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Sex ratio bias caused by endosymbiont infection in the dwarf spider *Oedothorax retusus*

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Abstract. Spiders exhibit a remarkable variety of reproductive phenotypes such as induced parthenogenesis and reproductive skew in primary sex ratio. However, observations of distorted sex ratios are mainly inferred from field catches of adult individuals, whereas detailed information on clutch primary sex ratio and sex ratio inheritance, resulting from multiple generations of laboratory rearing, is scarce. One of the potential causes of sex ratio variation is infection with maternally inherited endosymbiont bacteria that alter a mother's offspring sex ratio to increase their own fitness. Although studies show that spiders are infected with several endosymbiont species, it was only recently discovered that endosymbiont bacteria can cause a female sex ratio bias in this order. To explore the distribution of biased sex ratios and endosymbiont infection patterns, we investigated sex ratio variation and bacterial presence in *Oedothorax retusus* Westring 1851. Significant sex ratio variation was detected in six matrilines originating from wild-caught females, one of which consistently showed a female bias in offspring production. Congruent with a bacterial effect, the sex ratio bias showed a clear maternal inheritance, and treatment with antibiotics reversed the sex ratio to equal numbers of males and females. Female-biased clutches were found to exhibit a significantly lower number of hatched spiderlings than unbiased clutches, suggesting the occurrence of male-killing. All matrilines showed infection with the *Cardinium* endosymbiont, while two matrilines, including the female biased one, were additionally infected with *Wolbachia* and *Rickettsia.* These findings indicate that bacterial endosymbionts are responsible for the sex ratio variation in this species, and suggest that effects of endosymbiont bacteria in the order Araneae could be more widespread than previously assumed.

Keywords: Sex ratio distortion, solitary spider, endosymbiont bacteria, male-killing, *Wolbachia*

Spiders display a diversity of reproductive phenotypes, such as parthenogenesis and primary sex ratio distortion [sex ratio at the time of fertilization (overview in Goodacre et al. 2006; Martin & Goodacre 2009)]. Biases in offspring sex ratios have been most extensively studied in social spider species through both collections in the field and laboratory-based studies (Aviles & Maddison 1991; Rowell & Main 1992; Aviles et al. 2000; Lubin & Bilde 2007). However, in solitary species, existence of biased sex ratios is mainly inferred from population studies where the absence or low numbers of males could indicate either parthenogenesis and sex ratio distortion or differential mortality of one sex (Vollrath & Parker 1992; Baert & Jocque 1993; Levi 1996). Detailed information on primary sex ratio distortion in solitary spiders based on laboratory rearing is scarce (but see Gunnarsson & Andersson 1996; Gunnarsson et al. 2004; Vanthournout et al. 2011).

One of the mechanisms that can cause a distorted sex ratio is infection with endosymbiont bacteria. Endosymbiotic bacteria are maternally inherited microorganisms that may cause a variety of reproductive alterations in their hosts. Due to their almost exclusively maternal inheritance, induction of parthenogenesis, feminization, and male-killing, which all bias the offspring sex ratio towards females, and the occurrence of cytoplasmic incompatibility result in an increase of infected females in the population (Werren & Beukeboom 1998; Stouthamer et al. 1999; Charlat et al. 2003; Werren et al. 2008; Engelstadter & Hurst 2009). Owing to their obvious effects on host ecology and reproductive biology, endosymbiont bacteria have received increasing attention, and the combination of screening studies and meta-analyses provides mounting evidence that these endosymbionts are more widespread than previously thought (Goodacre et al. 2006; Duron et al. 2008a; Hilgenboecker et al. 2008). However, understanding of the phenotypic effects of a large number of such endosymbiont infections remains poorly investigated, particularly in the Araneae.

Although several studies show that spider species exhibit a high diversity and prevalence of endosymbiont species known to influence their hosts' reproductive biology (Goodacre et al. 2006; Baldo et al. 2008; Duron et al. 2008a, b; Martin & Goodacre 2009; Yun et al. 2011), their potential effects are verified in few cases. No effect of endosymbiont infection on spider host reproduction could be characterized in *Holocnemus pluchei* Scopoli 1763 (Stefanini & Duron 2012). In *Pityohyphantes phrygianus* C.L. Koch 1836, Gunnarsson et al. (2009) suggested that endosymbionts play a role in influencing offspring sex ratio, and recently Vanthournout et al. (2011) showed that the endosymbiont bacterium *Wolbachia* is a causative agent of a female-biased sex ratio distortion in the dwarf spider *Oedothorax gibbosus* Blackwall 1841.

Despite these documented cases, it remains at present unknown whether susceptibility to endosymbionts is confined to only a very limited number of spider species, or whether the effect is more widespread. To test this, we investigated the occurrence of sex ratio variation and presence of cytoplasmic sex ratio-distorting elements in a related species *Oedothorax retusus* Westring 1851 (Araneae: Linyphiidae: Erigoninae). This palearctic dwarf spider is found in a variety of habitats, usually in mosses, grasses and undergrowth, and is known to be infected with several endosymbiont species (Goodacre et al. 2006). Precise data on clutch sex ratio are currently lacking, however, making this a suitable candidate for examination.

In this study we investigate the diversity and prevalence of the endosymbiont community in *O. retusus* using endosymbiont-specific PCR assays. To quantify the potential effects of each endosymbiont on clutch sex ratio, we combined pedigree data resulting from several generations of laboratory rearing and results from a broad-spectrum antibiotic treatment. Furthermore, the phylogenetic position of the identified endosymbiont species was determined and compared with those found in *Oedothorax gibbosus*.

