

1 *Wolbachia cifB* induces cytoplasmic incompatibility in the malaria mosquito

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9

10 **Abstract:**

11 *Wolbachia* infections are a fascinating example of reproductive parasitism with strong potential to
12 combat vector-borne diseases, due to their combined ability to spread in insect populations and
13 block pathogen replication. Though the *Wolbachia* factors mediating the notable reproductive
14 manipulation cytoplasmic incompatibility (CI) have now been identified as prophage WO genes
15 *cifA* and *cifB*, the relative role of these genes is still intensely debated, with different models
16 claiming that CI requires either both factors or *cifB* alone. Here we investigated whether *cifA* and
17 *cifB* are sufficient to induce conditional sterility in the major malaria vector *Anopheles gambiae*, a
18 species that appears to have limited susceptibility to invasion by *Wolbachia*. We report that CI can
19 be fully recapitulated in these mosquitoes, and that *cifB* is sufficient to cause this reproductive
20 manipulation. *cifB*-induced sterility is fully rescued by high levels of *cifA* expression in females.
21 Surprisingly, however, when *cifA* is highly expressed in males alongside *cifB*, the CI phenotype is
22 attenuated. *cifB* strongly impairs fertility also when expressed in the female germline, again
23 mitigated by *cifA*. These data support a system whereby *cifB* and *cifA* must be fine-tuned to
24 exercise CI and rescue, respectively, possibly explaining the limited success of *Wolbachia* at

25 invading *Anopheles*. Our findings pave the way towards facilitating *Wolbachia* infections in
26 anopheline vectors, for use in malaria control strategies.

27

28 **Introduction:**

29 *Wolbachia* endosymbionts are extremely successful insect colonizers due to reproductive
30 manipulations they inflict on their insect hosts. One such notable manipulation is cytoplasmic
31 incompatibility (CI), which is the failure of *Wolbachia*-infected males to produce viable progeny
32 when mated to uninfected females¹. Fertility is rescued in females colonized by *Wolbachia*,
33 providing them with a reproductive advantage that, when paired with maternal transmission,
34 allows these bacteria to effectively invade insect populations². Recently the factors underscoring
35 CI were identified as two genes, *cifA* and *cifB*, located adjacent to one another within WO prophage
36 regions in the *Wolbachia* genome, with homologs in all known CI-inducing *Wolbachia* strains^{3,4}.
37 Since then, the activity and interactions between CifA and CifB have been disputed in the field.
38 While it is generally recognized that *cifA* expression in the female rescues fertility, different
39 theories (described in detail elsewhere⁵⁻⁸) hypothesize that either one (CifB) or both of these factors
40 are necessary for inducing CI. The model whereby CifB is the toxin and CifA the antidote
41 (generally referred to as the toxin-antidote model) is parsimonious as it implies only one role for
42 CifA in both sexes, and is supported by *cifB* toxicity in yeast⁴. In *Drosophila melanogaster*,
43 however, transgenic expression of *wMel cifB* alone does not cause infertility, which is instead
44 induced by co-expression of *cifA* and *cifB*. This finding lends support to a requirement for both
45 genes in CI, known as the Two-By-One model^{3,7}. While the toxin-antidote model can be modified
46 to fit within a Two-By-One model framework^{5,6}, its simplest interpretation does not, and there

47 remains a lack of consensus on whether CifA is purely a rescue factor or a required accessory in
48 CI.

49 Besides its role in reproductive parasitism, *Wolbachia* has attracted considerable attention for the
50 control of vector-borne diseases due to the capacity of some strains to combine CI with strong
51 pathogen-blocking effects. This is witnessed by large control programs based on the release of
52 *Wolbachia*-infected mosquitoes to reduce transmission of dengue and other arboviruses by *Aedes*
53 mosquitoes⁹⁻¹¹. Additionally, the infertility induced⁹ by *Wolbachia*-infected males when mating
54 with uninfected females can also be exploited to achieve suppression of insect populations, a
55 strategy called Incompatible Insect Technique (IIT) that is also successfully applied in field trials
56 of *Aedes* mosquitoes^{12,13}. The implementation of similar *Wolbachia*-based programs to combat
57 malaria transmission by *Anopheles* mosquitoes would be highly desirable, especially as
58 widespread insecticide resistance threatens the resurgence of this devastating infectious disease
59 ^{14,15}. Unfortunately the utility of *Wolbachia* for malaria control is limited by a paucity of stable
60 associations between *Anopheles* and these endosymbionts. Only one artificial *Wolbachia* infection
61 has been achieved in the germline of an anopheline species, where however the *wAlbB* strain in
62 *Anopheles stephensi* showed only partial rescue of CI and thus limited capacity for population
63 invasion¹⁶. Furthermore, natural *Wolbachia* infections in field populations of important malaria
64 vectors are reported sparsely and at low titers¹⁷⁻²² and there was no evidence for CI when
65 investigated²³. Such limited success at colonizing field and laboratory *Anopheles* populations has
66 prevented the use of these bacteria for reducing the burden of malaria, yet the biological
67 underpinnings of this phenomenon remain undetermined.

