote the manuscript: AK, LS, JH, JO; performed the experiments: AK, JO; analyzed the data: AK, LS; prokaryote sequence annotation and HGT detection: DW, JW; *Westeberhardia* genome assembly, functional annotation and phylogenomic analysis: AL, AM, AMM, RG; *Westeberhardia* localization (FISH): LF, MK. All authors read, reviewed and accepted the final version of the manuscript.

References

Akman L, Yamashita A, Watanabe H, Oshima K, Shiba T, Hattori M *et al.* (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies *Wigglesworthia glossinidia*. *Nat Genet* **32**: 402–407.

Alasaad S, Rossi L, Maione S, Sartore S, Soriguer RC, Pérez JM *et al.* (2008). HotSHOT Plus ThermalSHOCK, a new and efficient technique for preparation of PCR-quality mite genomic DNA. *Parasitol Res* **103**: 1455–1457.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Anders S, Pyl PT, Huber W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**: 166–169.

Andersen SO. (2012). Cuticular sclerotization and tanning. In: Gilbert LI (ed), *Insect Molecular Biology and Biochemistry*. Academic Press: Waltham, MA, USA, pp 167–192.

Balmand S, Lohs C, Aksoy S, Heddi A. (2013). Tissue distribution and transmission routes for the tsetse fly endosymbionts. *J Invertebr Pathol* **112**: S116–S122.

Baumann P. (2005). Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu Rev Microbiol* **59**: 155–189.

Benjamini Y, Hochberg Y. (1995). Controlling the false discovery rate: a pratical and powerful approach to multiple testing. *J R Stat Soc Ser B* **57**: 289–300.

Boetzer M, Henkel CV, Jansen HJ, Butler D, Pirovano W. (2011). Scaffolding pre-assembled contigs using SSPACE. *Bioinformatics* **27**: 578–579.

Brucker RM, Bordenstein SR. (2012). Speciation by symbiosis. *Trends Ecol Evol* **27**: 443–451.

Burge SW, Daub J, Eberhardt R, Tate J, Barquist L, Nawrocki EP *et al.* (2013). Rfam 11.0: 10 years of RNA families. *Nucleic Acids Res* **41**: D226–D232.

Caspi R, Altman T, Dreher K, Fulcher CA, Subhraveti P, Keseler IM *et al.* (2012). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res* **40**: D742–D753.

Chevreux B, Wetter T, Suhai S. (1999). Genome sequence assembly using trace signals and additional sequence information. *Proc German Conf Bioinf* **99**: 45–56.

Cordaux R, Bouchon D, Grève P. (2011). The impact of endosymbionts on the evolution of host sex-determination mechanisms. *Trends Genet* **27**: 332–341.

Dale C, Welburn SC. (2001). The endosymbionts of tsetse flies: manipulating host-parasite interactions. *Int J Parasitol* **31**: 628–631.

Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P *et al.* (2008). Paternal transmission of symbiotic bacteria in malaria vectors. *Curr Biol* **18**: R1087–R1088.

Douglas AE. (2009). The microbial dimension in insect nutritional ecology. *Funct Ecol* **23**: 38–47.

Engel P, Moran NA. (2013). The gut microbiota of insects—diversity in structure and function. *FEMS Microbiol Rev* **37**: 699–735.

Feldhaar H. (2011). Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecol Entomol* **36**: 533–543.

Feldhaar H, Straka J, Krischke M, Berthold K, Stoll S, Mueller MJ *et al.* (2007). Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*. *BMC Biol* **5**: 48.

Gil R, Latorre A, Moya A. (2010). Evolution of prokaryote–animal symbiosis from a genomics perspective. *Microbiol Monogr* **19**: 207–233.

Gil R, Sabater-Munoz B, Latorre A, Silva FJ, Moya A. (2002). Extreme genome reduction in *Buchnera* spp.: toward the minimal genome needed for symbiotic life. *Proc Natl Acad Sci USA* **99**: 4454–4458.

Gil R, Silva FJ, Peretó J, Moya A. (2004). Determination of the core of a minimal bacterial gene set. *Microbiol Mol Biol Rev* **68**: 518–537.

Gil R, Silva FJ, Zientz E, Delmotte F, González-Candelas F, Latorre A *et al.* (2003). The genome sequence of *Blochmannia floridanus*: comparative analysis of reduced genomes. *Proc Natl Acad Sci USA* **100**: 9388–9393.

