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Cytological analysis of cytoplasmic incompatibility induced by *Cardinium* suggests convergent evolution with its distant cousin *Wolbachia*

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Cytoplasmic incompatibility (CI) is a conditional sterility in numerous arthropods that is caused by inherited, intracellular bacteria such as *Wolbachia*. Matings between males carrying CI-inducing *Wolbachia* and uninfected females, or between males and females infected with different *Wolbachia* strains, result in progeny that die during very early embryogenesis. Multiple studies in diploid (*Drosophila*) and haplodiploid (*Nasonia*) insects have shown that CI-*Wolbachia* cause a failure of the paternally derived chromatin from resolving into distinct chromosomes. This leads to the formation of chromatin bridges and other mitotic defects as early as the first mitotic division, and to early mitotic arrest. It is currently unknown if CI-inducing symbionts other than *Wolbachia* affect similar cellular processes. Here, we investigated CI caused by an unrelated bacterium, *Cardinium*, which naturally infects a parasitic wasp, *Encarsia suzannae*. CI crosses in this host–symbiont system resulted in early mitotic defects including asynchrony of paternal and maternal chromosome sets as they enter mitosis, chromatin bridges and improper chromosome segregation that spanned across multiple mitotic divisions, triggering embryonic death through accumulated aneuploidy. We highlight small differences with CI-*Wolbachia*, which could be due to the underlying CI mechanism or host-specific effects. Our results suggest a convergence of CI-related cellular phenotypes between these two unrelated symbionts.

1. Background

Numerous arthropod species, particularly insects, carry vertically transmitted, intracellular bacterial symbionts that manipulate reproductive developmental processes of their hosts in ways that enhance symbiont transmission. By far the most common symbiont-induced reproductive manipulation is cytoplasmic incompatibility (CI). This form of sterility, best characterized in *Wolbachia* (α-Proteobacteria), results when infected males mate with uninfected females (unidirectional CI) or when mates harbour different strains of *Wolbachia* (bidirectional CI) [1]. The current understanding is that male chromosomes are marked during male gametogenesis to cause a lethal paternal effect (as *Wolbachia* are not present in the sperm). Paternal chromosomes are rescued in the egg if *Wolbachia* of a similar type are present in the egg cytoplasm (reviewed by [2,3]). The understanding of the molecular mechanism of *Wolbachia*-induced CI has been recently improved [4,5], supporting the modification–rescue framework, with two CI-inducing genes that appear to work together causing modification that can be rescued by CI-*Wolbachia*-infected host females.

Initial cytological studies of CI performed in *Wolbachia*-infected *Nasonia vitripennis* (jewel wasp) embryos revealed a tangled paternal chromatin mass next to the female chromosomes during metaphase of the first mitosis [6]. This abnormal mitotic behaviour in *Nasonia* was subsequently confirmed [7] and attributed to improper condensation of the paternal chromatin. Subsequently, mitotic defects were observed in the cleavage products of *Wolbachia*-infected *Drosophila simulans* embryos, such as the formation of a chromatin bridge during the first cleavage division (CD) and smaller, condensed chromatin bodies, presumably breakdown products resulting from defective subsequent cleavage divisions [8]. Later work confirmed the improper condensation of paternal chromosomes, causing some fragments to become incorporated into daughter nuclei (DN) and extensive chromosome bridging during anaphase in the first and later cleavage divisions [9–12].

The mitotic defects caused by *Wolbachia*-induced CI affect development in an organism-dependent manner. In diploid species, CI leads to early embryonic developmental arrest. By contrast, in haplodiploid organisms including *Nasonia*, CI results in either haploid male embryos (male development) or embryonic lethality (female mortality [13,14]). In *N. vitripennis*, defects in the male-derived pronucleus (PN) are so severe that the paternal chromosomes do not segregate, or segregate to one daughter nucleus and embryonic development proceeds from the remaining normal haploid nucleus at the end of the first mitotic division [15]. Embryonic lethality in *Drosophila* and *Nasonia* results from interference of the defective paternal chromatin with mitotic progression of the viable maternal chromosomes, with consequent aneuploidy in all cleavage nuclei [12,15]. The fact that CI-embryos from *Drosophila* and *Nasonia* exhibit very similar mitotic defects suggests that *Wolbachia* may be disrupting the same host targets that are conserved between these two organisms.

