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HOST MICROBE INTERACTIONS

# Effects, interactions, and localization of *Rickettsia* and *Wolbachia* in the house fly parasitoid, *Spalangia endius*

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## Abstract

Many insect species harbor facultative microbial symbionts that affect their biology in diverse ways. Here, we studied the effects, interactions, and localization of two bacterial symbionts—*Wolbachia* and *Rickettsia*—in the parasitoid *Spalangia endius*. We crossed between four *S. endius* colonies—*Wolbachia* only (W), *Rickettsia* only (R), both (WR), and none (aposymbiotic, APS) (16 possible crosses) and found that *Wolbachia* induces incomplete cytoplasmic incompatibility (CI), both when the males are W or WR. *Rickettsia* did not cause reproductive manipulations and did not rescue the *Wolbachia*-induced CI. However, when R females were crossed with W or WR males, significantly less offspring were produced compared with that of control crosses. In non-CI crosses, the presence of *Wolbachia* in males caused a significant reduction in offspring numbers. Females' developmental time was significantly prolonged in the R colony, with adults starting to emerge one day later than the other colonies. Other fitness parameters did not differ significantly between the colonies. Using fluorescence in situ hybridization microscopy in females, we found that *Wolbachia* is localized alongside *Rickettsia* inside oocytes, follicle cells, and nurse cells in the ovaries. However, *Rickettsia* is distributed also in muscle cells all over the body, in ganglia, and even in the brain.

**Keywords** Microbial symbionts · Reproductive manipulations · Cytoplasmic incompatibility · Fluorescence in situ hybridization · Hymenoptera · Pteromalidae

## Introduction

Many arthropods live in symbiosis with microorganisms. These microbial symbionts are generally divided into two groups: primary and secondary. Primary symbionts have an obligate relationship with their hosts, since they provide essential nutrients absent in the host's diet; in return, the host provides the symbiont with other nutrients or precursors, a stable environment, and a guaranteed route of maternal transmission to its offspring [1]. Secondary symbionts are facultatively associated with their hosts and are therefore generally not considered essential for host development and reproduction. Nevertheless, secondary symbionts can contribute directly to their host's fitness in various ways, such as conferring resistance to pathogens, natural enemies, and abiotic factors, thereby indirectly promoting their own fitness. On the other hand, some secondary symbionts promote their own spread by manipulating the host's reproduction to produce more females which will transmit them to their progeny. These reproductive manipulations include the induction of parthenogenesis, male killing (male embryos die before hatching), feminization (genetic males develop as females), and cytoplasmic incompatibility (CI; a cross between a symbiont-infected male and an uninfected female is incompatible, thereby reducing the proportion of uninfected individuals in the population). Secondary symbionts are usually maternally transmitted, but horizontal transmission can occasionally occur as well [2–5]. To date, five bacterial genera are known as reproductive manipulators (also commonly termed reproductive parasites): *Arsenophonus*, *Cardinium*, *Flavobacteria* relatives, *Rickettsia*, *Spiroplasma*, and

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*Wolbachia*, of which the latter is the most prevalent among insect species [6] and the only one known to induce all four types of manipulations [3, 7, 8].

Here, we studied the interactions between two bacterial secondary symbionts—*Rickettsia* and *Wolbachia* (both α-proteobacteria: Rickettsiales, both intracellular)—with the host *Spalangia endius* Walker (Hymenoptera: Pteromalidae). *Spalangia endius* is a parasitic wasp (= parasitoid) that attacks various fly species. The female wasp lays her eggs onto pupae of flies (usually one egg per pupa) and the wasp larva devours the fly pupa. This parasitoid species is common worldwide and often used as a biocontrol agent for controlling filth flies such as the house fly and the stable fly [9, 10]. The prevalence of *Rickettsia* and *Wolbachia* varies between wild populations of *S. endius*. For example, among four wild populations in Israel, two had no symbionts, whereas in two other populations, all individuals carried *Wolbachia* and some carried *Rickettsia* as well [11].

*Spalangia endius* harbors multiple strains of *Wolbachia* (when present as a sole symbiont, and when present in conjunction with *Rickettsia*) that collectively cause an incomplete CI, meaning that some daughters are still produced in a cross between *Wolbachia*-infected males and uninfected females [11]. Sons' production is unharmed in this CI cross, because the sex-determination system in *S. endius*, as in all hymenopterans, is haplodiploidy; thus, males develop from unfertilized eggs and are haploid.

*Rickettsia* is a diverse genus of obligate intracellular bacteria, associated with numerous hosts including arthropods, protozoa, algae, plants, and vertebrates. *Rickettsia* symbionts are less prevalent than *Wolbachia* among insect species [6], but similar to *Wolbachia*, some *Rickettsia* symbionts were found to be reproductive manipulators (causing male killing and parthenogenesis) or conferring fitness benefits to their hosts [3, 12]. The *Rickettsia* symbiont of *S. endius* belongs to the "transitional group" and is closely related to *R. felis*—a human pathogen transmitted mainly by flea species [13]. The *Rickettsia* symbiont of *S. endius* is closely related also to rickettsial symbionts of two other hymenopterans in which it induces parthenogenesis, and to a rickettsial primary symbiont of a booklouse [11]. The effect(s) of *Rickettsia* on *S. endius* is unknown, and this is one of the goals of the current study.