68 We investigated whether *cifA* and *cifB* are capable of inducing CI in *Anopheles gambiae*, the most
69 important malaria vector in Africa. After codon-optimization, we separately cloned the two Type

70 I *cif* genes from *wPip* (also referred to as *cidA* and *cidB*⁴) under the *zero population growth* (*zpg*)
71 promoter to drive expression in both male and female germlines²⁴ (**Fig. 1a**). Co-injection of *zpg-*
72 *cifA* and *zpg-cifB* constructs yielded F1 transgenics expressing either *cifA* alone, or both *cifA* and
73 *cifB* (*zpg-cifA;B*), but none that expressed *cifB* only, a suggestion that *cifB* may cause embryonic
74 toxicity alleviated by *cifA* co-expression.

75

76 **Results and Discussion:**

77 We set up crosses between *zpg-cifA;B* males and different female lines (*zpg-cifA;B*, *zpg-cifA*, and
78 wild type (WT) females), using WT males as control. In all crosses, females mated to *zpg-cifA;B*
79 males showed a striking degree of infertility (only 2–4% viable progeny) compared to controls,
80 suggestive of CI (**Fig. 1b**). The vast majority of infertile embryos were arrested early in
81 development, while a minority initiated development but did not hatch (**Extended Data Fig. 1**).
82 Embryo cytology revealed the hallmarks of CI^{3,4,25}, with most embryos showing early
83 developmental arrest, while others showed fewer nuclear divisions or were arrested later in the
84 blastoderm stage due to mitotic failures (**Fig. 1c**). Somewhat surprisingly, we did not observe any
85 significant rescue of this infertility when females expressed either *cifA* alone, or both *cifA* and *cifB*
86 (**Fig. 1b**). We also observed a minor (17%) decrease in fertility of *zpg-cifA;B* females compared
87 to their WT and *zpg-cifA* counterparts when mated with WT males (**Fig. 1b**), strengthening the
88 hypothesis of some reproductive toxicity due to *cifB*.

89 We speculated that the lack of fertility rescue by *zpg-cifA* could be due to insufficient expression
90 of *cifA* in females, as the rescue effect has been shown to be dose-dependent²⁶. To test this
91 possibility, we engineered *cifA* transgenic expression using the *vasa* promoter (**Fig. 1a**), which
92 showed considerably higher expression levels in the germline than the *zpg* promoter (**Fig. 2a**).

93 When mated to *zpg-cifA;B* males, high levels of infertility were observed in both *zpg-cifA* and WT
94 females as above, while fertility was fully restored in crosses with *vasa-cifA* females,
95 demonstrating effective rescue by this transgene (**Fig. 2b**). Combined, these results reveal that CI
96 can be entirely recapitulated in *An. gambiae* mosquitoes by transgenic expression of *cifA* and *cifB*.
97 Incidentally, we also attempted co-injections of *vasa-cifA* and *vasa-cifB* constructs (**Fig. 1a**), but
98 failed to isolate any *cifB*-expressing progeny, a further confirmation of *cifB*'s possible embryonic
99 toxicity.

100 Following our observations of potential *cifB* toxicity while establishing the transgenic lines, we
101 investigated whether *cifB* alone is capable of causing CI. Although we could not maintain a *zpg-*
102 *cifB* colony in the absence of *cifA*, we were able to isolate a limited number of F1 *zpg-cifB* males
103 from natural colony matings between heterozygous individuals. We found that *zpg-cifB* males
104 induced high infertility when mated to WT females, comparable to the infertility levels induced
105 by *zpg-cifA;B* males (**Fig. 3a**). Progeny sired by *zpg-cifA* males were instead fully fertile (**Fig. 3a**).
106 CI induction did not differ depending on whether *zpg-cifB* males were isolated from *zpg-cifA;B* or
107 *vasa-cifA;zpg-cifB* colonies (denoted (*z*)*zpg-cifB* or (*v*)*zpg-cifB*, respectively) (**Fig. 3b**). Once
108 again, *vasa-cifA* expression in females was sufficient to fully rescue sterility caused by *zpg-cifB*
109 males, ruling out CI-independent effects (**Fig. 3b**). Embryo cytology confirmed the results
110 obtained with *zpg-cifA;B* males, revealing the canonical patterns of CI (**Extended Data Fig. 2**).
111 These findings represent the first conclusive report of conditional sterility induced by *cifB* alone
112 in insects, supporting its independent role as an inducer of CI.