The entirety of what is known regarding the cellular basis of CI stems from studies on *Wolbachia*–host systems. Interestingly, *Cardinium*, an unrelated bacterial symbiont in the Bacteroidetes that appears to infect 9% of arthropods [16], also causes CI [17–19]. *Cardinium* strains, like *Wolbachia* strains, can elicit a range of effects on host development in addition to CI, including parthenogenetic female development [20,21] and feminization of genetic male embryos [22,23]. The sequencing of the genome (*c*Eper1) of a CI-inducing *Cardinium* strain infecting the parasitic wasp *Encarsia pergandiella*, now known as *Encarsia suzannae* [24], found little homology and no evidence of lateral gene transfer between these two symbionts [25], despite records of co-infections in other species [18,26,27]. This lack of homology between *Wolbachia* and *Cardinium* genomes indicates CI has arisen independently in these two bacterial lineages [25]. A compelling question, then, is how similar are the underlying cellular and molecular mechanisms of CI-induced by these bacteria? In *E. suzannae* infected by CI-causing *Cardinium*, CI-affected embryos do not hatch into larvae, but the cytological mechanism and specific developmental window of *Cardinium*-induced mortality has remained unknown [17].

Here, we performed the first detailed cytological analysis of CI-*Cardinium*-affected embryos in *Encarsia*. Our work suggests that *Cardinium*-induced CI results from mitotic defects occurring during the first and subsequent mitotic divisions. There is a large degree of overlap in the specific cytological phenotypes of CI between *Cardinium* and *Wolbachia* (e.g. mitotic asynchrony, chromatin bridging), with some differences (e.g. absence of mortality at the first mitotic division in *Cardinium*), pointing to a possible convergence of *Wolbachia* and *Cardinium* on the same targeted pathway(s) in their respective hosts.

2. Material and methods

(a) Insect cultures

We used laboratory cultures of *Cardinium*-infected and uninfected *E. suzannae*. The infected line is fixed for *Cardinium* and was originally collected from its natural whitefly host *Bemisia tabaci* in the Rio Grande Valley in Texas in 2003. An uninfected line was obtained by curing adult wasps of a subpopulation of the infected line with 50 mg ml^{-1} rifampicin in honey for three generations. Both cultures were maintained on *B. tabaci* reared on cowpea plants (*Vigna unguiculata*) in a climatic chamber at 27°C, 60–70% relative humidity and 16 L : 8 D photoperiod. *Encarsia suzannae* has an unusual biology; only diploid female eggs are laid in whiteflies (male eggs are laid in parasitoid pupae developing within the whitefly [28]). All eggs examined in this study were dissected from whiteflies and were therefore diploid, incipient females.

(b) Egg collection

Three types of matings were set up in glass vials: the CI cross between infected males and cured females of *E. suzannae*, and two control crosses, cured males × cured females and infected males × infected females. Cytological events of embryogenesis were studied from the eggs taken from small oviposition arenas prepared by laying a leaf disc hosting third and fourth instar nymphs of whiteflies into a Petri dish (50 mm diameter) containing a moist filter paper disc. Several mated *E. suzannae* females were allowed to oviposit under observation for about 30 min, parasitized whiteflies were marked and the time of each oviposition recorded on a map of the whiteflies in the dish. After removing the parasitoids, the wasp eggs were left to develop by incubating arenas at room temperature (25 ± 2°C) until shortly before the chosen fixation time. Each hydropic egg, measuring 70–80 µm at oviposition [29,30], was extracted from whitefly nymphs by dissection under a binocular microscope and immediately fixed. Varying the incubation time yielded embryos that ranged in age from 15 min to 96 h post-oviposition, although we focused, in particular, on the first 24 h of embryo development.

(c) Cytological technique

Eggs were dissected from nymphs on a microscope slide in 50 µl of tris-buffered saline (TBS), and, in order to remove most of the whitefly debris, subsequently transferred to 50 µl of 3.7% formaldehyde in TBST (TBS plus Tween 20) for fixation, using a glass Pasteur pipette that had been pulled to a narrow capillary end. The slide with the drop of fixative was then placed in a humid chamber for 1 h, followed by three washes in TBST at 5 min intervals with fluid removed by either a slightly moist twisted Kimwipe (Kimtech Science) or the modified pipette. After the third wash, eggs were stained with 1 µg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole) (Molecular Probes) in TBST for 5 min and washed three more times in TBST as above. Each stained egg was mounted on a Superfrost Plus slide (Fisher Scientific) or on a slide previously rubbed with siliconized paper (Bausch and Lomb). The mounting medium was a solution of 80% glycerol, 20% TBST with 2% *n*-propyl-gallate (Sigma), and the coverslip was sealed with clear nail polish. Slides were then stored at 4°C in the dark for at least one day to allow sufficient stain penetration.

(d) Confocal microscopy

Meiosis and early embryonic development of DAPI-stained eggs were imaged by optically sectioning embryos using a laser scanning confocal inverted microscope, either a Zeiss 510 LSCM or Nikon C1si. DAPI fluorescence was generated with a 405 nm excitation beam, using either a 40× dry objective or a 63× immersion oil objective at 1 to 2× zoom.