METHODS

1) Field collection, rearing conditions and breeding design.—Six matrilines were set up in the laboratory by collecting adult females by hand catches at Damvallei (Belgium) in the summer of 2010. We placed females individually in plastic vials of 5 cm diameter and 2.5 cm height with plaster bottoms. Moistening the plaster with tap water kept humidity levels at 100%. A piece of moss was added to allow the construction of a functional web. We provided fruit flies (*Drosophila* sp.) in overabundance and checked food and humidity levels several times a week. Vials were placed in a climate chamber with a constant temperature of 20°C and a light-dark regime of 16L–8D. Females were allowed to deposit up to three egg sacs before being preserved in ethanol. Offspring were reared individually as described above, except that juvenile spiders received collembolans as a food source until the third molt. After the final molt, we determined the sex of the spiders by visual inspection using a stereomicroscope. This allowed assessment of the tertiary sex ratio (number of adult male offspring/total number of adult offspring).

Adult females were mated with unrelated males (n = 22 females, F1 generation) to investigate the inheritance pattern of the sex ratio trait and for the application of antibiotics (see 2). We reared offspring under standard conditions and again determined tertiary sex ratio. Adult female offspring were further mated with unrelated males to increase sample size and to investigate the underlying mechanism (n = 19 females, F2 generation; see 4).

2) Antibiotic treatment.—It has been previously shown that application of antibiotics is effective in eliminating endosymbionts in spiders (Goodacre et al. 2009; Vanthournout et al. 2011). To test whether administering antibiotics restores an equal sex ratio (Morimoto et al. 2006; Gotoh et al. 2007), we exposed F1 females from the distorted line (M1: Table 2) to the broad-spectrum antibiotic, tetracycline. After reaching adulthood, six haphazardly chosen F1 females were treated by moistening the plaster on the bottom of the vial with the antibiotic solution (0.1%, w/v; 0.002 M). After approximately seven days, females were allowed to copulate with first-generation unrelated males. Offspring were reared individually as described above with the continuous use of an antibiotic solution. We used other F1 females from the distorted matriline as a control treatment (n = 6 females). Sex ratio and survival of the clutches were determined and compared between the treatments with a generalized linear mixed model (proc GLIMMIX in SAS v. 9.1.2). To account for dependence in sex ratio among mothers, mother ID was included as a random effect.

3) Endosymbiont detection and phylogenetic relationship.—We investigated the infection status of wild-caught females by means of a PCR assay for five endosymbionts: *Wolbachia*, *Rickettsia*, *Cardinium*, *Spiroplasma* and *Arsenophonus*. All potentially cause reproductive alterations in arthropods. Since one female died before being stored in ethanol, three of her daughters were used in the PCR assay to determine maternal infection status. All three daughters gave consistent results for every endosymbiont tested.

Whole spiders were used for DNA extraction using the Nucleospin Tissue kit (©Machery Nagel) following the manufacturer's recommended protocol. We used primers for five endosymbiont bacteria: *Wolbachia*, *Rickettsia*, *Cardinium*, *Spiroplasma* and *Arsenophonus* (Table 1). PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing (for temperature, see Table 1), 30 sec, extension (72°C, 90 sec) and a final extension at 72°C for 5 min. Electrophoresis was performed on a 1.5% agarose gel. Gels were stained in a solution of GELRED for approximately 15 min. Bands were visualized by UV-fluorescence.

As *Cardinium* tested positive for all individuals, we were able to confirm the reliability of the PCR detection of bacterial infection; hence, no further positive control was necessary. Prey items can be ruled out as a potential source of *Wolbachia* contamination in the samples, as it has previously been shown that our fruit fly and springtail breeding stocks were uninfected (Vanthournout et al. 2011). To test the significance of a relationship between endosymbiont infection and sex ratio, we used a generalized linear mixed model (Proc GLIMMIX in SAS v.9.1.2.) with endosymbiont presence/absence as a fixed effect. Dependence in sex ratio among matrilines was accounted for by including matriline identity as a random factor. The estimates obtained from the sex ratios for infected and uninfected females were compared with an even sex ratio using a t-test. Differences in sex ratio between matrilines were analyzed using a chi-square test (RxC).

In order to check primer specificity and to investigate strain diversity, we sequenced the PCR products using the BigDye v.1.1 Terminator Sequencing mix and ran them on an ABI 3710 automated sequencer. For *Wolbachia,* only the *wsp* gene was sequenced. We used BLAST searches to identify the closest relatives of the endosymbiont sequences obtained. The ClustalW algorithm implemented in MEGA5 (Tamura et al. 2011) was used to align the sequences obtained with those from other endosymbionts available in Genbank, which mainly originate from the studies reported in Rowley et al. (2004), Goodacre et al. (2006), Duron et al. (2008a) and Wang et al. (2010). We then compared the phylogenetic position of the endosymbiont *wsp* (*Wolbachia*), 16S (*Cardinium*) and *citrate* (*Rickettsia*) gene sequences with those found in spiders and other host species. For the *wsp* phylogeny, *Wolbachia* supergroup delimitation was used as reported in Rowley et al. (2004) and Goodacre et al. (2006). We constructed a p-distance-based neighbor joining tree as implemented in MEGA 5 (Tamura et al. 2011). Bootstrap percentage support was calculated for the nodes by generating 10,000 bootstrap values.

4) Mechanism.—Infection with male-killing endosymbionts typically lowers the clutch size to about one half, compared to clutches produced by uninfected females. Feminization and parthenogenesis induction do not influence clutch size. Based

Table 1.—Primers used for detection of five endosymbiont genera.