Co-existence of multiple symbionts within the same host is not uncommon. Prominent examples include the pea aphid and the sweet potato whitefly, which in addition to an obligatory primary symbiont, carry several lineages of facultative secondary symbionts, each with a certain effect on the host [14, 15]. Multiple symbiont infections were found also in *Drosophila* flies [16], parasitoid wasps [17], and elsewhere. When multiple symbionts co-occur within the same host, the host can be viewed as a microhabitat, in which the symbionts likely compete for the (limited) host resources—space and nutrients. A host may suffer higher fitness costs if the symbionts over exploit its resources. On the other hand, such fitness costs may be compensated by the coexisting symbionts if they confer fitness advantage(s) to their host. Thus, the symbiotic community will vary according to the ecological selection pressures that operate in each environment, leading to either a fixation of different symbiotic communities in different environments, or to variation in symbiotic communities within a population [18–20]. The occurrence of two (or more) reproductive manipulators in the same host raises additional questions such as: do both symbionts manipulate the host's reproduction? Does one symbiont affect the manipulation of the other? *Wolbachia* often co-occurs with another reproduction manipulating bacterium—*Cardinium*; the outcome of this duo differs from host to host [21–26]. *Rickettsia* spp. and *Wolbachia* cohabiting the same host is less common: *Wolbachia* co-occurs with *R. bellii* in aphids [15] and whiteflies [14], with *R. asemboensis* in fleas [27] and with *Rickettsia* sp. in beetles [28, 29]. Hence, another goal of this research was to study whether *Rickettsia* is a reproductive manipulator of *S. endius*, and whether it modifies the *Wolbachia*-induced CI.

The third goal of our research was to study the localization of the two symbionts inside the body of *S. endius*. Secondary symbionts are typically found in females' reproductive organs including the oocytes, as an essential prerequisite for maternal transmission. Secondary symbionts of hosts that carry also primary symbionts (e.g., aphids, whiteflies), are usually localized inside bacteriocytes together with the primary symbiont (see review in [30]). *Wolbachia* has been found also in somatic tissues in some hosts [31], but for *Rickettsia* (excluding pathogenic species), there are few example of localization in somatic tissues [32–34].

## Materials and methods

### Insect rearing

**House flies** Adult house flies were held in net cages with water and a diet of sugar, milk powder, and egg yolk powder mixture (2:2:1 by weight, respectively). The larvae were reared on a medium of wheat bran mixed with calves' food pellets, wetted with water to 60–65% moisture. The flies were maintained at 26 ± 1°C, 60 ± 20% RH, and 14:10 light/dark photoperiod.

**Parasitoid colonies** *Spalangia endius* were collected in 2014 from an egg-laying poultry facility in Hazon, Israel (32° 54′ 25.8″ N; 35° 23′ 49.0″ E), using sentinel pupae as described in Chiel and Kuslitzky (2016). The parasitoids that emerged from the sentinel pupae were separated to iso-female lines: using a fine brush, each female parasitoid was placed individually in a plastic cup (30 $cm^3$ volume, with a perforated lid to allow ventilation) with 50 house fly pupae (48-h-old) for

oviposition for 3 days, then retrieved, identified to the species level [35, 36], and symbiont infection was determined by PCR, as described in [11]. The infection status was verified by testing two of the emerging offspring in each cup, and subsequently, cohorts with identical infection status were pooled to establish two colonies: *S. endius* carrying *Wolbachia* only (W) and *S. endius* carrying *Wolbachia* and *Rickettsia* (WR). As mentioned above, *S. endius* harbors multiple *Wolbachia* genotypes, which vary between individuals within the colony [11], and it is currently not possible to generate colonies with identical *Wolbachia* strain(s). Since the W and the WR colonies may harbor multiple different strains of *Wolbachia*, we interpret all results with extra caution. An aposymbiotic (symbiont-free) colony was established from the WR colony via an antibiotic treatment, as follows: 0.5 g rifampicin was added to 100 g of a house fly larval food mixture with about two hundred 2$^{nd}$-stage larvae. Upon pupation (3-4 days later), the puparia were supplied as hosts to WR *S. endius*. The emerging offspring were found to be all aposymbiotic by PCR and were maintained as a separate colony. Similarly, we attempted to establish a colony carrying *Rickettsia* only (R) by applying different antibiotics (rifampicin, tetracycline, ofloxacin) at various concentrations as described above, but all the antibiotic/concentration combinations yielded either aposymbiotic, W, or WR parasitoids. Eventually, an R colony was established via horizontal transmission, as follows: 300 house fly pupae were exposed to 30 WR parasitoids for 24 h; the parasitized pupae were incubated at 26 ± 1 °C for 13 days (until parasitoids had reached the prepupal or pupal stage). Then, these puparia were divided to 27 groups of ten, each group in a separate cup, and one aposymbiotic parasitoid female was introduced into each cup. Normally, *S. endius* refrains from parasitizing fly pupae that are already parasitized by con- or heterospecifics, unless it has no other options. Hence, this setup forced aposymbiotic *S. endius* to parasitize WR conspecifics, generating the opportunity for horizontal transmission of one or both of the symbionts. The aposymbiotic females were retrieved after 24 h, and the puparia were incubated until $F_1$ offspring emergence. Offspring emerged only in two replicates: one female in one replicate, one female plus one male in the second replicate. These wasps were placed separately in new cups (the single female was supplied with an aposymbiotic male) with 30 fresh house fly pupae, allowed to parasitize for 3 days and then their infection status was checked by PCR. The three wasps were found positive for both symbionts. Upon emergence of their offspring ($F_2$) this procedure was repeated, and it was found that six (four females and two males) out of 20 offspring of the single female had *Rickettsia* only, while all the offspring of the second replicate had none of the symbionts. Subsequent generations of the *Rickettsia*-infected wasps showed a stable vertical transmission of this symbiont to all progeny, and they are maintained as a separate colony to this date (a separate publication on this issue is currently in preparation).