(e) Data analysis

Stacked images and three-dimensional videos of the different optical sections were obtained from multifocal Z-stack imaging using the microscopes' native software: Zeiss's LSM Image Browser and Nikon's EZ-C1. Images were then edited and assembled using GIMP. Cytological events occurring during embryo development were compared between CI crosses and cured and infected compatible crosses.

3. Results

(a) Normal development of compatible embryos

We observed embryogenesis in 102 eggs from the control crosses (infected female × infected male and cured female × cured male), most in stages before the end of the fourth CD. We found no difference in meiosis and early embryonic development between the two control crosses. By 50–70 min after egg deposition (AED), the second division of meiosis produced four haploid nuclei. One of these products became the haploid female PN that moved towards the interior of the egg's cytoplasm, while the other three products formed haploid polar bodies (PB), remaining at a position near the plasma membrane (figure 1*a*,*b*). Simultaneously, the sperm nucleus changed from a needle-like shape to become more ovoid and decondensed, and became the paternal PN. By 75–90 min AED, the male PN had migrated towards the female PN in the middle of the egg. By 01.30–01.50 h AED, the two PN in prophase were physically apposed (figure 1*c*,*d*), forming the synkaryon (figure 1*e*), while the PB coalesced into a single polar body derivative (PD) (figure 1*c*). At the end of metaphase through early anaphase of the first CD, four groups of chromosomes, two from each PN, were visible (figure 1*e*). The first CD was completed by 02.15–02.30 h AED (figure 1*f*,*g*), ultimately resulting in two DN and two PDs arising from the division of the first one. The second CD was completed by 03.00–03.30 h AED, resulting in four DN, while the two PDs did not undergo a further division (figure 1*h*,*i*). Within 04.00–04.30 h AED, the third CD resulted in eight DN, while the two PDs remained apparently inactive (figure 1*j*). The DN at the pole close to the PDs then lost synchrony with the other DN, so that at the end of the fourth CD (05.00–05.30 h AED) there were 14 DN and one apical blastomere (AB), a mitotic product that loses synchrony with the other DN and does not contribute to the formation of the multinucleated embryo (blastula) [30]. The AB completed a division 01.00–01.30 h later, forming two ABs, while the other 14 DN approached the prophase of the fifth CD. At the same time, the two PDs underwent polyploidization, each of them giving rise to a giant nucleus (figure 1*k*–*n*). Later observations of infected embryos at 24 and 48 h AED showed a blastula surrounded by big extra-embryonic nuclei stretched against the chorion (figure 1*o*). These giant nuclei, forming an extra-embryonic membrane, are known as teratocytes and have been found in the yolkless (hydropic) eggs of many families of Hymenoptera [30]. The normal development of compatible embryos that we observed in *E. suzannae* closely matched the embryonic development of the sibling species *Encarsia gennaroi* (*E. pergandiella* in [30]), to which the reader is referred for a detailed description of embryonic development in *Encarsia*, and the normal development of the sibling species *Encarsia marthae* (*E. pergandiella* in [31]).

(b) Abnormal development of cytoplasmic incompatibility embryos

We observed 112 eggs from the incompatible cross (cured female × infected male), representative of each stage of embryonic development until the end of the fifth CD (7–9 h AED) and of later embryonic development occurring at 16–96 h AED. As about 70% of progeny die in the CI cross [32], we expected a minority of eggs to exhibit normal embryogenesis. For all eggs examined, events leading to the apposition of female and male PN (figure 2*a*) did not differ between control and CI-embryos. Like eggs from control crosses, meiosis was completed by 60–75 min, and in the same time frame, the sperm underwent apparently normal modifications. At pronuclear apposition (90–120 min), the two PN seemed to be at the same condensation level during prophase as in the control embryos (figure 2*b*). Aberrant mitotic events were observed immediately following synkaryon formation (02.00–02.10 h). During prometaphase and metaphase (02.10–02.40 h AED) chromosomes of one PN appeared less condensed than the other (figure 2*c*,*d*) and at the end of the first CD (02.40–03.00 h) one set of chromosomes lagged behind the two DN (figure 2*e*), often along with the formation of a chromatin bridge between the two DN (figure 2*f*). Occasionally along with, or instead of, bridge formation, we observed misshapen DN at the end of mitosis (figure 2*g*), probably a result of bridge formation. Chromatin bridges and misshapen nuclei are both considered products of improper chromosome segregation [15]. Overall, of the 28 eggs observed during the first CD, only two did not show any mitotic defects.