Endosymbiont	Primer	Gene	Annealing temperature	Reference
Wolbachia	WSP81F WSP691R	*wsp*	54°C	Braig et al. 1998
	16Swolb99F 16Swolb99R	16S rRNA		Oneill et al. 1992
Rickettsia	RICS741F RCIT1197R	*citrate*	54°C	Davis et al. 1998 Majerus et al. 2000
Cardinium	CLO-F1 CLO-R1	16S rRNA	54°C	Gotoh et al. 2007
Spiroplasma	SP-ITS-J04 SP-ITS-N55	Spacer region between 16S rRNA and 23S rRNA	52°C	Majerus et al. 1999
Arsenophonus nasoniae	ArsF, ArsF3 ArsR2	16S rRNA	52°C	Duron et al. 2008a

on this difference, we can discriminate among these mechanisms using two different approaches: testing for correlations between number of offspring and sex ratio and censusing the number of offspring as eggs and at hatching.

First, we tested for a correlation between number of adult offspring and egg sac sex ratio among *Wolbachia*/*Rickettsia*-infected (M1, M2; Table 1) and among uninfected (M3–M6: Table 1) females by means of a Pearson correlation on all clutches, weighted for number of adult offspring. This was further explored by investigating the relationship between the egg sac sex ratio and the infection status of the mother, total number of adult offspring and their interaction using a generalized linear mixed model (Proc GLIMMIX in SAS v. 9.1.2). Dependence in sex ratio among mothers was taken into account by adding the identity of the mother as a random effect. Moreover, if feminization occurs in this species, females producing a biased clutch should produce more female offspring than females producing offspring in equal numbers of males and females. Therefore, we compared the number of female offspring produced by *Wolbachia*/*Rickettsia*-infected and uninfected females by means of a generalized linear mixed model (Proc GLIMMIX in SAS v. 9.1.2). To account for dependence in sex ratio among matrilines, matriline ID was included as a random effect.

Second, we determined the number of offspring at two different census times: at the egg stage and at hatching from the egg sac. Females that were used to produce the second-generation offspring were allowed to oviposit up to three egg sacs before being stored in ethanol. The spiderlings from the first egg sac were allowed to emerge, and offspring were reared to adulthood to determine the total number of emerged spiderlings and tertiary sex ratio. The second and third egg sacs were stored in ethanol six days after oviposition to allow sufficient development of the eggs. Afterward, the proportion of fertile eggs to the total number of eggs produced was determined. Using a generalized linear mixed model (proc GLIMMIX in SAS v.9.1.2), we compared the total number of emerged spiderlings and number of eggs produced for the *Wolbachia*/*Rickettsia* infection status of the mother, census time (before hatching versus after hatching), and their two-way interaction. Identity of the mother was included as a random effect to correct for dependence between clutches.

RESULTS

1) Sex ratio variation among maternal lines.—A highly significant difference in average clutch sex ratio was detected among matrilines (Table 2: $df = 5$, $X^2 = 65.6$, $P < 0.0001$). This difference was primarily attributed to a single matriline, in which the wild-caught female and the daughter offspring of at least two subsequent generations produced significantly female-biased offspring. In the other five matrilines, equal numbers of males and females were produced, even in subsequent generations (M2–6: Table 2). Given that daughters of the M1 line were in many cases crossed with males from the other lines, persistence of the sex ratio distortion over several generations strongly suggests a maternal inheritance (M1:

Table 2.—Sex ratio data and endosymbiont infection status grouped by matriline.

Matriline	Endosymbiont infection status			Number of crosses	Number of males	Total number[1]	Sex ratio	*P*-value[2]
	Cardinium	*Rickettsia*	*Wolbachia*					
M1	+	+	+	16	123	434	0.29	<0.0001
M2	+	+	+	5	62	135	0.46	0.38
M3	+	-	-	6	102	208	0.49	0.8
M4	+	-	-	5	73	165	0.44	0.16
M5	+	-	-	4	88	156	0.56	0.13
M6	+	-	-	5	123	225	0.55	0.18
Tetracycline treatment								
M1				6	71	165	0.43	0.09

[1] Denotes the sum of the number of adult males and females.
[2] Denotes the probability value of difference from an even sex ratio as calculated by a binomial test.

Table 2). Conversely, three females from the undistorted lines mated with three male offspring of M1 produced sex ratios that were not significantly different from 0.5 (mean ± SE: 0.54 ± 0.05, $P = 0.22$). Therefore, since no effect of males was observed in the reciprocal crosses, this demonstrates that the sex ratio distortion is not heritable through males and confirms the exclusive maternal inheritance.

2) Antibiotic treatment.—Treatment of female offspring of the distorted matriline with antibiotics significantly affected the tertiary sex ratio ($F_{1,\ 10} = 6.46$, $P = 0.03$). Untreated females produced a significantly female-biased sex ratio (mean ± SE: 0.21 ± 0.05, $t_{10} = -4.47$, $P = 0.0012$), while tetracycline treatment returned the sex ratio to an equal proportion of males and females (mean ± SE: 0.43 ± 0.07, $t_{9.92} = -0.97$, $P = 0.4$). Applying antibiotics did not influence offspring survival, as no difference was found ($F_{1,1} = 2.6$, $P = 0.4$) in survival between tetracycline-treated (mean ± SE: 0.93 ± 0.02) and control offspring (mean ± SE: 0.97 ± 0.01).

3) Endosymbiont detection and phylogenetic relationship.—Screening of six individual females showed infection with up to three different endosymbionts known to cause reproductive alterations in arthropods. All females were infected with *Cardinium*, while two females were infected with both *Wolbachia* and *Rickettsia*. Over all generations, *Wolbachia*/*Rickettsia* infection status had a significant effect on the sex ratio produced by a female ($F_{1,35} = 4.62$; $P = 0.04$), with females infected with *Wolbachia* and *Rickettsia* producing a significantly more distorted sex ratio than uninfected females (mean ± SE: 0.35 ± 0.06, $t_{35} = -2.46$, $P = 0.02$; 0.51 ± 0.04, $t_{35} = 0.25$, $P = 0.8$, respectively).