Next, we conducted a series of introgression crosses to homogenize the nuclear background of the colonies. This was done by crossing aposymbiotic males with virgin females from the other three colonies (W, R, WR, each colony separately) for six consecutive generations (generation time is 21 days at 26 °C). In doing so, we introgressed the aposymbiotic nuclear background into the three symbiont(s)-carrying colonies. The infection status of all the colonies was verified by PCR every ~ 3 months.

## Fitness measurements of *S. endius*

All the experiments described hereinafter were performed in 26 ± 1°C, 60 ± 10% RH, and 14:10 light/dark photoperiod.

## Developmental time

Ten house fly pupae were placed in plastic cups with one female and one male *S. endius* (24–48-h-old), 20 replicate cups for each colony. The parasitoids were retrieved after 8 h and the pupae were incubated. Twenty-one days later, when offspring started to emerge, the number and sex of the emerging parasitoids were recorded daily. The results were analyzed by a linear mixed effects model for the effect of symbiont(s) species on development time, using the identity of the mother as a random intercept term. The model was fit in R (R Core Team 2018) using the lme4 package [37]. Additionally, to evaluate differences in emergence dynamics between parasitoids with different symbionts, we used a nonlinear logistic model approach on the aggregated emergence data. We first fit a logistic model to all emergence data points together (i.e., disregarding symbiont treatment). The model had the following structure: cumulative emergence = $A_{sym}/(1 + \exp((X_{mid} - N_{days})/\text{scale}))$; where $A_{sym}$, $X_{mid}$, and scale are model parameters and $N_{days}$ is the number of days until emergence. We then fit four reduced models, each one omitting the data points of a single symbiont treatment (i.e., the reduced models were nested within the full model). We then used an F test to compare model fit between the full model and reduced models, to identify if the inclusion of a specific symbiont treatment had a significant effect on the parameters of the full emergence model. We did this process for the males and females separately. Unfortunately, we were not able to conduct this test at the level of the individual mothers because of model convergence issues. Hence, we opted to aggregate emergence numbers across all replicates within each treatment, thus reducing the degrees of freedom of the analysis.

### Longevity and fecundity

Newly-emerged parasitoids (up to 24-h-old) from the four colonies were placed in plastic cups, one female and one male with 40 house fly pupae in each cup. The parasitoids were monitored daily and alive/dead was recorded. Throughout their lifetime, the parasitoids were transferred every 72 h to new cups with 40 new house fly pupae for oviposition. All the pupae were incubated and the emerging offspring were counted. The results were analyzed by ANOVA using SPSS (IBM statistics, version 24).

### Testing for reproductive manipulations

Pupae from the four colonies were placed individually in 1-ml glass vials before the parasitoids had emerged, to prevent uncontrolled mating. On emergence, the adults were sexed, randomly assigned to one of the 16 possible crosses, and left for 24 h to mate as a group in cups. After the mating period, the adults were transferred to cups with 40 house fly pupae, one female and one male per cup and allowed to oviposit for 4 days. Subsequently, the cups were incubated until all offspring had emerged, and then the offspring were sexed and counted. Offspring sex ratio (proportion of females) was analyzed as a logistic regression (SPSS), regarding the ♀APS/♂APS as control cross to which all other crosses were compared. Additionally, we compared specific crosses by ANOVA if data (number of offspring) met the criteria for parametric analyses, or non-parametric tests if these criteria were not met, even after various transformations. In the "Results" section, we specify the test used in each comparison.

### Determining the localization of *Wolbachia* and *Rickettsia*

Adult females and larvae of $W^+R^+$ *S. endius* were fixed in 4% formaldehyde (in 1× PBS) for 24 h and then embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany). Semithin sections (8 μm) were obtained on a rotary microtome (Microm HM355S) with a glass blade and transferred to silanized microscope slides. Samples were hybridized for 90 min at 50 °C in hybridization buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS) containing 25 nM of each of the symbiont-specific probes as well as 5 μg/ml DAPI for counterstaining of host cell nuclei. Residual probes were removed by a 20-min wash step at 50 °C with pre-warmed wash buffer (0.1 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS, 5 mM EDTA), followed by a 20-min washing step in $dH_2O$. After short rinsing in $dH_2O$ and shaking off the excess liquid, slides were covered with VectaShield H-1400 (Vector, Burlingame, USA) and inspected on an AxioImager.Z2 fluorescence microscope with Apotome (Zeiss, Jena, Germany). The *Rickettsia* probe was 5′-Cy5-CCGGCATTACCCGC TGGCAA-3′ [38], and the *Wolbachia* probe was 5′-Cy3-CTTCTGTGAGTACCGTCATTATC-3′ [39], both targeting the 16S rRNA. Aposymbiotic parasitoids were used as negative control.

## Results

Development time from egg to adult was significantly longer in females from the R colony than females from the three other colonies (type II Wald's chi square test, $\chi^2 = 27.25$, df = 3, $p < 0.0001$; Table 1), which can be attributed to the fact that R females started to emerge 1 day later than females from the other colonies (Fig. 1a). The development time of males was also longer in the R colony than the other colonies, but not statistically significant (type II Wald's chi square test, $\chi^2 = 5.22$, df = 3, $p = 0.16$; Fig. 1b, Table 1). Females from the R colony had a significantly different emergence dynamics compared with that of other symbiont treatments ($F_6 = 26.6$, $p < 0.0001$; Fig. 1a). In contrast, male parasitoids did not exhibit variation in cumulative emergence dynamics across symbiont types. Female longevity was similar across the four colonies (ANOVA, $F_3 = 0.512$; $p = 0.67$; Table 1), and the survival curves did not differ significantly (Kaplan-Meier analysis (Tarone-Ware test); females, $\chi^2 = 2.0$, df = 3, $p = 0.57$; males, $\chi^2 = 3.44$, df = 3, $p = 0.33$). Lifetime fecundity also did not differ between the colonies (ANOVA, $F_3 = 2.45$; $p = 0.08$; Table 1).