During the second CD, which was completed at 03.15–03.40 h AED, we observed more mitotic defects, including improper segregation of one set of chromosomes, chromatin bridges and/or small, condensed chromatin bodies (figure 2*h*–*k*). Abnormal segregation and asynchrony of one chromosome set continued in the third CD (04.20–05.30 h AED). At this stage, there would normally be eight DN and two PDs, but we sometimes observed fewer than eight DN, as some nuclei may have stopped dividing. In addition, we recorded many small, condensed chromatin bodies resulting from irregular divisions, chromatin bridges sometimes connecting several pairs of sister nuclei, and misshapen nuclei with a distinct peduncle (figure 2*l*–*o*). However, the severity of the aberration was variable, with instances of normal numbers of DN and only slight defects, like some misshapen nuclei or a few unsegregated chromosomes scattered in the egg cytoplasm (figure 2*l*,*m*). In the fourth CD (05.30–07.00 h AED), only 10% of observed embryos reached the 17–18 nuclei stage (14 DN + 1–2 AB + 2PDs) seen in the compatible control embryos without apparent mitotic defects (figure 2*p*). The remaining 90% included either embryos with the normal number of nuclei and small mitotic defects or embryos with

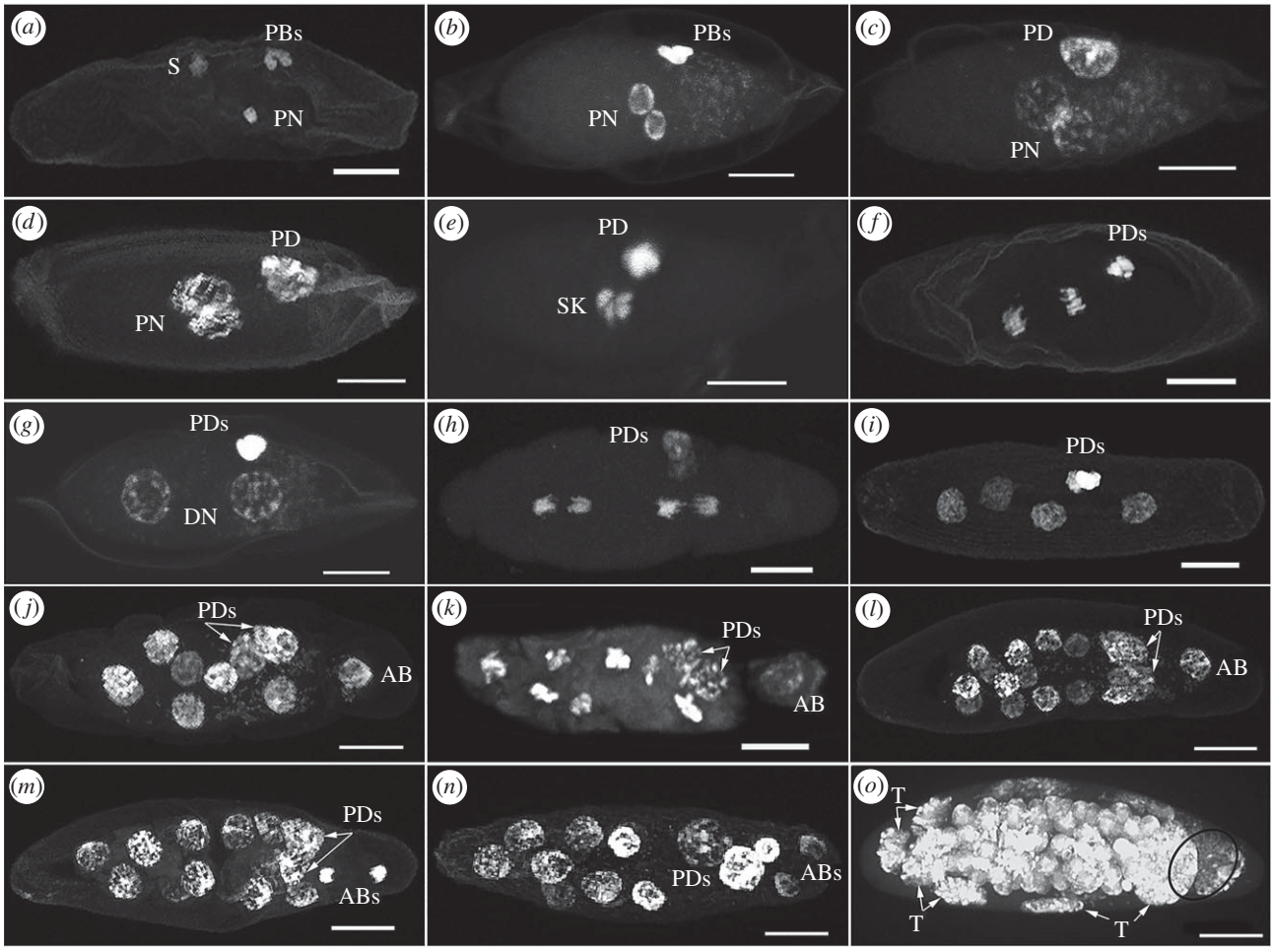

Figure 1. Normal embryo development of *E. suzannae* in compatible crosses. (*b*,*c*,*g*,*j*,*l*,*m*,*n*,*o*) Eggs of infected females mated with infected males, containing the bacterial endosymbiont *Cardinium* (bright small dots). (*a*,*d*,*e*,*f*,*h*,*i*,*k*) Eggs of cured females mated with cured males. (*a*) Fertilized egg at the end of meiosis, containing the female pronucleus (PN) and three polar bodies (PBs) and the peripheral sperm nucleus (S). (*b*) Female and male pronuclei approaching at interphase, three PBs. (*c*,*d*) Apposition of female and male pronuclei at prophase of the first cleavage division (CD), one polar body derivative (PD) is occurring. (*e*) First CD: synkaryon (SK) at the end of metaphase–early anaphase with four groups of chromosomes, a pair from each pronucleus. (*f*) First CD, end of anaphase and two PDs. (*g*) First two daughter nuclei (DN) and two PDs. (*h*) Anaphase of second CD and two PDs. (*i*) At the end of second CD, there are four DN. (*j*) End of third CD: eight DN of which one migrating to the pole close to the two PDs forms the apical blastomere (AB). (*k*) Fourth CD: seven DN in metaphase while the asynchronous AB is still in interphase, the two PDs polyploidize. (*l*) End of fourth CD: 14 DN, 1 AB in prophase and two PDs. (*m*) End of fourth CD: 14 DN, the AB at the end of anaphase, two PDs. (*n*) End of fourth CD: 14 DN, two ABs and two PDs. (*o*) Embryo after 24 h surrounded by stretched polyploid nuclei (teratocytes: T) forming the extra-embryonic membrane and with a high density of *Cardinium* bacteria near the right (posterior) pole in the black circle.