For the two females testing positive for *Wolbachia* infection, both the *wsp* and *Wolbachia*-specific 16S rDNA primer gave consistent results. Sequencing of the *wsp* primer revealed no individual variation. BLAST searches revealed high similarity with available *Wolbachia* sequences (E-values < 1e-199). The *wsp* sequence [Genbank: JN889706] was most similar, with sequences from the spiders *Cybaeus penedentatus* Bennet 2009 [Genbank: GQ480746], *Araneus diadematus* Clerck 1757 [Genbank: DQ231505] and *Pityohyphantes phrygianus* C.L. Koch 1836 [Genbank: DQ231504], and clustered with high support within supergroup B (Fig. 1: neighbor joining tree of *Wolbachia* sequences).

The females with *Wolbachia* infection also tested positive for *Rickettsia*. BLAST searches showed homology with previously published *Rickettsia* sequences (E-values < 1e-199). The *Rickettsia* sequence [Genbank: JN889707] showed high similarity with the sequences of the spiders *Oedothorax gibbosus* [Genbank: HQ286289], *Hylyphantes graminicola* Sundevall 1830 [Genbank: DQ231487] and a *Theridiidae* sp. [Genbank: DQ231486] (Fig. 2: neighbor joining tree of *Rickettsia* sequences).

The *Cardinium* endosymbiont was found in all of the females tested. Alignment of the sequences obtained revealed no individual variation, and BLAST searches yielded high similarity with available *Cardinium* sequences (E-values < 1e-199). Sequences [Genbank: JN889705] were closely related to the *Cardinium* sequence of the spider *Holocnemus pluchei* Scopoli 1763 [Genbank: EU333930] and clustered with high support together with the sequence of the spider *Oedothorax gibbosus* [Genbank: HQ286292] (Fig. 3: neighbor joining tree of *Cardinium* sequences). We detected bands for *Arsenophonus* in the two females infected with *Wolbachia* and *Rickettsia*. However, sequencing and BLAST searches revealed that these were amplifications of *Rickettsia* and thus constituted false positives.

4) Mechanism.—We found a significant relationship between the number of adult offspring and egg sac sex ratio in *Wolbachia*/*Rickettsia* infected females, with a significantly lower proportion of males in smaller egg sacs (weighted Pearson correlation: $r = 0.57$, $P = 0.005$: Fig. 4). For uninfected females, no such correlation could be detected (weighted Pearson correlation: $r = 0.02$, $P = 0.95$: Fig. 4). There was a significant effect found for the total number of adult offspring ($F_{1,11} = 7.05$, $P = 0.02$) and *Wolbachia*/*Rickettsia* infection ($F_{1,11} = 13.02$, $P = 0.004$) on the egg-sac sex ratio. Moreover, when *Wolbachia*/*Rickettsia*-infected mothers produced a high number of offspring, the egg-sac sex ratio was not biased; if a lower number was produced, the egg sac sex ratio became significantly female biased, suggesting the occurrence of male-killing. This was not observed in *Wolbachia*/*Rickettsia* uninfected mothers ($F_{1,11} = 6.60$, $P = 0.03$).

There was no significant effect of infection status of the female on the total number of female offspring ($F_{1,3.3} = 0.02$, $P = 0.9$, mean ± SE: 11.9 ± 1.7 and mean ± SE: 12.2 ± 1.3 for infected and uninfected females, respectively). The total number of spiderlings was smaller than the total number of eggs produced, irrespective of the *Wolbachia*/*Rickettsia* infection status of the mother ($F_{1,31} = 47.58$, $P < 0.0001$). However, significantly fewer spiderlings emerged when the female was infected with *Wolbachia* and *Rickettsia* (mean ± SE: 19.4 ± 1.8) than did uninfected mothers (mean ± SE: 32.8 ± 3.2, $F_{1,31} = 15.32$, $P = 0.0005$: Fig. 5). *Wolbachia*/*Rickettsia* infection status significantly lowered the effect over the total number of spiderlings and number of eggs ($F_{1,13.41} = 6.76$, $P = 0.02$). Again, for this subset of mothers (F2 generation, see 1) a significant effect was found for *Wolbachia*/*Rickettsia* infection status on the offspring sex ratio ($F_{1,13} = 11.67$, $P = 0.005$). *Wolbachia* and *Rickettsia*-infected mothers produced a significant female-biased sex ratio (mean ± SE: 0.30 ± 0.04, $t_{13} = -5.08$, $P = 0.0002$), while uninfected mothers produced an even sex ratio (mean ± SE: 0.48 ± 0.04, $t_{6.78} = -0.65$, $P = 0.54$).

DISCUSSION

In this study, we report the presence of a maternally inherited, sex-ratio-distorting bacterium in the solitary dwarf spider *Oedothorax retusus*. This is deduced from several lines of evidence: 1) one matriline produced significantly female-biased offspring sex ratios, and several generations of outcrossed laboratory rearing did not diminish the biased sex ratio, 2) administering antibiotics to females of this distorted matriline resulted in equalized sex ratios and 3) three endosymbionts known to cause sex-ratio biases in their hosts were found: *Wolbachia, Rickettsia* and *Cardinium.* Differential mortality of the sexes during juvenile development is unlikely to contribute to the sex ratio distortion, as average juvenile survival is generally high (92%), and some highly distorted clutches had almost 100% juvenile survival. All females were infected with *Cardinium*, while only two females were infected with *Wolbachia* and *Rickettsia*. Since 4 of the 5 undistorted