### Reproductive manipulations

The number of progeny and their sex ratio differed significantly between crosses: the four crosses in which males carried *Wolbachia* (either W or WR) and the females did not carry *Wolbachia* (either R or APS) (crosses # 2, 4, 14, 16 in Fig. 2) had fewer offspring than the other twelve crosses, and the sex ratio was male-biased (Fig. 2, Table 2). Specific questions and their results are as follows:

- Does *Wolbachia* induce CI? Yes. Among the four possible crosses between the APS and the W colonies (crosses # 1, 4, 5, 8 in Fig. 2), fewer offspring were produced in the W♂/APS♀ cross (Kruskal-Wallis test: $p = 0.063$), and the sex ratio was male-biased, resulting from a sharp decrease in female progeny (Kruskal-Wallis test: $p < 0.001$) and a significant increase in male progeny (ANOVA, $F_3 = 16.9$; $p < 0.001$).
- Does *Rickettsia* induce CI? No. The number of offspring and their sex ratio did not differ among the four possible crosses between the APS and the R colonies (crosses # 1, 3, 13, 15 in Fig. 2) (number of offspring, $F_3 = 0.53$, $p = 0.66$; sex ratio, $F_3 = 2.6$, $p = 0.07$).

**Table 1** Fitness components of *S. endius* carrying either *Wolbachia* (W), *Rickettsia* (R), both (WR), or no symbionts (aposymbiotic, APS)

| | Colony | | | | |
|---|---|---|---|---|---|
| | APS | W | R | WR | Statistics |
| Female development time (days) | 23.5 ± 0.1 (81) | 23.17 ± 0.1 (70) | 23.9 ± 0.1 (56) | 23.2 ± 0.1 (76) | $\chi^2 = 27.25$, df = 3, $p < 0.0001$ |
| Male development time (days) | 21.6 ± 0.1 (22) | 21.73 ± 0.1 (15) | 22.0 ± 0.2 (15) | 21.6 ± 0.1 (22) | $\chi^2 = 5.22$, df = 3, $p = 0.16$ |
| Female longevity (days) | 11.5 ± 0.5 (10) | 10.9 ± 0.6 (10) | 10.7 ± 0.6 (9) | 11.5 ± 0.5 (10) | $F_3 = 0.512$; $p = 0.67$ |
| Lifetime fecundity (offspring/female) | 37.0 ± 2.1 (10) | 31.9 ± 2.8 (10) | 34.4 ± 1.4 (9) | 39.9 ± 2.1 (10) | $F_3 = 2.45$; $p = 0.08$ |

Values are means ± s.e, numbers of replicates in parentheses

- Does the presence of *Rickettsia* in males modify the *Wolbachia*-induced CI? No. In the four possible crosses between the APS and the WR colonies (crosses # 1, 2, 9, 10 in Fig. 2), fewer offspring were produced in the WR♂/APS♀ cross (ANOVA, $F_3 = 6.3$; $p = 0.002$), and the sex ratio was male-biased (ANOVA, $F_3 = 34.4$, $p < 0.001$). However, the results of crosses WR♂/APS♀ and W♂/APS♀ (crosses 2 and 4, respectively) did not differ (total offspring number:, $F_1 = 0.27$, $p = 0.6$; number of male offspring, $F_1 = 0.009$, $p = 0.93$).
- Does the presence of *Rickettsia* in females modify the *Wolbachia*-induced CI? Yes. In the two CI crosses in which females carried *Rickettsia* (crosses 14 and 16, combined), there were significantly less offspring (ANOVA, $F_1 = 10.7$, $p = 0.002$), particularly males (ANOVA, $F_1 = 5.97$, $p = 0.02$) compared with the CI crosses in which the females were aposymbiotic (crosses 2 and 4).
- Do symbionts affect the number of offspring, regardless of CI? For this analysis we excluded the CI crosses (# 2, 4, 14, and 16) and compared the total offspring numbers grouped by either the mother's or father's symbionts. The results were analyzed by a two-way ANOVA, assigning the mother's and father's infection status as main effects. The father's infection status had a significant effect on total offspring number ($F_3 = 4.74$, $p = 0.004$): higher numbers of offspring were produced in crosses in which the fathers had no *Wolbachia* (Fig. 3). The mother's infection status did not affect the total offspring number ($F_3 = 0.35$, $p = 0.79$) and the interaction between these two main factors was not significant either ($F_5 = 0.61$, $p = 0.69$).

Also, notably, in the four crosses with WR mothers (#9–12), fewer offspring were produced when the fathers were W or WR (#10 + 12 vs. 9 + 11, $F_3 = 2.66$, $p = 0.064$), but the sex ratio was not affected.

## Localization of the symbionts

In *S. endius* adult females, *Wolbachia* and *Rickettsia* co-inhabit the oocytes and the accompanying nurse cells and follicle cells (Fig. 4a). However, while *Wolbachia* was found only in these cells, *Rickettsia* was found also in other tissues: abdominal and thoracic muscles, ganglia, salivary glands, and even in the brain (Fig. 4a–c). In *S. endius* larvae, both symbionts were observed in fat cells, trophocytes, and other cells (Fig. 4d–e).