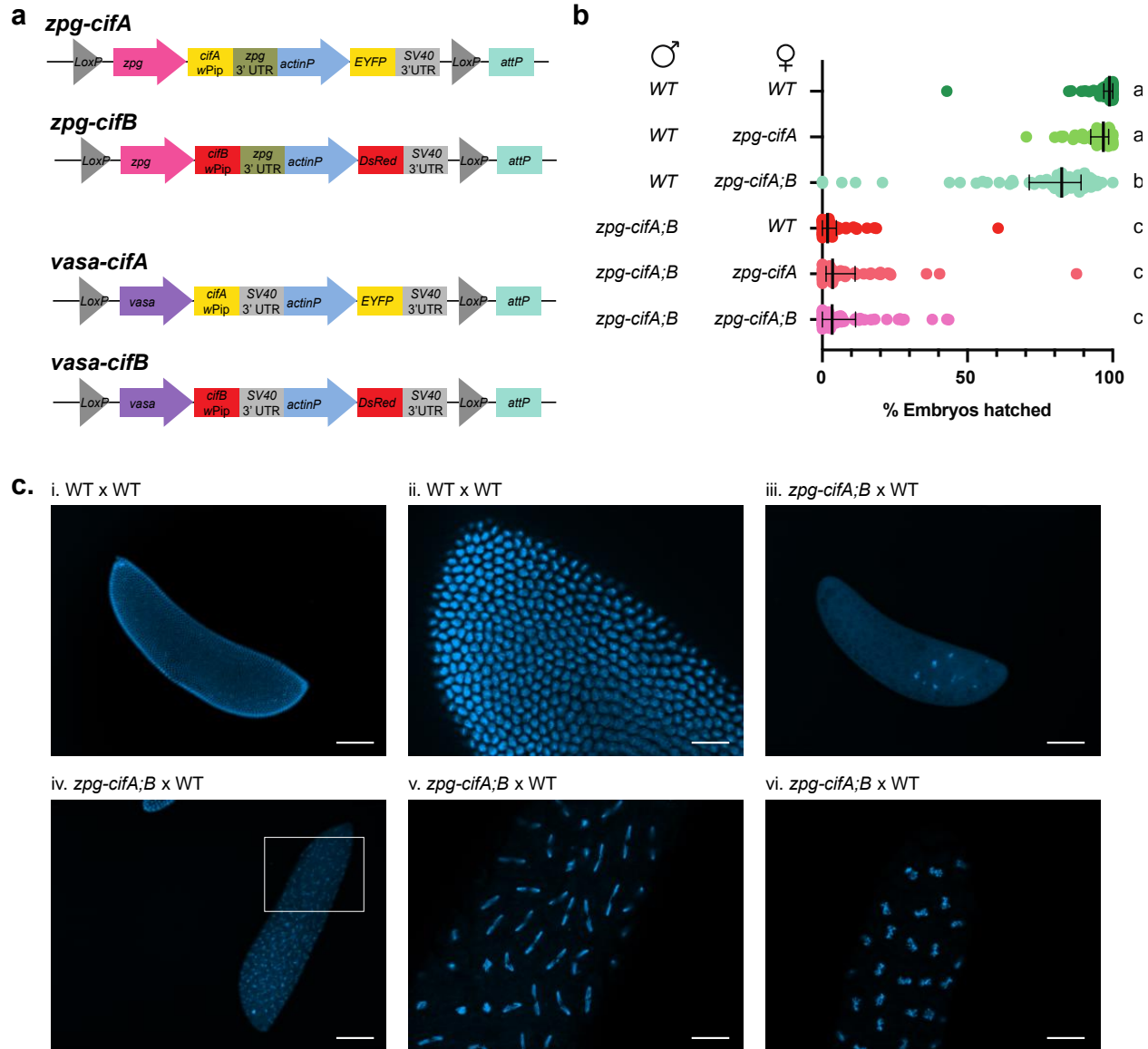
113 Given that *vasa-cifA* rescues inviability caused by *cifB* in the embryo while *zpg-cifA* does not, we
114 next asked whether expression levels of *cifA* in males may impact the strength of CI. To this end,
115 we compared fertility of crosses between males expressing either low (*zpg-cifA;B*) or high (*vasa-*

116 *cifA;zpg-cifB* *cifA* levels and WT females (**Fig. 3c**). Intriguingly, *vasa-cifA;zpg-cifB* males were
117 considerably more fertile (median of 48% hatched embryos) compared to *zpg-cifA;B* males
118 (median of 0% hatched embryos) (**Fig. 3d**), despite similar *cifB* expression levels in these male
119 groups (**Fig. 3c**). These data indicate that high expression of *cifA* in males reduces CI penetrance
120 rather than favoring it, possibly either by limiting CifB activity within the male germline, or by
121 rescuing CifB toxicity in the embryo following transfer of CifA in sperm²⁷.

122 Our finding that *cifB* expression in males is sufficient to induce significant sterility prompted us to
123 investigate toxicity of this factor in females. We designed crosses between WT males and either
124 *zpg-cifA;B* or *vasa-cifA;zpg-cifB* females (**Fig. 4a**) and then characterized egg development and
125 fertility of the *zpg-cifB* F1 female progeny after mating to WT males. We noticed that, in contrast
126 to males (**Fig. 3b**), *cifB*-mediated effects were dependent on the colony of origin. When derived
127 from *zpg-cifA;B* mothers, most F1 *zpg-cifB* females (called (*z^{mat}*)*zpg-cifB*) failed to develop eggs
128 following a blood meal, and only a few females yielded fertile progeny (**Fig. 4b, c**). Additionally,
129 morphological analysis of these ovaries before and after blood feeding showed that follicles were
130 largely absent, suggestive of defects in germline development (**Fig. 4d, e**). On the other hand,
131 when derived from *vasa-cifA;zpg-cifB* mothers, F1 females ((*v^{mat}*)*zpg-cifB*) showed intermediate
132 phenotypes, with substantial follicle development, although both fecundity and fertility were
133 reduced compared to WT females (**Fig. 4b-e**). However, when the *cifB* transgene was inherited
134 from *vasa-cifA;zpg-cifB* fathers (**Fig. 4a**), the majority of F1 females ((*v^{pat}*)*zpg-cifB*) had ovaries
135 similar to the ones observed in (*z^{mat}*)*zpg-cifB* females, showing remarkably reduced follicle
136 development (**Fig. 4d, e**). As the *zpg-cifB* insertion site and promoter is the same in all these
137 groups, these results reveal rescue effects possibly caused by maternal deposition of CifA from
138 *vasa-cifA*-expressing mothers. *cifB* expression in females is therefore highly deleterious in the