severe aberrations. In general, the defects observed consisted of different condensation levels among nuclei, abnormal segregation of chromosomes, irregularly shaped nuclei, small condensed chromatin bodies or chromatin bridges (figure 2*q*,*r*). Similarly, at the fifth CD (07.00–09.00 h AED), we found aborted embryos and a few developed embryos with slight defects (figure 2*s*–*t*). In eggs fixed at 16–20 h AED, all embryos were defective, with 42% showing normal development (multinucleated blastula surrounded by big stretched extra-embryonic nuclei) associated with slight mitotic defects consisting of one to four very dense chromatin bodies of much smaller size than the blastula nuclei (figure 2*u*). In the remaining 58% of embryos, the deleterious effects of the accumulation of mitotic defects in previous divisions resulted in aberrant embryos that did not complete normal development (figure 2*v*,*w*). Even in these later embryos, we could sometimes observe chromatin bridges (figure 2*w*). Eggs imaged from 48 to 96 h from oviposition did not complete embryogenesis (figure 2*x*), and in only one case did we observe a first instar larva at 96 h (they normally hatch within 48 h). The frequencies of anomalies at different developmental stages remained fairly constant throughout development, suggesting that all mitotic defects follow from what happened at the first mitosis, following pronuclear apposition, but not necessarily as a result of chromatin bridging, which was evident only in 28% of defective embryos (table 1).

Therefore, *Cardinium* causes widespread mitotic defects that are nearly identical to those caused by *Wolbachia*, but unlike *Wolbachia*, the defects span a much wider range of cleavage divisions, leading not to early (i.e. at first CD) but to late embryonic death, probably through the accumulation of mitotic defects in the first several divisions.

4. Discussion

Our results showed a remarkable resemblance of cellular phenotypes caused by CI-*Cardinium* (Bacteroidetes) to the distantly related *Wolbachia* (α-Proteobacteria). Hallmarks of *Wolbachia*-induced CI in *Drosophila* and *Nasonia* parasitic wasp species are the asynchrony of the paternal and maternal