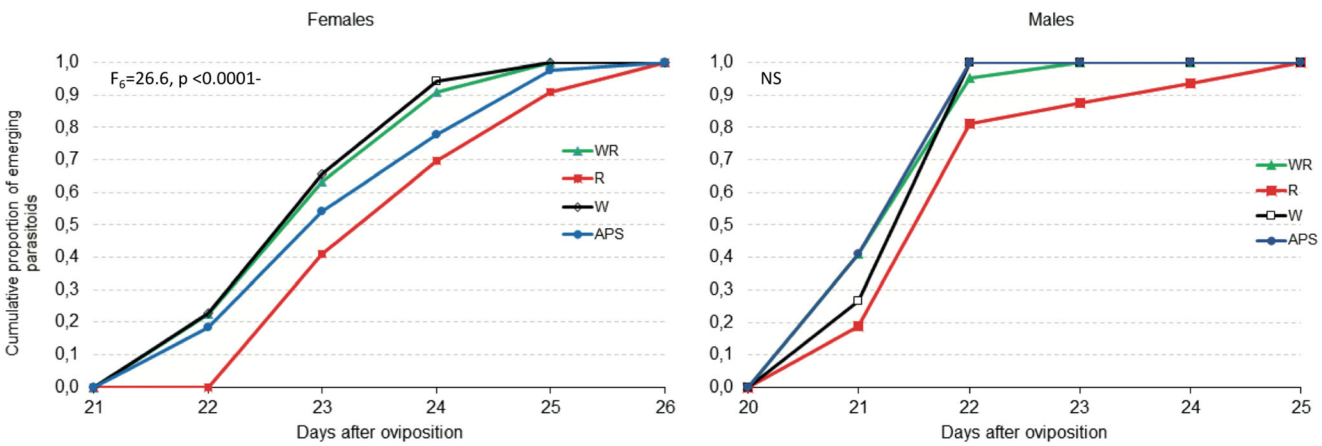


**Fig. 1** Cumulative proportions of emerging *S. endius* females (left) and males (right) from the four colonies. *Wolbachia* (W), *Rickettsia* (R), both (WR), none (aposymbiotic, APS). See text for statistical analyses

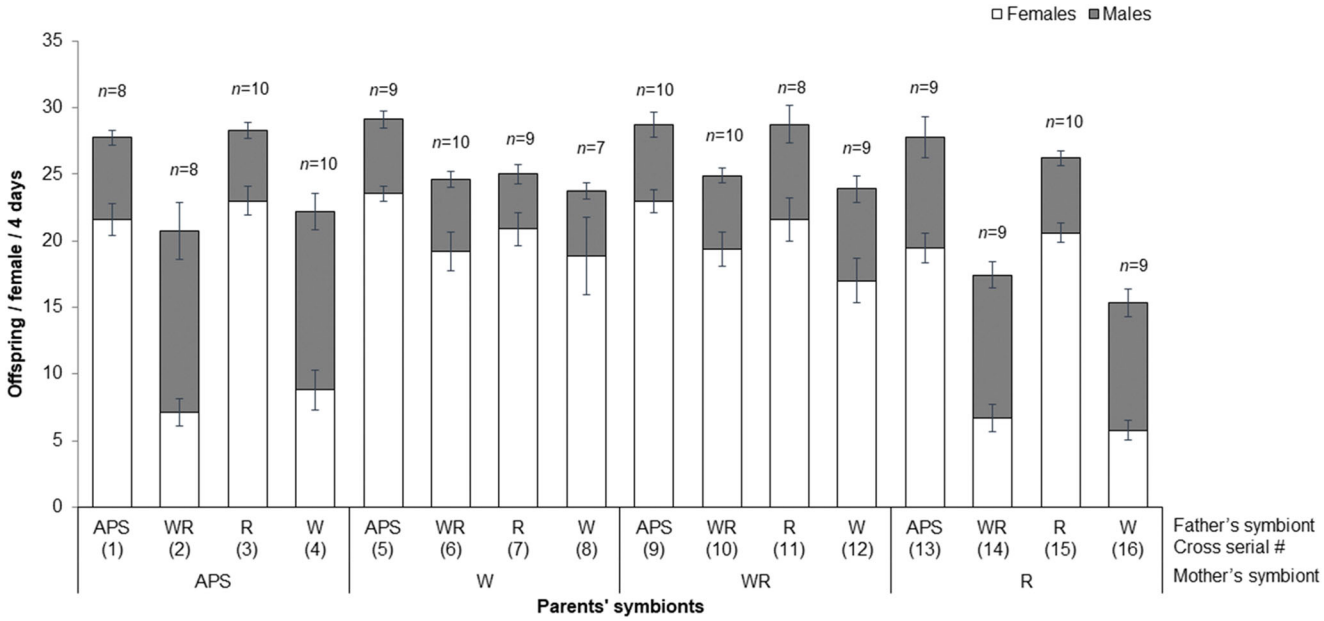


**Fig. 2** Number of offspring (means ± s.e) produced during 4 days in the 16 possible crosses between *S. endius* carrying either *Wolbachia* (W), *Rickettsia* (R), both (WR), or none (aposymbiotic, APS)