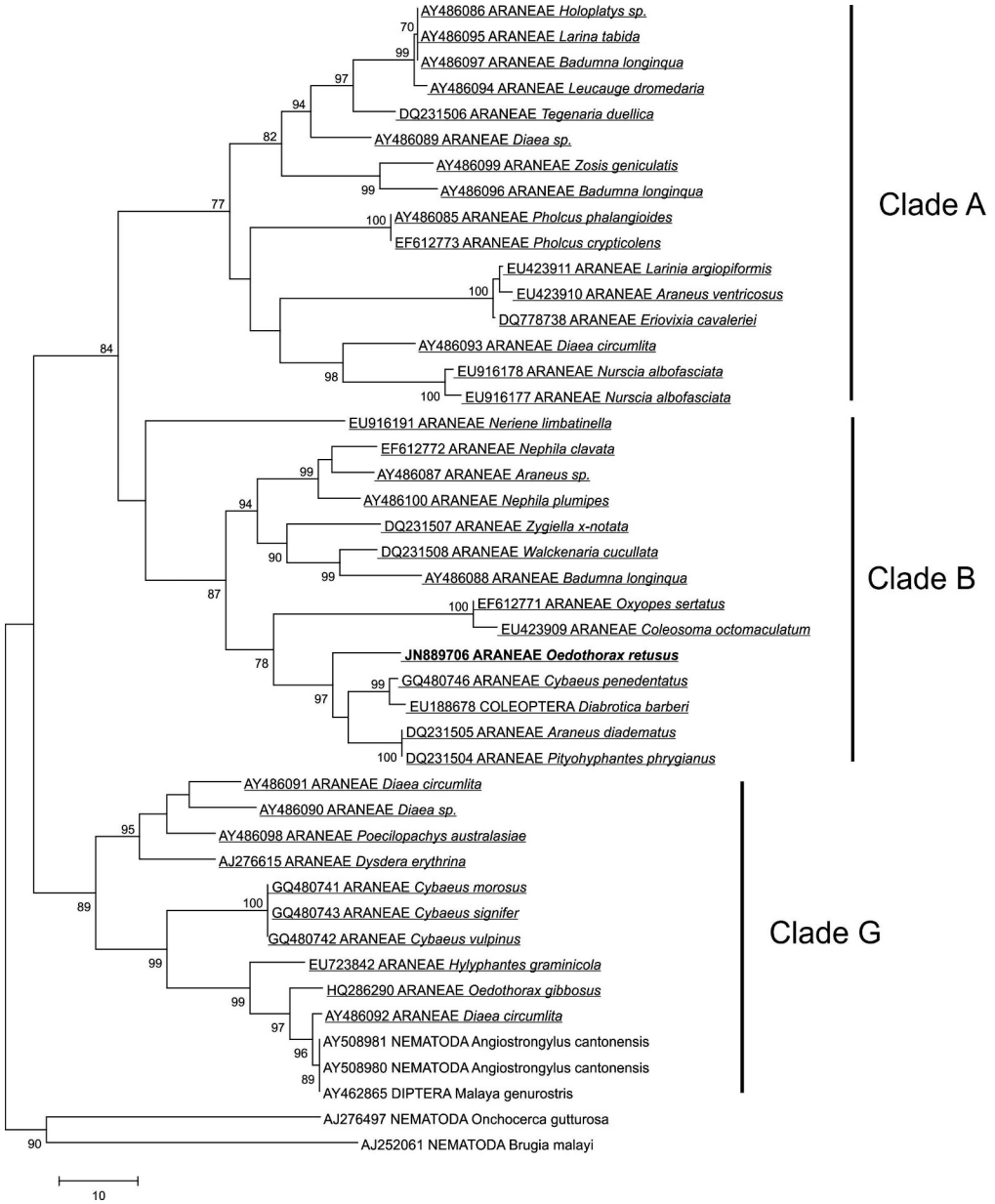

Figure 1.—Phylogenetic position of *Wolbachia wsp* sequence of *Oedothorax retusus* [GenBank: JN889706]. Terminal taxa represent host species. A p-distance based neighbor joining tree was constructed as implemented in MEGA 5 (Tamura et al. 2011) on a subset of *Wolbachia wsp* sequences available at GenBank, with indication of the major *Wolbachia* supergroups (as reported in Rowley et al. 2004; Goodacre et al. 2006). Percentage bootstrap support was calculated for the nodes. Genbank accession numbers are given in front of the taxonomic group to which the host species belongs. Sequences that originate from spider hosts are underlined. *Oedothorax retusus* is shown in bold.

lines were not infected, and a significant relationship was found between *Wolbachia*/*Rickettsia* infection and occurrence of the sex ratio bias within this matriline, infection with these endosymbionts is the most plausible causative agent of the sex ratio distortion.

However, the relationship between bacterial presence and sex ratio effect is not completely clear-cut. A significant difference in sex ratio was found between M1 and M2, both *Wolbachia* and *Rickettsia* infected matrilines (Table 1: $df = 2$; $X^2 = 14.51$; $P < 0.0002$). This variable pattern of bacterial expression of sex ratio distortion could be due to differences in endosymbiont density (Breeuwer and Werren 1993; Hurst et al. 2000; Bordenstein et al. 2006). Alternatively, the presence of host suppressor genes could produce variation in the effect on the sex ratio. Such suppressor genes are expected to evolve in the framework of general sex ratio theory. The discovery of such genes in butterflies and ladybirds indeed provides empirical confirmation (Hornett et al. 2006; Majerus and Majerus 2010a). Performing planned crosses to investigate the precise mode of action of a proposed suppressor gene will be necessary to demonstrate their presence in this species (Majerus and Majerus 2010a).