139 female germline when unchecked by the presence of *cifA*, and it appears to act during early stages
140 in germline development based on the capacity for maternally derived *cifA* to rescue these defects.
141 Using *cif* genes from *wPip* in *An. gambiae*, our data show that *cifB* expression is sufficient to
142 induce CI. Previous efforts to generate *cifB^{wPip}*-expressing flies were unsuccessful⁴, consistent with
143 our own difficulties of isolating *cifB*-expressing individuals and with our results demonstrating
144 *cifB* toxicity. Our findings are in contrast with results in *D. melanogaster* where both *cifA* and *cifB*
145 from *wMel* were required to induce CI, and where a *cifB* transgenic line was isolated in the absence
146 of *cifA*³. Strain-dependent and/or host-dependent differences may explain the dissimilar findings
147 between CI induction by the Type I *cif* genes in *wMel* and *wPip*, but our data nonetheless call into
148 question the universality of the Two-By-One model. In our view, our findings are supportive of a
149 parsimonious toxin-antidote model where CifB is the toxin and CifA is the antidote. Lending
150 support to this, other studies have shown some infertility induced by *cifB* alone in flies, with both
151 *wPip*'s Type IV *cifB* homolog (also called *cinB*) and *wRec*'s Type I *cifB* gene, though neither study
152 demonstrated rescue of these effects and thus could not conclude that they were CI related^{28,29}.
153 While it is plausible that in some systems CifA may stabilize or even potentiate CifB activity, our
154 results rule out that CifA is always necessary for CI. In fact, *cifA* may even hinder CI induction,
155 as shown by reduced sterility when *cifA* is highly expressed alongside *cifB* in males.
156 Importantly, we conclusively show that it is possible to both induce and rescue CI in *An. gambiae*,
157 suggesting that it may be feasible to eventually utilize *Wolbachia* to invade these mosquitoes.
158 However, the reproductive toxicity observed in both sexes upon *cifB* expression may partially
159 explain why infections using CI-inducing *Wolbachia* strains have been difficult to establish in
160 laboratory colonies and why these endosymbionts have been detected at low prevalence and
161 intensity in field populations of *Anopheles* mosquitoes¹⁷⁻²². Combined with our evidence that high

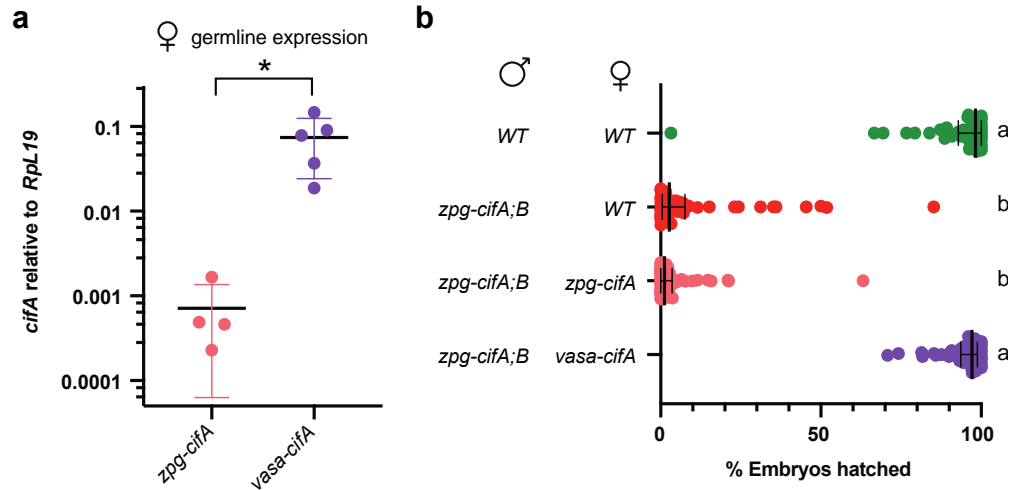
162 levels of *cifA* are needed to rescue CI in females but attenuate *cifB* activity in males, it emerges
163 that *Wolbachia* may have to fine-tune the relative expression of these two genes in males and
164 females to successfully colonize *Anopheles* mosquitoes. Such a balance undoubtedly would
165 present a hurdle in *Wolbachia*'s invasion of these insect hosts, one that may result in silencing the
166 toxic *cifB* gene through mutation. This hypothesis is supported by the discovery of *cifB* nonsense
167 mutations through sequencing from *wAnM* and *wAnD* strains recently discovered in some
168 *Anopheles* species³⁰ as well as by the low prevalence^{18,21-23} and lack of observed CI in *An. gambiae*
169 field populations²³. With this knowledge, in the future it may be possible to facilitate successful
170 and stable *Wolbachia* colonization of *Anopheles*, for instance by limiting *cifB* toxicity in these
171 mosquitoes via *cifA* germline expression. This would create an avenue to screen *Wolbachia* strains
172 that can block transmission of *Plasmodium* parasites, therefore paving the way to exploit these
173 endosymbionts for malaria control. Finally, the remarkable sterility induced by *cifB* could be
174 utilized for sterile male releases to suppress *Anopheles* populations even in the absence of
175 *Wolbachia* infection, similar to the IIT programs implemented in *Aedes* mosquito control^{12,13}. At
176 a time when novel malaria control strategies are urgently needed, our data presents a step towards
177 utilizing *Wolbachia*, or *Wolbachia*-derived genes, in either population replacement or suppression
178 programs targeting *Anopheles* mosquitoes.