Gotoh T, Noda H, Ito S. (2006). *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. *Heredity* **98**: 13–20.

Hansen AK, Jeong G, Paine TD, Stouthamer R. (2007). Frequency of secondary symbiont infection in an invasive psyllid relates to parasitism pressure on a geographic scale in California. *Appl Environ Microbiol* **73**: 7531–7535.

Heddi A, Grenier AM, Khatchadourian C, Charles H, Nardon P. (1999). Four intracellular genomes direct weevil biology: nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. *Proc Natl Acad Sci USA* **96**: 6814–6819.

Heinze J, Cremer S, Eckl N, Schrempf A. (2006). Stealthy invaders: the biology of *Cardiocondyla* tramp ants. *Insect Soc* **53**: 1–7.

Hopkins TL, Kramer KJ. (1992). Insect cuticle sclerotization. *Annu Rev Entomol* **37**: 273–302.

Husník F, Chrudimský T, Hypša V. (2011). Multiple origins of endosymbiosis within the Enterobacteriaceae (γ-Proteobacteria): convergence of complex phylogenetic approaches. *BMC Biol* **9**: 87.

Husník F, Nikoh N, Koga R, Ross L, Duncan RP, Fujie M *et al.* (2013). Horizontal gene transfer from diverse bacteria to an insect genome enables a tripartite nested mealybug symbiosis. *Cell* **153**: 1567–1578.

Hyatt D, Chen G-L, LoCascio PF, Land ML, Larimer FW, Hauser LJ. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinform* **11**: 119.

Jiménez N, González-Candelas F, Silva FJ. (2000). Prephenate dehydratase from the aphid endosymbiont (*Buchnera*) displays changes in the regulatory domain that suggest its desensitization to inhibition by phenylalanine. *J Bacteriol* **182**: 2967–2969.

Kaltenpoth M, Gottler W, Herzner G, Strohm E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Curr Biol* **15**: 475–479.

Kaltenpoth M, Yildirim E, Gürbüz MF, Herzner G, Strohm E. (2012). Refining the roots of the beewolf—*Streptomyces* symbiosis: antennal symbionts in the rare genus *Philanthinus* (Hymenoptera, Crabronidae). *Appl Environ Microbiol* **78**: 822–827.

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo TY, Stubblefield JW *et al.* (2014). Partner choice and fidelity stabilize coevolution in a Cretaceous—age defensive symbiosis. *Proc Natl Acad Sci USA* **111**: 6359–6364.

Karp PD, Paley SM, Krummenacker M, Latendresse M, Dale JM, Lee TJ *et al.* (2010). Pathway Tools version 13.0: integrated software for pathway/genome informatics and systems biology. *Brief Bioinform* **11**: 40–79.

Keseler IM, Mackie A, Peralta-Gil M, Santos-Zavaleta A, Gama-Castro S, Bonavides-Martínez C *et al.* (2013). EcoCyc: fusing model organism databases with systems biology. *Nucleic Acids Res* **41**: D605–D612.

Koga R, Meng XY, Tsuchida T, Fukatsu T. (2012). Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. *Proc Natl Acad Sci USA* **109**: E1230–E1237.

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D *et al.* (2009). Circos: An information aesthetic for comparative genomics. *Genome Res* **19**: 1639–1645.

Lai CY, Baumann L, Baumann P. (1994). Amplification of *trpEG*: adaptation of *Buchnera aphidicola* to an endosymbiotic association with aphids. *Proc Natl Acad Sci USA* **91**: 3819–3823.

Lapidus A, LaButti K, Foster B, Lowry S, Trong S, Goltsman E. (2008). POLISHER: An effective tool for using ultra short reads in microbial genome assembly and finishing. AGBT, Marco Island, FL, USA.

Lartillot N, Lepage T, Blanquart S. (2009). PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics* **25**: 2286–2288.

Laslett D, Canback B. (2004). ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences. *Nucleic Acids Res* **32**: 11–16.

Li H. (2011). A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics* **27**: 2987–2993.

Lowe TM, Eddy SR. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* **25**: 955–964.

Luo R, Liu B, Xie Y, Li Z, Huang W, Yuan J *et al.* (2012). SOAPdenovo2: an empirically improved memory-efficient short-read *de novo* assembler. *Gigascience* **1**: 18.