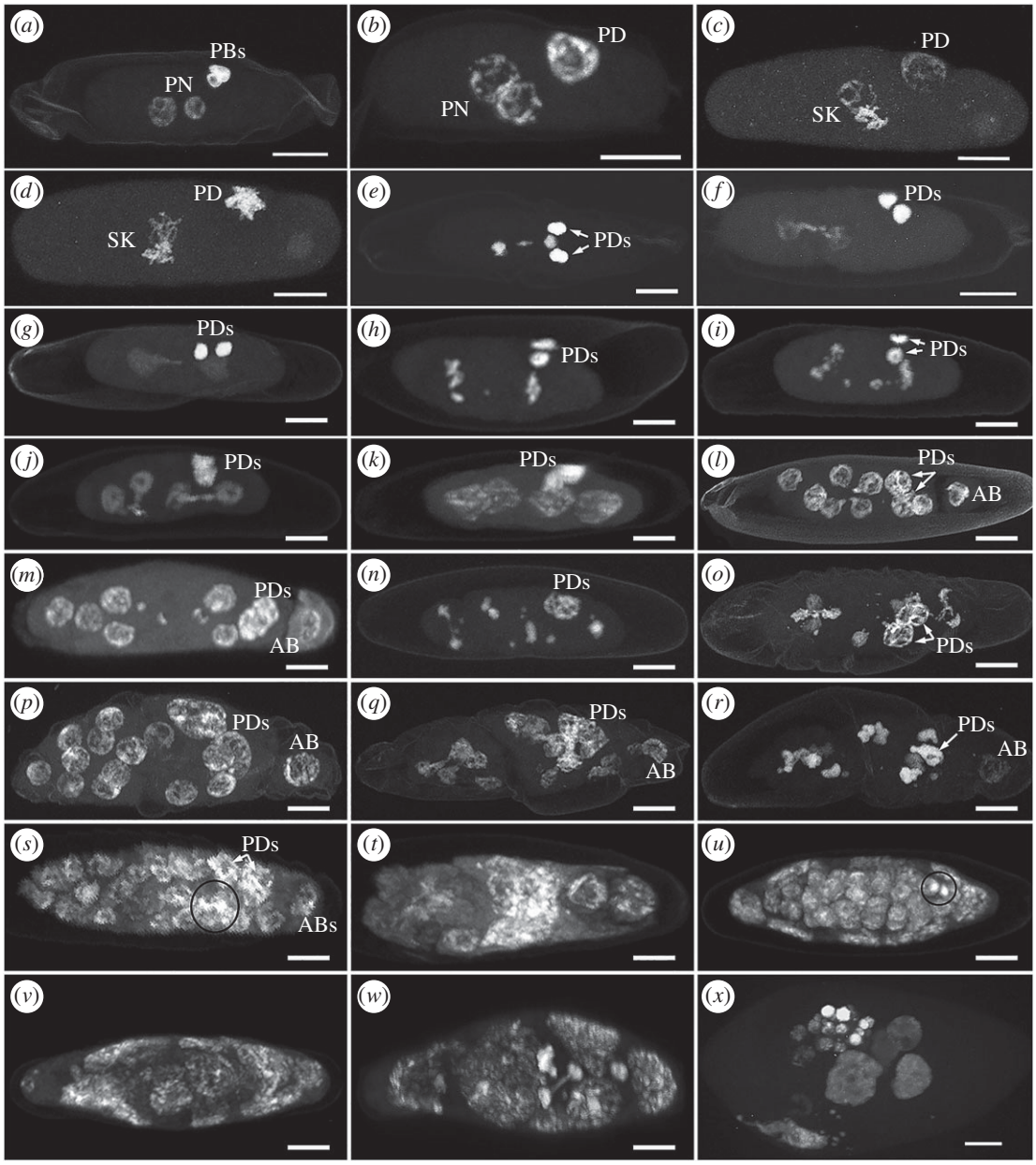

Figure 2. Abnormal embryo development of *E. suzannae* in CI crosses. (*a*) Fertilized egg containing the male pronucleus (PN) approaching the female PN and three polar bodies (PBs). (*b*) Apposition of female and male PN showing synchronous prophase, one polar body derivative (PD). (*c*,*d*) At prometaphase (*c*) and metaphase (*d*) one PN is less condensed than the other one, and the PD is dividing. (*e*) At the end of telophase, one set of chromosomes lags between the segregating maternal chromosomes and two PDs occur. (*f*) Irregularly segregating chromosomes form a bridge between the first two daughter nuclei (DN). (*g*) Misshapen first two DN and two PDs much more condensed. (*h*) Slightly aberrant second cleavage division (CD): the two DN are in metaphase and only one chromosome lags between them. (*i*) Highly aberrant second CD: many chromosomes do not segregate correctly lagging between the DN or are scattered in the egg cytoplasm. (*j*,*k*) Highly aberrant second CD: the two pairs of sister DN are connected by a chromatin bridge (*j*) and the resulting four DN are misshapen (*k*). (*l*,*m*) Third CD results in the normal number of CN (eight) but misshapen (*l*) or unsegregated chromosomes lag in the egg cytoplasm (*m*), the apical blastomere (AB) is differentiated. (*n*,*o*) Third CD aborted: unsegregated chromosomes lag in the egg cytoplasm and nuclei are connected by chromatin bridges; four nuclei connected in (*o*). (*p*) Normal embryo development at the end of the fourth CD: 14 DN, one AB and two PDs. (*q*) Irregular fourth CD producing 14 DN, misshapen or connected by chromatin bridges. (*r*) Aborted fourth CD: highly condensed and some less condensed groups of chromosomes, and unsegregated chromosomes scattered in the egg cytoplasm. (*s*) The end of the fifth CD: highly condensed chromatin bodies (inside the black circle) co-occur with nuclei of regular shape. (*t*) At the end of the fifth CD, embryonic development arrested, only few nuclei are clearly recognizable and largely diffuse chromatin is visible. (*u*–*w*) At 16–20 h from oviposition a percentage of the embryos appear normally developed and surrounded by the polyploid teratocytes forming the extra-embryonic membrane, nevertheless bearing the products of defected mitosis like few small condensed chromatin bodies, here inside the black circle (*u*); in most of the embryos, development has stopped, showing only few nuclei (*v*) or fragmented nuclei originating many condensed chromatin bodies (*w*) together with diffuse chromatin, while peripheral teratocytes are still clearly visible. (*x*) Embryo in which development has arrested, observed at 72 h after egg deposition: within the hydropic egg, a few small nuclei and large chromatin masses occur.

chromosome sets as they enter into mitosis, and abnormal chromosome segregation, usually revealed by chromatin bridging, which leads to the formation of aneuploid nuclei during early embryonic development [2,3]. Here, we showed that *Cardinium* also causes such phenotypes, although the prevalence of chromatin bridges seems to be somewhat lower than in *Wolbachia*-mediated CI. Owing to the lack of chromosomal differences between the sexes in Hymenoptera, our current experimental approach does not allow us to distinguish between the paternal and maternal