179

180 **Figure 1: Co-expression of *cifA* and *cifB* in male *An. gambiae* causes embryonic lethality in**
 181 **progeny.** (a) Construct design of *zpg-cifA*, *zpg-cifB*, *vasa-cifA*, and *vasa-cifB*. (b) Males that
 182 express *zpg-cifA;B* produce largely inviable progeny, regardless of whether their female mate
 183 expresses *zpg-cifA*. Expression of *zpg-cifA;B* in females causes a decrease in female fertility
 184 compared to WT, but *cifA* alone does not (Dunn's multiple comparisons, $p \leq 0.0071$ for a vs b,
 185 $p < 0.0001$ for a vs c and b vs c). Median and interquartile ranges are shown. For each group (top
 186 to bottom) the n (number of broods) is as follows: 58, 52, 59, 51, 53, 62. Kruskal-Wallis test:

187 H=265, $p < 0.0001$, $df = 5$. (c) Embryos from *zpg-cifA;B* males crossed with WT females (or WT
188 crosses, as controls in i. and ii.) were fixed and imaged with DAPI 3-4 hours post oviposition,
189 showing developmental arrest of most CI embryos during early nuclear divisions (iii.), while some
190 embryos complete multiple rounds of nuclear division but show mitotic defects such as chromatin
191 bridging (iv. with a close-up in v.) and other chromosomal abnormalities resulting in delayed or
192 arrested development (vi). Scale bars represent 100 μ m for 100X images (i., iii., iv.) and 400 μ m
193 for 400X images (ii., v., vi.).
194



195

196 **Figure 2: High expression of female *cifA* rescues *cifA;B*-induced CI in *An. gambiae*.** (a)

197 Transcript abundance of *cifA* is higher in *vasa-cifA* females compared to *zpg-cifA* females, relative
198 to *Rpl19* (Unpaired t-test (two-tailed), $p=0.0232$, mean and SD are displayed). For each group the

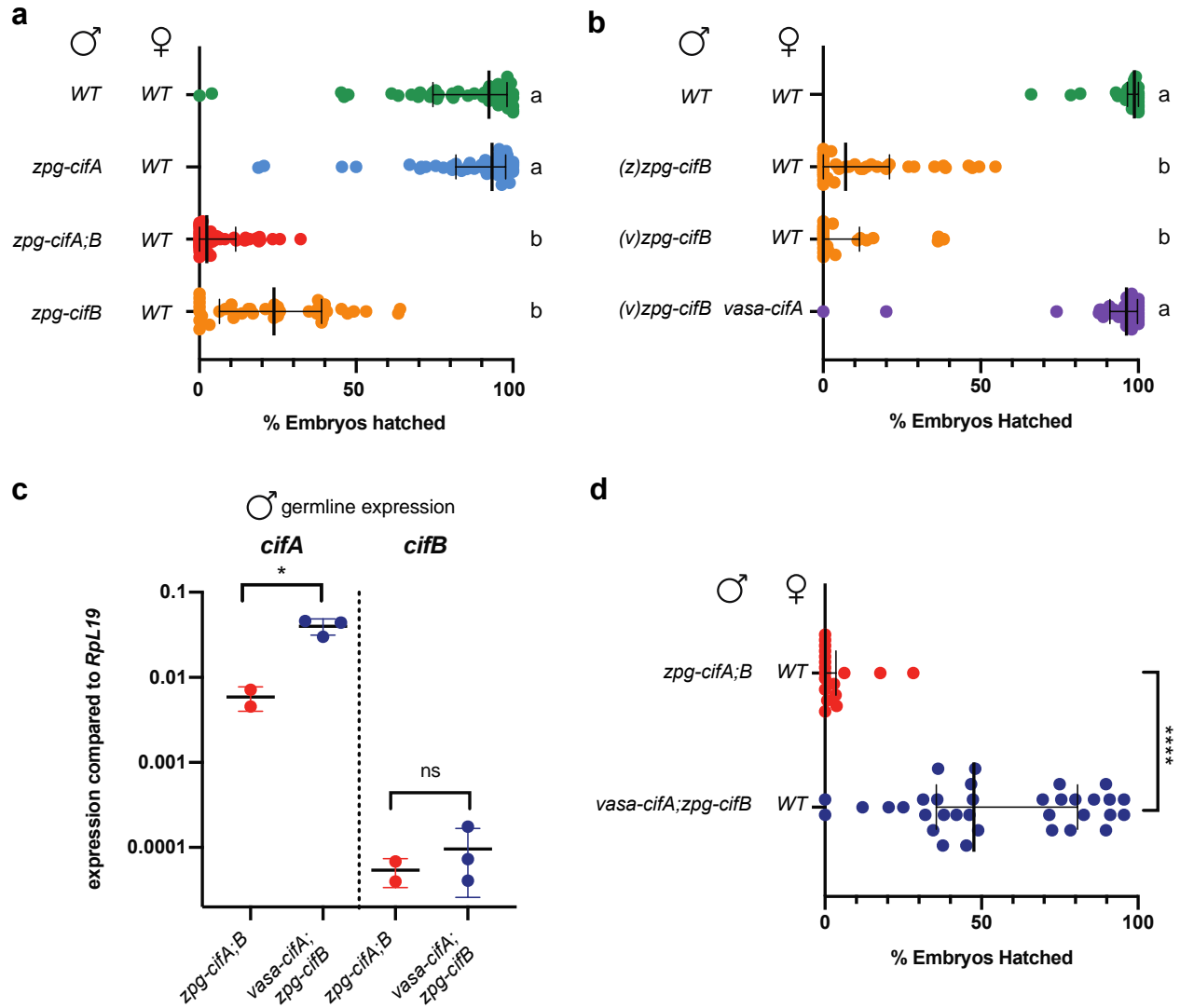
199 n is as follows, from left to right: 64, 80. (b) The expression of *vasa-cifA* in females rescues