Mahajan-Miklos S, Cooley L. (1994). Intercellular cytoplasm transport during *Drosophila* oogenesis. *Dev Biol* **165**: 336–351.

Manzano-Marín A, Latorre A. (2014). Settling down: the genome of *Serratia symbiotica* from the aphid *Cinara tujafilina* zooms in on the process of accommodation to a cooperative intracellular life. *Genome Biol Evol* **6**: 1683–1698.

Maynard Smith J, Szathmáry E. (1997). *The Major Transitions in Evolution*. Oxford University Press: Oxford, UK.

McCutcheon JP, Moran NA. (2012). Extreme genome reduction in symbiotic bacteria. *Nat Rev Microbiol* **10**: 13–26.

Michalkova V, Benoit JB, Weiss BL, Attardo GM, Aksoy S. (2014). Vitamin B6 generated by obligate symbionts is critical for maintaining proline homeostasis and fecundity in tsetse flies. *Appl Environ Microbiol* **80**: 5844–5853.

Moran NA, Dunbar HE. (2006). Sexual acquisition of beneficial symbionts in aphids. *Proc Natl Acad Sci USA* **103**: 12803–12806.

Moran NA, Russell JA, Koga R, Fukatsu T. (2005). Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Appl Environ Microbiol* **71**: 3302–3310.

Moya A, Peretó J, Gil R, Latorre A. (2008). Learning how to live together: genomic insights into prokaryote-animal symbioses. *Nat Rev Genet* **9**: 218–229.

Murray RG, Stackebrandt E. (1995). Taxonomic note: implementation of the provisional status *Candidatus* for incompletely described procaryotes. *Int J Syst Bacteriol* **45**: 186–187.

Nakabachi A, Shigenobu S, Sakazume N, Shiraki T, Hayashizaki Y, Carninci P *et al.* (2005). Transcriptome analysis of the aphid bacteriocyte, the symbiotic host cell that harbors an endocellular mutualistic bacterium, *Buchnera*. *Proc Natl Acad Sci USA* **102**: 5477–5482.

Nawrocki EP, Eddy SR. (2013). Infernal 1.1: 100-fold faster RNA homology searches. *Bioinformatics* **29**: 2933–2935.

O'Reilly C, Turner PD, Smith-Keary PF, McConnell DJ. (1984). Molecular cloning of genes involved in purine