The combination of these factors does not allow us to identify the actual causal agent for sex ratio distortion in this

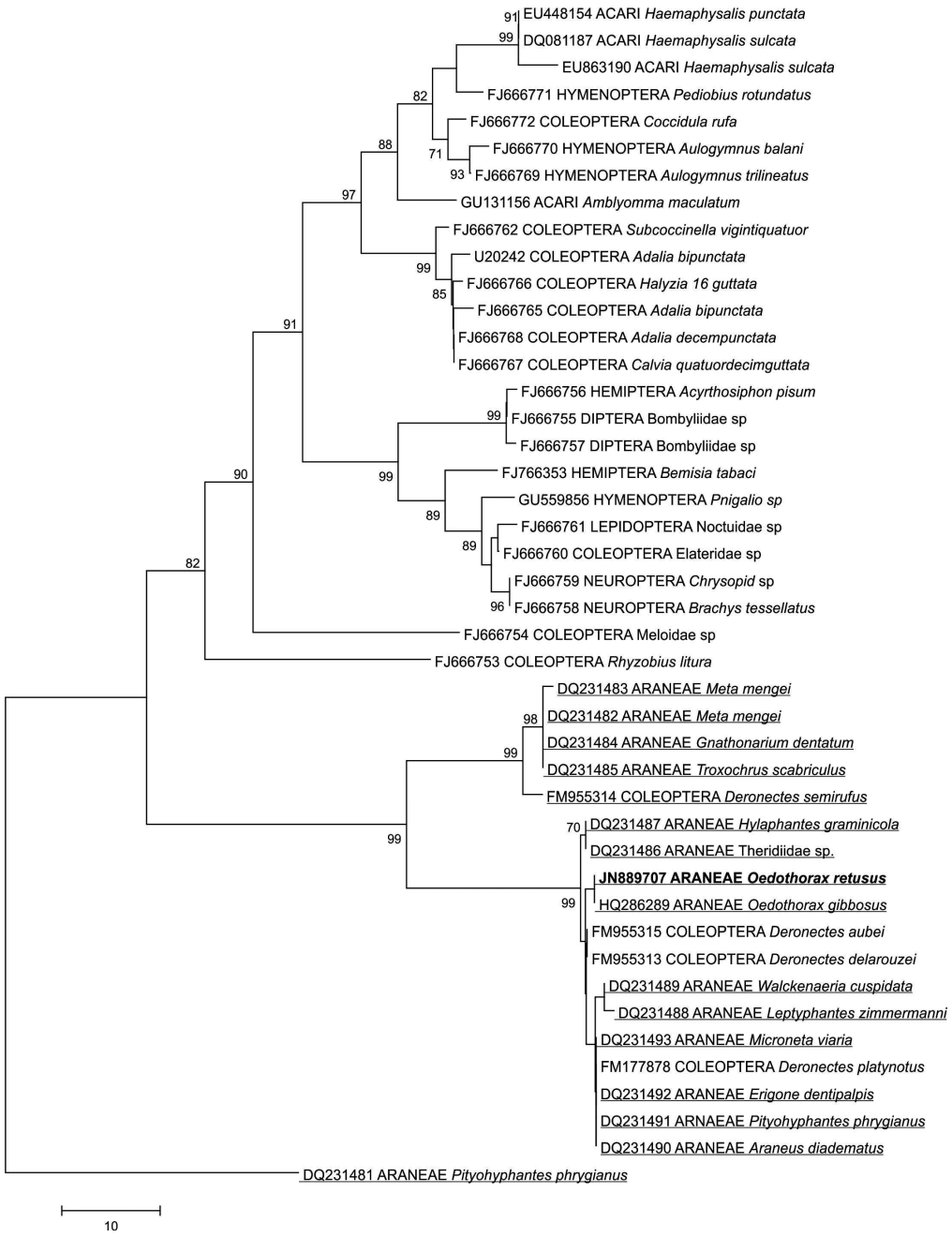

Figure 2.—Phylogenetic position of the *Rickettsia* (partial *citrate* sequence) endosymbiont of *Oedothorax retusus* [GenBank: JN889707]. Terminal taxa represent host species. A p-distance based neighbor joining tree was constructed as implemented in MEGA 5 (Tamura et al. 2011) on a subset of *Rickettsia* sequences available at GenBank. Percentage bootstrap support was calculated for the nodes. Genbank accession numbers are given in front of the taxonomic group to which the host species belongs. Sequences that originate from spider hosts are underlined. *Oedothorax retusus* is shown in bold.

spider. A first indication of the identity of the sex ratio distorter as well as a possible explanation for the apparently variable sex ratio effect can be obtained by analyzing the different densities of *Wolbachia* and *Rickettsia*, using quantitative PCR (Goto *et al.* 2006). Obtaining females singly infected with either endosymbiont could lead to more conclusive evidence on the exact roles of each endosymbiont and their possible interactions. This might be realized by increasing the number of field-caught females if natural variation is present between females infected with either *Wolbachia* or *Rickettsia*. Extending the current study by increasing the sample size of collected females and by including multiple populations would equally allow for an accurate assessment of the occurrence of the sex ratio bias in natural populations.

Alternatively, treatment of doubly infected females with low doses of antibiotics (Sasaki et al. 2005) or transfection of endosymbionts (Sasaki et al. 2002) can establish such single