## Discussion

In the last two decades, ample studies have demonstrated the diverse influences of microbial secondary symbionts on their eukaryote hosts: some contribute directly to the host's fitness, some manipulate the host's reproduction, and some do both [3, 40]. However, the effect of two putative reproductive parasites on host fitness is less well-explored (with the exception of aphids). In the current study, we investigated the nature of the symbiosis between *S. endius* and *Rickettsia*, and asked whether *Rickettsia* modifies the CI induced by *Wolbachia* [11]. Our findings reveal neither beneficial effects nor reproductive manipulations of *Rickettsia* in *S. endius*. The fitness parameters we measured—longevity, fecundity, and development time—were mostly similar in the four colonies, but notably, the R females' development time was slower than the other colonies (Fig. 1, Table 1). We interpret this result as a negative effect of *Rickettsia* on the parasitoid fitness, but the normal development time of the WR females indicates that *Wolbachia* somehow counterbalances *Rickettsia*'s negative effect. Perhaps this effect is quantity-dependent, i.e., when coinciding with *Wolbachia*, the quantity of *Rickettsia* may go down and consequently the negative effect diminishes. In our previous study [11], we found that the *Rickettsia* symbiont of *Spalangia* is part of the *Rickettsia* "transitional group" and is closely related to *R. felis*—a mammalian pathogen vectored mainly by fleas, and to a lesser extent also by *Anopheles* mosquitos [13]. Healy *et al.* [41] found no effects of *R. felis* on the development or reproductive fitness of cat fleas. It is possible then that *Rickettsia* enhances the fitness of *S. endius* in context-dependent ways, such as pesticide-resistance [42] or heat-tolerance [43], although such benefits may be conditional [44]. In addition to *R. felis*, the *Rickettsia* symbiont of *Spalangia* is closely related also to rickettsial symbionts of two other hymenopterans: a leaf-miner parasitoid [45] and a gall wasp [46, 47]; in both, these hymenopterans *Rickettsia* induces parthenogenesis, whereas in *S. endius*, *Rickettsia* does not induce any reproductive manipulation. One possible reason for this difference might be the evolutionary history of the association between *Rickettsia* and each of these hosts, which is substantially younger in *Spalangia* (Fig. 3 in [11]).

In contrast to *Rickettsia*, *Wolbachia* manipulates the reproduction of *S. endius*, by inducing an incomplete CI (Fig. 2). This result is not surprising and was recently published [11], but here, the CI phenotype is somewhat different: the CI crosses yielded significantly fewer female offspring and significantly more male offspring, compared with the other crosses. These results indicate that some of the fertilized eggs developed normally to a female offspring, some fertilized eggs died due to CI (hence fewer female offspring), and some eggs

**Table 2** Parameter estimates of the logistic regression of female proportions in the four CI crosses, in comparison with the APS♂/APS♀ cross

| Cross (# in Fig. 2) | Effect size ± S.E | Odds ratio | *p* value |
|---|---|---|---|
| WR♂/APS♀ (#2) | − 2.24 ± 0.22 | 0.107 | < 0.001 |
| W♂/APS♀ (#4) | − 1.68 ± 0.21 | 0.186 | < 0.001 |
| WR♂/R♀ (#14) | − 1.99 ± 0.22 | 0.137 | < 0.001 |
| W♂/R♀ (#16) | − 2.06 ± 0.23 | 0.127 | < 0.001 |

in which fertilization failed due to CI developed into haploid males (hence more male offspring) [8]. Alternatively, the increase in numbers of male offspring may be a result of the mothers laying more unfertilized eggs because the sperm of W males is of lower quality and/or quantity [48–50]. This postulation is supported also by the finding that fewer offspring were produced in the non-CI crosses in which the fathers carried *Wolbachia*, reflecting the cost imposed on males carrying a reproductive parasite. Koehncke et al. [51] predicted that in cases like this, selection will favor *Wolbachia*-free males and consequently *Wolbachia* is "likely to be lost from host populations on long evolutionary time scales due to reduction of CI levels in males" (see also [2]). Another possibility, although highly speculative and hard to test, is that mothers in CI crosses "intentionally" produce more (*Wolbachia*-free) sons in order to mitigate the impact of *Wolbachia*-inflicted CI on reproductive output. Whatever the mechanism may be, the outcome is a slower pace of *Wolbachia* spread in the population. Fewer offspring were produced when both parents carried *Wolbachia* (crosses # 6, 8, 10, 12) compared with the controls, but the offspring` sex ratio was unaffected. This may reflect a milder or different CI phenotype between different *Wolbachia* strains. Isolating colonies with identical strain(s) of *Wolbachia* is challenging due to the collection of strains in each individual wasp, some of which may result from ongoing recombinations between strains [11]. Therefore, the minor differences found between the W and WR colonies (Fig. 3) may be attributed to this factor and conclusions should be taken cautiously.

On top of the negative effect of *Wolbachia* in males, we found that in the CI crosses between *Wolbachia*-infected males (either W or WR) and *Rickettsia*-infected females (crosses 14 and 16), the number of offspring decreased significantly (compared with crosses 2 and 4, or 6 and 8, or 10 and 12). In other words, *Rickettsia* does not rescue fertilized eggs from *Wolbachia*-induced CI; on the contrary, it further diminishes reproductive success. The physiological basis for this phenomenon awaits further research, but a recent finding of a toxin–antitoxin module with putative deubiquitylating (DUB) activity in *R. felis* symbiont of the booklouse *Liposcelis bostrychophila* [52], suggests that *Rickettsia* DUB enzymes are incompatible with those of *Wolbachia*, thereby worsening the CI outcome. We found no previous reports of similar antagonism; a similar experiment [17] was performed with the parasitoid *Encarsia inaron* harboring *Wolbachia* and/or *Cardinium*; in that study, CI was induced by *Wolbachia*, whereas *Cardinium* did not induce CI nor modify the *Wolbachia*-CI (contrary to *Rickettsia* in our study).