biosynthetic and salvage pathways of *Salmonella typhimurium*. *Mol Gen Genet* **196**: 152–157.
Oettler J, Suefuji M, Heinze J. (2010). The evolution of alternative reproductive tactics in male *Cardiocondyla* ants. *Evolution* **64**: 3310–3317.
Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. (1999). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* **27**: 29–34.
Oliver KM, Russell JA, Moran NA, Hunter MS. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci USA* **100**: 1803–1807.
Patino-Navarrete R, Piulachs M-D, Belles X, Moya A, Latorre A, Peretó J. (2014). The cockroach *Blattella germanica* obtains nitrogen from uric acid through a metabolic pathway shared with its bacterial endosymbiont. *Biol Lett* **10**: 20140407.
Petersen TN, Brunak S, Heijne von G, Nielsen H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Meth* **8**: 785–786.
Pérez-Brocal V, Gil R, Ramos S, Lamelas A, Postigo M, Michelena JM *et al.* (2006). A small microbial genome: the end of a long symbiotic relationship? *Science* **314**: 312.
Ratzka C, Gross R, Feldhaar H. (2013). Gene expression analysis of the endosymbiont-bearing midgut tissue during ontogeny of the carpenter ant *Camponotus floridanus*. *J Insect Physiol* **59**: 611–623.
Reuter M, Pedersen JS, Keller L. (2005). Loss of *Wolbachia* infection during colonisation in the invasive Argentine ant *Linepithema humile*. *Heredity* **94**: 364–369.
Romiguier J, Lourenco J, Gayral P, Faivre N, Weinert LA, Ravel S *et al.* (2014). Population genomics of eusocial insects: the costs of a vertebrate-like effective population size. *J Evol Biol* **27**: 593–603.
Russell JA, Moran NA. (2006). Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proc Biol Sci USA* **273**: 603–610.
Russell JA, Moreau CS, Goldman-Huertas B, Fujiwara M, Lohman DJ, Pierce NE. (2009). Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proc Natl Acad Sci USA* **106**: 21236–21241.
Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream M-A *et al.* (2000). Artemis: sequence visualization and annotation. *Bioinform Appl Note* **16**: 944–945.
Sambrook J, Russell DW. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: New York, NY, USA.
Sauer C, Stackebrandt E, Gadau J, Hölldobler B, Gross R. (2000). Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus* Blochmannia gen. nov. *Int J Syst Evol Microbiol* **50**: 1877–1886.
Scheer M, Grote A, Chang A, Schomburg I, Munaretto C, Rother M *et al.* (2011). BRENDA, the enzyme information system in 2011. *Nucleic Acids Res* **39**: D670–D676.
Schrader L, Kim JW, Ence D, Zimin A, Klein A, Wyschetzki K *et al.* (2014). Transposable element islands facilitate adaptation to novel environments in an invasive species. *Nat Comms* **5**: 5495.
Schrempf A, Aron S, Heinze J. (2006). Sex determination and inbreeding depression in an ant with regular sib-mating. *Heredity* **97**: 75–80.
Schrempf A, Heinze J, Cremer S. (2005). Sexual cooperation: mating increases longevity in ant queens. *Curr Biol* **15**: 267–270.
Staden R, Beal KF, Bonfield JK. (2000). The Staden package, 1998. *Methods Mol Biol* **132**: 115–130.
Stoll S, Feldhaar H, Fraunholz MJ, Gross R. (2010). Bacteriocyte dynamics during development of a holometabolous insect, the carpenter ant *Camponotus floridanus*. *BMC Microbiol* **10**: 308.
Suzek BE, Ermolaeva MD, Schreiber M, Salzberg SL. (2001). A probabilistic method for identifying start codons in bacterial genomes. *Bioinformatics* **17**: 1123–1130.
Tåquist H, Cui Y, Ardell DH. (2007). TFAM 1.0: an online tRNA function classifier. *Nucleic Acids Res* **35**: W350–W353.
Van Domselaar GH, Stothard P, Shrivastava S, Cruz JA, Guo A, Dong X *et al.* (2005). BASys: a web server for automated bacterial genome annotation. *Nucleic Acids Res* **33**: W455–W459.
Vigneron A, Charif D, Vincent-Monégat C, Vallier A, Gavory F, Wincker P *et al.* (2012). Host gene response to endosymbiont and pathogen in the cereal weevil *Sitophilus oryzae*. *BMC Microbiol* **12**: S14.
Vigneron A, Masson F, Vallier A, Balmand S, Rey M, Vincent-Monégat C *et al.* (2014). Insects recycle endosymbionts when the benefit is over. *Curr Biol* **24**: 2267–2273.
Wang J, Weiss BL, Aksoy S. (2013). Tsetse fly microbiota: form and function. *Front Cell Infect Microbiol* **3**: 69.
Watanabe K, Yukuhiro F, Matsuura Y, Fukatsu T, Noda H. (2014). Intrasperm vertical symbiont transmission. *Proc Natl Acad Sci USA* **111**: 7433–7437.
Wenseleers T, Sundstrom L, Billen J. (2002). Deleterious *Wolbachia* in the ant *Formica truncorum*. *Proc Biol Sci* **269**: 623–629.
Werren JH, Baldo L, Clark ME. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol* **6**: 741–751.
West-Eberhard MJ. (2005). Developmental plasticity and the origin of species differences. *Proc Natl Acad Sci USA* **102**: 6543–6549.
Wheeler D, Redding AJ, Werren JH. (2013). Characterization of an ancient lepidopteran lateral gene transfer. *PLoS One* **8**: e59262.
Wolschin F, Hölldobler B, Gross R, Zientz E. (2004). Replication of the endosymbiotic bacterium *Blochmannia floridanus* is correlated with the developmental and reproductive stages of its ant host. *Appl Environ Microbiol* **70**: 4096–4102.
Worning P, Jensen LJ, Hallin PF, Staerfeldt H-H, Ussery DW. (2006). Origin of replication in circular prokaryotic chromosomes. *Environ Microbiol* **8**: 353–361.
Wu D, Daugherty SC, Van Aken SE, Pai GH, Watkins KL, Khouri H *et al.* (2006). Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. *PLoS Biol* **4**: e188.
Zientz E, Beyaert I, Gross R, Feldhaar H. (2006). Relevance of the endosymbiosis of *Blochmannia floridanus* and carpenter ants at different stages of the life cycle of the host. *Appl Environ Microbiol* **72**: 6027–6033.

Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)