Table 1. Frequency of mitotic defects (md), in general, and of chromatin bridging (cb), in particular, over the first five mitotic divisions and subsequent divisions up to 96 h from oviposition in incompatible embryos. *n*, number of embryos observed; AED, after egg deposition.

mitotic division																			
1st			2nd			3rd			4th			5th			greater than 5th (16–96 h AED)				
n	md	cb	*n*	md	cb	*n*	md	cb	*n*	md	cb	*n*	md	cb	*n*	md	cb		
28	26	8	8	7	3	15	13	3	11	9	3	9	9	2	29	28	5		

chromosome sets. Genetic studies in CI-*Wolbachia*-influenced *N. vitripennis* embryos have shown that it is the paternal genome that is excluded from the developing embryo, as haploid males resulting from CI-embryos express maternal but not paternal markers [6,7]. Molecular markers of paternal chromatin confirmed *Wolbachia* CI-induced defects are limited to paternal chromosomes in *Drosophila* [33]. It is highly likely that the paternal chromatin is the one affected also in CI-*Cardinium*-affected embryos in *Encarsia*, as the hypercondensation of one of the two nuclei is very consistent with *Wolbachia*-induced CI in all *Drosophila* and *Nasonia* species examined thus far [11,15]. In *E. suzannae*, the expression of *Cardinum*-induced CI matches the phenotypic expression of *Wolbachia*-induced CI, that is, sterility when an uninfected female crosses with an infected male, and fertility recovery when an infected female mates with an infected male [16]. In *Drosophila* and *Nasonia* systems, this effect is driven by *Wolbachia* products that mark male chromosomes during gametogenesis, causing mitotic abnormalities and arrest during early embryogenesis and rescue of paternal chromosomes in the egg infected by *Wolbachia* of a similar type [4,5]. Similarly, it is highly unlikely that in the unidirectional CI of our system the modification mechanism targets the female nucleus and spares the male nucleus, making our hypothesis that the paternal set is the one affected the most plausible at this time. However, this hypothesis needs to be tested in future experiments.

The first mitosis in insects is unique, because the female and male pronuclei do not fuse upon apposition but stay on two separate regions of the metaphase plate, and enter independently into anaphase [12,34]. It is thus essential that the female and male pronuclei enter and exit mitosis synchronously to successfully produce DN that contain both maternal and paternal chromosomes. Instead, in *Encarsia* embryos affected by CI-*Cardinium*, chromosome condensation of the two pronuclei is defective, just as it is in both *Drosophila* and *Nasonia* infected by *Wolbachia* [12,35]. Although similar phenotypes could have different genetic bases, the striking similarity of pronuclear asynchrony and abnormal chromosome segregation caused by both symbionts suggests that, despite their phylogenetic distance and evidence of independent evolution of CI [25], *Wolbachia* and *Cardinium* affect mitotic progression in similar ways during very early embryo development, possibly aiming at conserved molecular targets in their hosts.