Nonetheless, while crosses between *Wolbachia*-infected males and *Rickettsia*-infected females were detrimental for offspring production, the measured fitness parameters of *S. endius* that harbored both symbionts (WR) were similar to

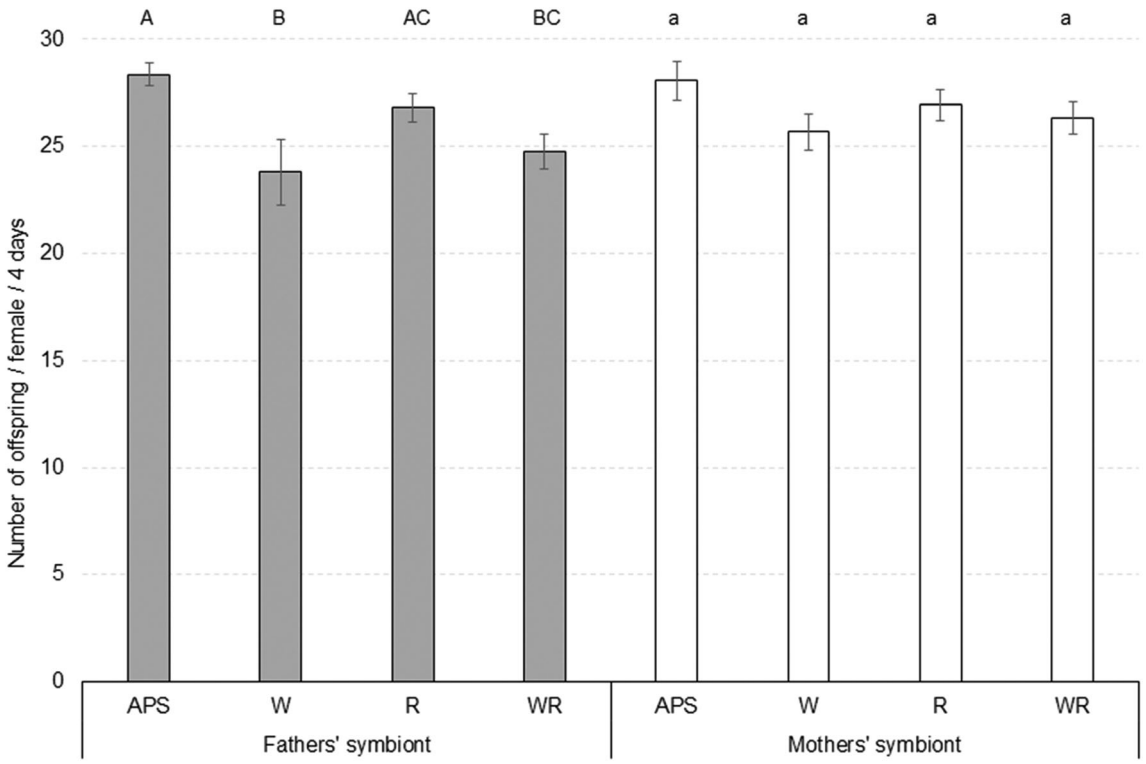


**Fig. 3** Number of offspring produced in 12 crosses (means ± s.e; the four CI crosses were omitted from this comparison), pooled either by the fathers' symbionts (grey bars) or mothers' symbionts (white bars). Letters above bars denote statistical differences between groups. (W-*Wolbachia*, R-*Rickettsia*, WR-both, APS-none)

the other three colonies. This result suggests that the *Wolbachia–Rickettsia-S. endius* three-way symbiosis has gone through sufficient time and selection pressures that alleviated the initial costs of *Wolbachia* and *Rickettsia* coinfection and stabilized it.

The microscopic observations revealed an interesting pattern: while *Wolbachia* is localized only in the ovaries of adult *S. endius* (oocytes, nurse cells, follicle cells, and germarium), *Rickettsia* is distributed in multiple organs and tissues, including the ovaries, muscles, and nerves (Fig 4). The presence of both symbionts in the oocytes conforms to their vertical, transovarial transmission, as in most other symbionts [30, 53]. A non-specific tissue tropism is typical among pathogenic *Rickettsia* species within their arthropod vectors [12, 13], but among non-pathogenic *Rickettsia*, such localization pattern was so far reported only from whiteflies [32] and a few beetles [54, 55].

To briefly summarize the findings from the current study and our previous one [11]: (1) *Wolbachia* is fixed in some natural *S. endius* populations, whereas *Rickettsia* is not fixed [11]; (2)