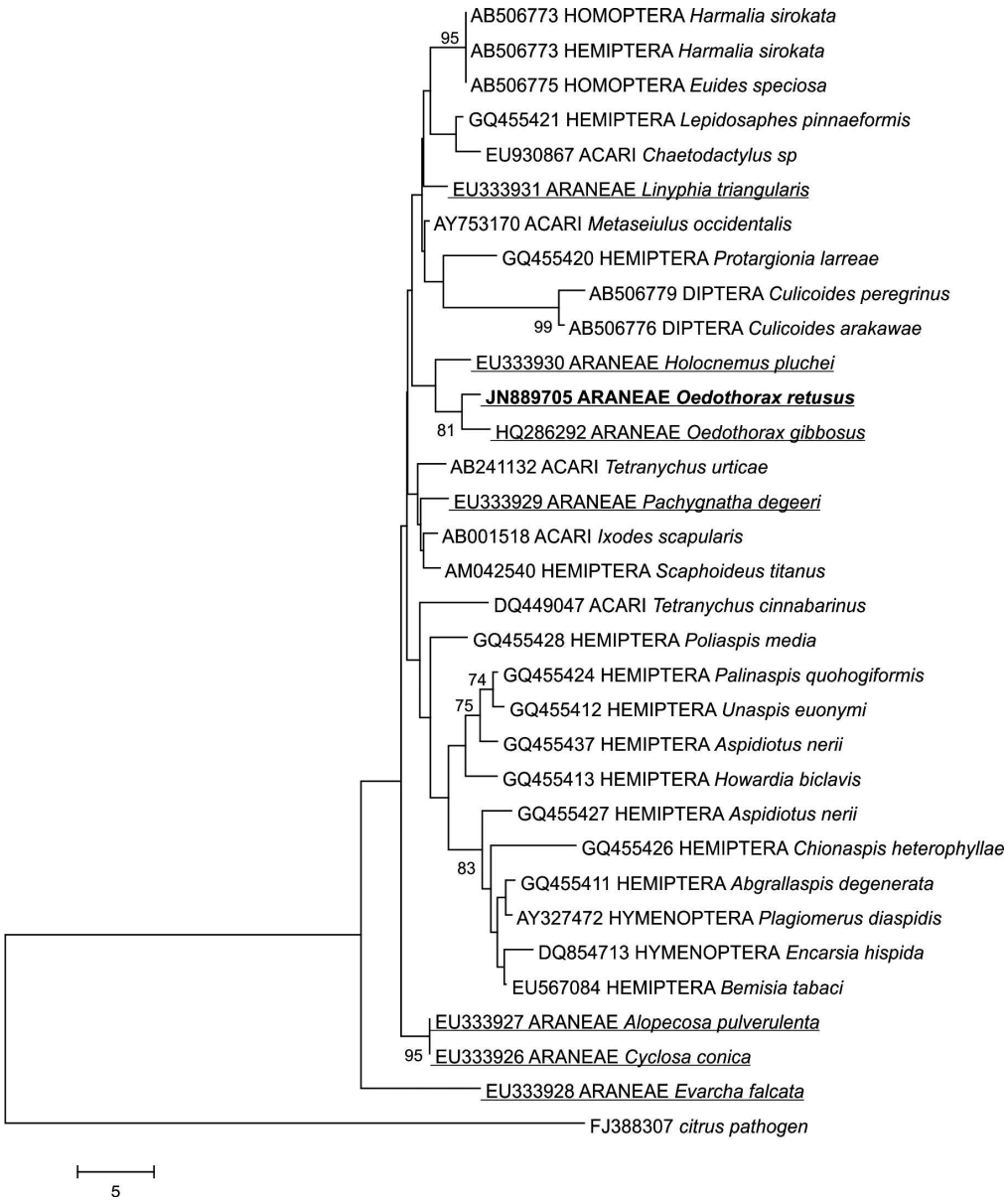

Figure 3.—Phylogenetic position of the *Cardinium* (16S rRNA gene) endosymbiont of *Oedothorax retusus* [GenBank: JN889705]. Terminal taxa represent host species. A p-distance based neighbor joining tree was constructed as implemented in MEGA 5 (Tamura et al. 2011) on a subset of *Cardinium* sequences available at GenBank. Percentage bootstrap support was calculated for the nodes. Genbank accession numbers are given in front of the taxonomic group to which the host species belongs. Sequences that originate from spider hosts are underlined. *Oedothorax retusus* is shown in bold.

infections. Also, we cannot exclude the possibility that the observed sex ratio bias is caused by an as yet unidentified endosymbiont distorter. A next generation sequencing approach would allow us to obtain a broad assessment of the endosymbiont diversity in this spider species (Andreotti et al. 2011; Hirsch et al. 2012).

The strong correlation between number of adult offspring and egg-sac sex ratio supports the hypothesis that the killing of males is the most plausible mechanism of sex ratio distortion. Feminization is highly unlikely, as infected and uninfected females produced equal numbers of female offspring. The occurrence of male-killing is supported by comparing the total number of spiderlings with the total number of eggs produced. This is the most favorable approach for directly linking the number of emerged spiderlings, egg number, and corresponding offspring sex ratio of one female, since development of eggs within the egg sac hampers visual inspection of egg development and hatching rates. Moreover, hatching from the egg and first molt of the spiderlings occur inside the egg sac. This causes a time lag between hatching of the eggs and emergence of the spiderlings from the egg sac, resulting in the inability to single out any undeveloped eggs. As expected, spiderling number is significantly smaller than egg number for both infected and uninfected mothers. This can be caused by mortality during egg hatching and early juvenile cannibalism occurring inside the egg sac. However,