200 infertility caused by *zpg-cifA;B* expression in males to WT levels, while expression of *zpg-cifA* in

201 females does not (Dunn's multiple comparison tests, $p<0.0001$ for differences between all

202 statistical groups). Median and interquartile ranges are shown. For each group (top to bottom) the

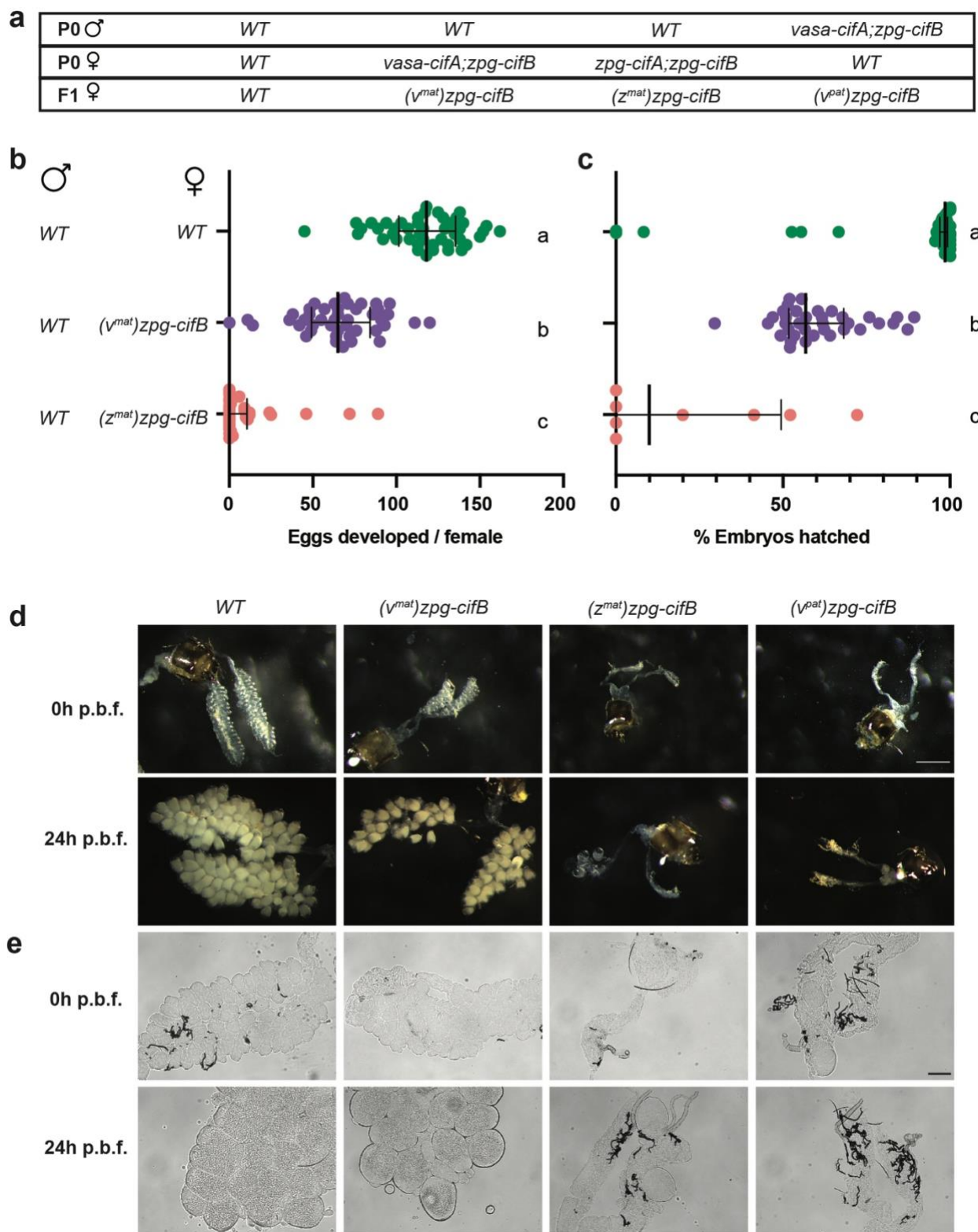
203 n is as follows: 51, 50, 52, 52. Kruskal-Wallis results: $H=153.1$, $p<0.0001$, $df=3$.