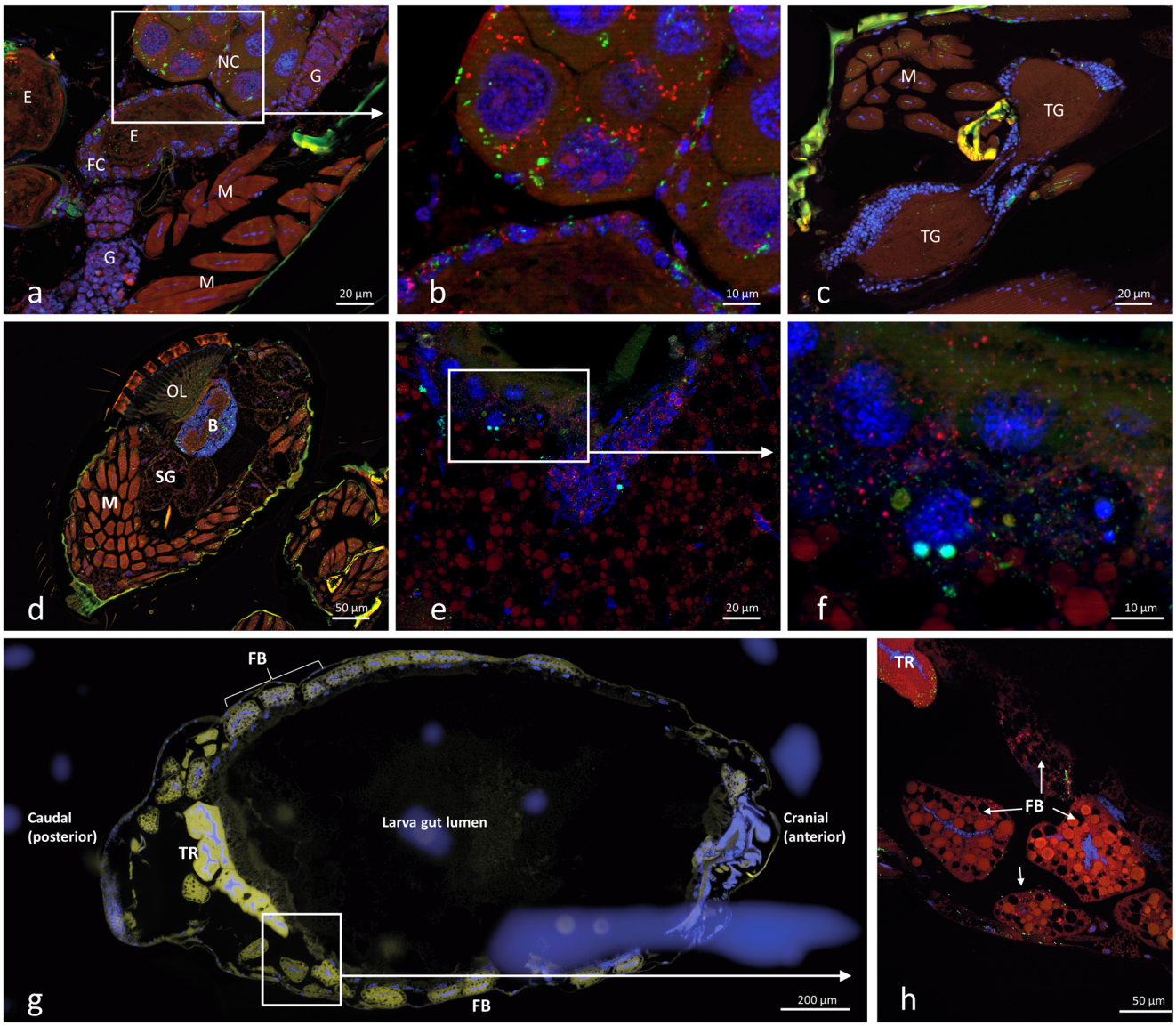


**Fig. 4** Localization of *Wolbachia* (red signals) and *Rickettsia* (green signals) in various organs and cells of *S. endius*. Cells nuclei are stained by DAPI (blue-purple). **a** Both symbionts can be seen in the females' reproductive tissues: eggs (E), follicle cells (FC) surrounding the eggs, nurse cells (NC, note the typical big nuclei), and the germarium (G). *Rickettsia* signal can be seen also in the abdominal muscles (M); **b** Higher magnification of the area marked in **a**; **c** *Rickettsia* signals in thoracic ganglion (TG) and surrounding muscles (M) in *S. endius* female; **d** Head and prothorax of *S. endius* female. *Rickettsia* signals can be seen in the optic lobe (OL), brain (B), salivary glands (SG), and muscles (M); **e** Nurse cells of *S. endius* female pre-pupa; **f** Higher magnification of the area marked in **e**; **g** Longitudinal section in *S. endius* larva. FB-fat body, GC-gland cells; **h** Higher magnification of the area marked in **g**. *Rickettsia* and *Wolbachia* signals in fat body (FB) cells and trophocyte (TR) of *S. endius* larva

the association of *Rickettsia* with *S. endius* is relatively recent [11]; (3) *Wolbachia* induces CI, *Rickettsia* does not induce any reproductive manipulation (current study); (4) *Rickettsia* slightly slows down the development rate of *S. endius* (current study); (5) no fitness advantages of *Rickettsia* were found (current study); and (6) *Wolbachia* is localized only in the ovaries of *S. endius*, whereas *Rickettsia* is distributed in multiple organs and tissues (current study). Taken together, we conclude that *Wolbachia* has spread in the population, until fixation, by means of CI. Next, one or a few individuals of *Wolbachia*-infected *S. endius* acquired *Rickettsia* (in our field samples, we found no *S. endius* that harbored *Rickettsia* only), possibly from a fly host with a transstadial infection of *R. felis* or a closely related *Rickettsia*. As many other species in this genus, *Rickettsia* has the ability to disperse in various tissues of the host, and has managed to infect the host's germ line, hence securing its transmission to the following generations. This step is not trivial, as evidenced by a study on a whitefly's parasitoid, in which the whitefly's *Rickettsia* infected the parasitoid during its larval development and subsequently migrated to the ovaries, but has reached a dead end because it failed to penetrate to the oocytes [56]. *Rickettsia* does not manipulate the reproduction of *S. endius*, but it may spread in the population by utilizing *Wolbachia*'s CI. In populations where *Wolbachia* has already reached fixation, *Rickettsia* may spread further either by horizontal transmission and/or by conferring fitness benefits that were not investigated here, such as resistance to biotic or abiotic factors.

To summarize, our study demonstrates the complexity of host-symbiont and symbiont-symbiont interactions, highlights the importance of studying these interactions for applied biocontrol programs, and sheds a light on the role of symbionts on this ecologically and economically important parasitoid.

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**Author contributions** All authors prepared and collected data. Data analysis was done by Benjamin Weiss, Martin Kaltenpoth, and Elad Chiel. The first draft of the manuscript was written by Elad Chiel, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Compliance with ethical standards

**Conflicts of interest** The authors declare that they have no conflict of interests.

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