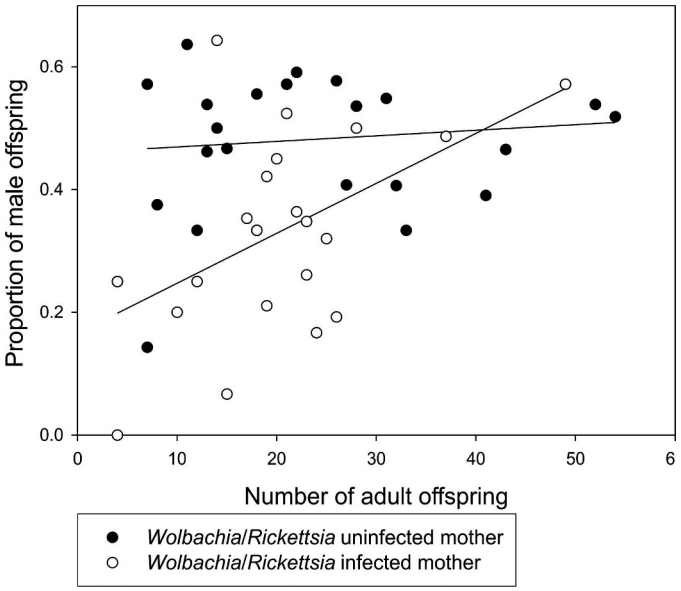

Figure 4.—Relationship between number of adult offspring and proportion of male offspring in the egg sac. Open circles: *Wolbachia* and *Ricketssia*-infected females, filled circles: *Wolbachia* and *Ricketssia*-uninfected females. The solid line visualizes the linear correlation.

significantly fewer spiderlings emerge from egg sacs produced by *Wolbachia* and *Rickettsia*-infected females. The reduction in offspring being produced correlates with the bias toward female offspring in these clutches, suggesting that the offspring that do not emerge from the egg sac are predominantly males. This is again strong evidence for the occurrence of male-killing. Because almost all eggs showed signs of embryonic

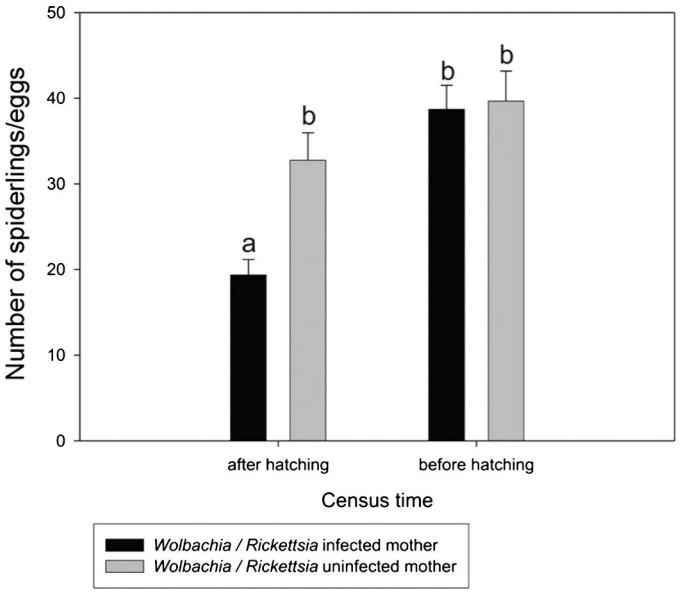

Figure 5.—Clutch size number produced by *Wolbachia* and *Rickettsia* infected (black bars) and uninfected (grey bars) females in first egg sacs (after hatching, number of spiderlings) and second and third egg sacs (before hatching, number of eggs). Bars with the same letter annotation indicate values that are not significantly different.

differentiation (95.7%, $n = 349$) in the egg sacs of infected mothers, male-killing is most likely occurring late in embryonic development or during hatching.

As the sister species *O. gibbosus* is infected with similar genera of endosymbionts, establishing the phylogenetic position of the 16S sequences could present valid information on the relatedness of the endosymbionts. This reveals a close relatedness between the *Cardinium* endosymbionts infecting both species. This is also the case for the *citrate* gene sequence in *Rickettsia*, which is closely related to the sequence of the *Rickettsia* endosymbiont of *O. gibbosus*. In contrast, a clear dissimilarity is found between the *wsp* sequences of *Wolbachia*. The *O. retusus wsp* sequence clusters within supergroup B, while the *wsp* sequence of *O. gibbosus* clusters within supergroup G. Therefore, these data suggest that *Cardinium* and *Rickettsia* infection predates the divergence of these species, followed by independent invasions of different strains of *Wolbachia* in the two species. However, to gain more insight into the routes of infection in the different species and the relatedness between endosymbionts in the genus *Oedothorax*, a more detailed analysis by applying a multilocus comparison (Baldo et al. 2006) would be most suitable.

Although our research is based on a small sample size, the similarity of these results to a prior study of endosymbionts of *Oedothorax gibbosus* is striking (Vanthournout et al. 2011). For the populations investigated, both species seem to be fixed for the *Cardinium* endosymbiont, while the *Wolbachia* infection shows a more variable pattern with approximately half of the individuals infected. The infection pattern of the *Rickettsia* endosymbiont is different for the two species; *O. gibbosus* seems to be fixed, while *O. retusus* shows infection for half of the individuals.

The effect of male-killing endosymbionts in several species of ladybirds shows high variation in the production of offspring sex ratios, ranging from all-female broods to the production of significant numbers of males, (Hurst et al. 1992; Majerus & Majerus 2010b). This is similar to the male-killing effect in both *O. retusus* and *O. gibbosus*, with infected females showing a high variation in sex ratio among clutches, which is of the same order of magnitude [*O. gibbosus*: 0.36 ± 0.04 (Vanthournout et al. 2011); *O. retusus*: 0.35 ± 0.06].

In contrast, for the butterfly species *Hypolimnas bolina* and *Acraea encedon*, infection with male-killers exhibits a higher level of penetrance with the production of only all female broods (Jiggins et al. 2001; Dyson et al. 2002). The evolutionary significance of this difference in pattern of expression of sex ratio distortion remains to be investigated.

Our findings suggest that the phenotypic effects of endosymbiont bacteria on reproductive characteristics could be more widespread in the Araneae order. This confirms the use of a bacterial model as one possible mechanism of different reproductive phenotypes found in many spider species. Further studies into the effects in other spider taxa are necessary to determine their general susceptibility to endosymbiont bacteria and the effects on their hosts' ecology and evolution.

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