Despite the similar cellular phenotypes between these two symbiont–host systems, we also observed several differences. *Wolbachia*-induced incompatible crosses in *Drosophila* cause the arrest of almost 50% of embryos during very early mitosis, with the remaining part arrested at the syncytial blastoderm stage or before hatching [11]. In haplodiploids, the most common phenotype of *Wolbachia*-induced CI is the arrest of embryo development as early as the first mitotic division in *N. longicornis* and *N. giraulti*, while in *N. vitripennis* more variants are observed due to the possible expression of both types of CI (male development and female mortality) [15]. However, in *Encarsia*, most of the *Cardinium*-induced CI-embryos do not arrest during the first mitotic division. In fact, the mitotic anomalies we observed during the first mitosis did not prevent embryos from progressing in their development. We also did not observe other phenomena seen in *Wolbachia* CI in *Nasonia*: embryos with two morphologically normal nuclei and one

highly condensed genome that does not segregate, or embryos where one chromosome set segregates to only one daughter nucleus, which in *N. vitripennis* results in normal development of haploid males [15]. The absence of such embryos provides cytological evidence that *E. suzannae* does not have the male development type of CI, confirming the evidence of crossing experiments [17]. The lack of abnormal embryos that will develop as males signals a difference from the *Wolbachia*–*Nasonia* system, where the two species with predominantly female mortality type of CI (*N. longicornis* and *N. giraulti*) also have a few such 'male development' embryos [15]. However, like the *Cardinium*–*Encarsia* system, 30% of the *N. giraulti* CI-embryos were found to reach the blastoderm stage with evidence of aneuploid cells [14].

Recently, the understanding of the molecular mechanism of *Wolbachia*-induced CI has been substantially improved [4,5]. Homologous genes to the 'CI genes' identified in *Wolbachia* are absent in *Cardinium*, and the conserved domains of these genes across taxa are still not characterized [5]. Given that the molecular mechanism of CI-*Cardinium* is probably different, the observed phenotypic differences could be due to functional disparity in how these two symbionts cause CI. However, the differences seen could also be attributed to symbiont density or host-species effects. Defective embryos that develop as males in *Nasonia* are thought to be associated with the highest levels of *Wolbachia* modification and potentially density-dependent, with higher titres of the symbiont resulting in complete condensation and exclusion of the paternal set in embryogenesis [15]. The observed differences in cytological events between *Wolbachia*- and *Cardinium*-induced CI could also be influenced by interactions of the symbiont with particular host species, as is well known for *Wolbachia* CI in different hosts [36].

CI in *E. suzannae*, as well as in other systems with CI-*Cardinium*, is often incomplete, with egg hatching reduced but not eliminated [17,18,26]. We speculate that the disparity in the overall frequency of embryos with mitotic defects (mild or severe) observed here (90%) and the known lethality of CI in *E. suzannae* (73%) [32] may result from the likelihood that a small portion of embryos can survive mild mitotic defects. In such embryos, the affected nuclei may be those not contributing to the blastula formation. It has been demonstrated that *Drosophila* embryos can undergo substantial mitotic defects and still survive to adulthood [37,38].

The prevailing model for *Wolbachia*-induced CI is that *Wolbachia* modify sperm chromatin during spermatogenesis and these modifications render the sperm unable to successfully participate in embryonic development. It has been hypothesized that the fate of the paternal genome during the first mitosis of the zygote is determined by the extent of *Wolbachia* modification present in the sperm [2,39]. Thus, sperm nuclei that have been strongly altered by *Wolbachia* may produce chromosomes that will not segregate, resulting in embryos that will develop as haploid males, whereas sperm nuclei that have been only moderately modified may produce chromosomes that mis-segregate, resulting in detrimental aneuploidy. It would be interesting to know if this model also holds for *Cardinium*. If so, the sperm nuclei mis-segregate, allowing the embryo to survive for a time while ensuring it ultimately dies from the accumulated insults of incomplete segregation.

Data accessibility. All data necessary to replicate the experiments have been provided. The best images of cytological events have been selected. However, additional images are available upon request to the corresponding author.

Authors' contributions. M.S.H. conceived and coordinated the study; M.Ge., M.Gi., S.E.K., M.S.H., P.M.F. participated in the design of the study; M.Ge., S.E.K., M.R.D. carried out the experiments; M.Ge., M.Gi. analysed the data; P.M.F., M.S.H. helped with interpretation of the data; M.Ge., M.Gi., M.S.H. drafted the manuscript; P.M.F. revised it critically for important intellectual content. All authors commented on drafts of the manuscript and gave final approval for publication.

Competing interests. We declare no conflicting or competing interests.

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