204

205 **Figure 3: Male *cifB* expression is sufficient to cause CI, while male *cifA* attenuates it.** (a) *zpg-*
 206 *cifB* males cause infertility in WT females, while *zpg-cifA* males do not (Dunn's multiple
 207 comparisons, $p \leq 0.0001$ for differences between all statistical groups). Median and interquartile
 208 ranges are shown. For each group (top to bottom) the n is as follows: 55, 55, 44, 39. Kruskal-
 209 Wallis results: $H=133.8$, $p < 0.0001$, $df=3$. (b) The expression of *vasa-cifA* in females rescues
 210 infertility caused by (*v*)*zpg-cifB* expression in males, which induce CI to the same extent as (*z*)*zpg-*
 211 *cifB* males. (Dunn's multiple comparisons, $p \leq 0.0001$ for differences between all statistical groups).
 212 Median and interquartile ranges are shown. For each group (top to bottom) the n is as follows: 36,

213 39, 24, 32. Kruskal-Wallis results: $H=95.08$, $p<0.0001$, $df=3$. (c) Expression of *cifA* in the male
214 germline is higher in *vasa-cifA* than *zpg-cifA*, (Unpaired t- test (two-tailed), $p=0.0135$, mean and
215 SD are shown), while the expression of *cifB* is similar (Unpaired t- test (two-tailed), $p=0.4882$,
216 mean and SD are displayed). For each group (left to right) the total n is as follows: 32, 48, 32, 48.
217 (d) Expression of *vasa-cifA; zpg-cifB* in males causes only partial induction of CI. (Mann-Whitney
218 test (two-tailed), $p<0.0001$. Median and interquartile ranges are shown. For each group (top to
219 bottom) the n is as follows: 18, 34.



220

221 **Figure 4: *cifB* expression in females causes severely impaired follicle development in the**

222 **absence of *cifA*.** (a) Crosses were set up to isolate *zpg-cifB* females, F1 progeny derived from

223 either mothers who also expressed *vasa-cifA* ($(v^{mat})zpg-cifB$) or *zpg-cifA* ($(z^{mat})zpg-cifB$), or fathers
224 also expressing *vasa-cifA* ($(v^{pat})zpg-cifB$). (b) Egg development is nearly abolished in $(z^{mat})zpg-$
225 *cifB*-expressing females, while nearly all $(v^{mat})zpg-cifB$ females show egg development, although
226 with decreased numbers of eggs compared to WT. (Dunn's multiple comparisons tests. $p < 0.0032$
227 for differences between all statistical groups). Medians and interquartile ranges are shown. For
228 each group (top to bottom) the n is as follows: 23, 20, 24. Kruskal-Wallis test results: $H=39.85$,
229 $p < 0.0001$, $df=2$. (c) $(z^{mat})zpg-cifB$ and $(v^{mat})zpg-cifB$ females show impaired fertility compared to
230 WT females. (Dunn's multiple comparisons, $p < 0.0001$ for differences between all statistical
231 groups). Median and interquartile ranges are shown. For each group (top to bottom) the n is as
232 follows: 45, 44, 33. Kruskal-Wallis test: $H=91.88$, $p < 0.0001$, $df=2$. (d, e) Ovaries from *cifB*
233 females show severely impaired follicle development unless derived from a *vasa-cifA*-expressing
234 mother, when imaged (d) at either 0h or 24h post blood feeding (p.b.f.) prior to fixing under
235 brightfield microscopy (size marker: 800 μ m) or (e) after fixing using Differential Interference
236 Contrast (size marker: 100 μ m).



237

238 **Extended Data Figure 1: Embryos from *zpg-cifA;B* males show either early or late arrest.**

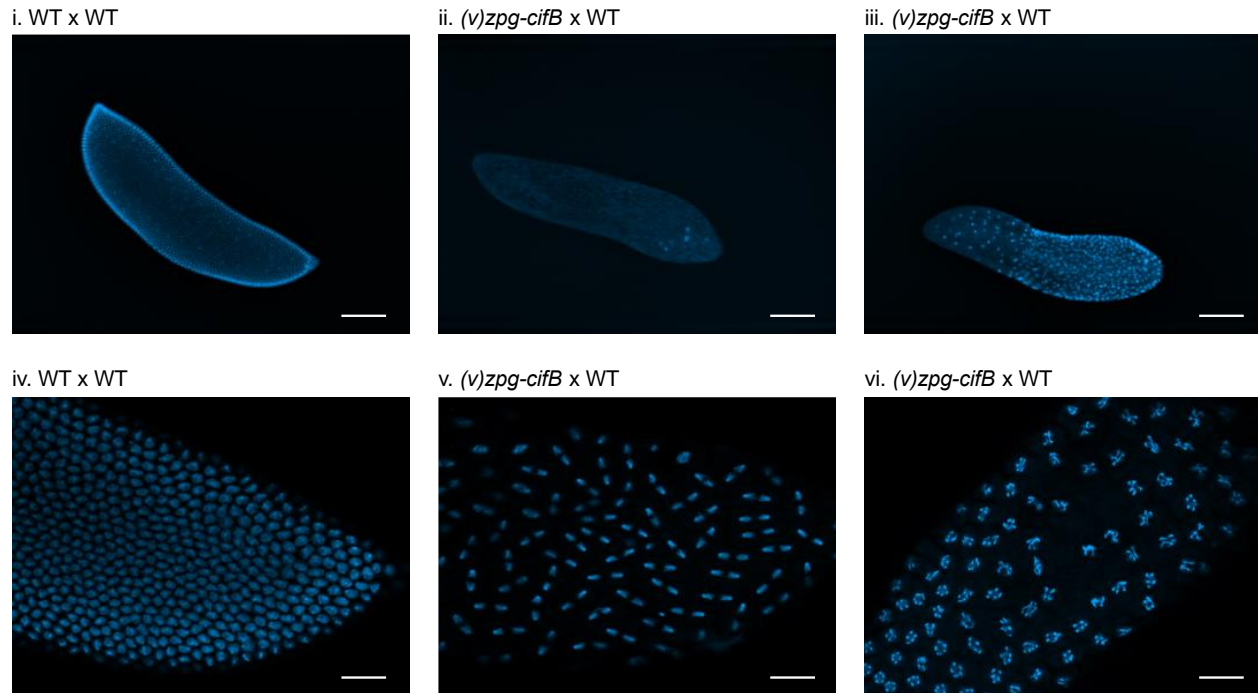
239 Brightfield images of eggs 5 days after oviposition from crosses between WT mosquitoes (left) or

240 *zpg-cifA;B* males and WT females (right). While WT embryos show full development and the

241 standard opening of the hatching cap following larval hatching, embryos from *zpg-cifA;B* males

242 are inviable and arrested either during early development (EA) with a pale brown color, or late

243 development (LA), which show stemmata, but also present severe abnormalities and do not hatch.



244

245 **Extended Data Figure 2: Cytology of the progeny of *cifB* males shows hallmarks of CI. F1**

246 embryos of crosses between either WT or (*v*)*zpg-cifB* males with WT females were stained for

247 DAPI and imaged 3-4 hours after oviposition. At 100X, while WT controls (i. and iv.) show normal

248 development, (*v*)*zpg-cifB* embryos show various hallmarks of CI, including (ii.) early arrest, (iii.)

249 regional mitotic failure, and (v., vi.) chromatin bridging or chromosomal abnormalities and

250 delayed or arrested nuclear division. Scale bars represent 100 μ m for 100X images (i.-iii.) and

251 400 μ m for 400X images (iv.-vi.).

252

253 **Methods:**

254 **Generation of constructs:**

255 The amino acid sequences for *cifA* (wPa_0282) and *cifB* (wPa_0283) coding regions from the
256 published wPipI Pel strain of wPip from *Culex pipiens*³¹ were codon-optimized by hand for
257 expression in *An. gambiae* using published codon bias information³². Gene blocks were ordered
258 from Integrated DNA Technologies (Coralville, IA) using Custom Gene Synthesis to create the
259 desired DNA fragments. Transgenesis constructs were engineered to express the wPip CI genes
260 *cifA* and *cifB* under the control of the germline-specific promoters *zpg* (*zpg*, AGAP006241) and
261 *vasa* (*vasa2*, AGAP008578)³³. The constructs also express a fluorescent marker under control of
262 the ubiquitous *actin* promoter to enable selection of transgenic mosquitoes. Integration into the
263 mosquito genome was mediated by *piggyBac* transposition and rearing lines to homozygosity was
264 accomplished through pupae sorting via fluorescence intensity.

265

266 **Embryonic microinjection:**

267 *PiggyBac* transgenic construct pairs corresponding to each germline promoter (*zpg-cifA-EYFP* and
268 *zpg-cifB-DsRed*; or *vasa-cifA-EYFP* and *vasa-cifB-DsRed*) were co-injected into the posterior of
269 freshly laid embryos from *An. gambiae* at a concentration of 250ng/μL. Pupae that survived
270 injection were separated according to sex, reared to adulthood, and backcrossed to wild-type G3
271 to identify and isolate transgenics. A total of 17 EYFP/DsRed double positive F1 transgenics were
272 recovered from the *zpg* promoter-driven CI constructs injections. In contrast, only *vasa-cifA-EYFP*
273 positive transgenics were recovered from the *vasa-cifA/cifB* co-injections. Irrespective of germline
274 promoter, no *cifB* transgenic mosquitoes were identified post-injection.

275

276 **Mosquito lines and rearing:**

277 *An. gambiae* mosquitoes (species verified by PCR³⁴) from the G3 strain and transgenic derivatives
278 of the G3 strain were maintained in a 27°C insectary environment with 70-80% humidity and a
279 12h light: 12h dark cycle. Adults are given 10% glucose and water *ad libitum* and fed on human
280 blood (Research Blood Components, Boston, MA). Larvae are fed a mixture of Tetramin fish
281 flakes and pellets.

282 Separate colonies were maintained containing the following transgenes: Colony 1: *zpg-cifA*
283 (Chromosome 3R insertion). Colony 2: *zpg-cifA* (3R); *zpg-cifB* (Chromosome X insertion).
284 Colony 3: *vasa-cifA* (putative Chromosome 2L insertion (2L*)). Colony 4: *vasa-cifA* (2L*); *zpg-*
285 *cifB* (X). Colony 5: *zpg-cifA* (unknown insertion); *zpg-cifB* (X). To establish Colony 4, *zpg-cifB*
286 males isolated from Colony 2 or 5 were crossed with *vasa-cifA* females from Colony 3. All
287 colonies were maintained as heterozygotes, and screened for fluorescent markers as pupae to select
288 for the presence of transgenes. For all experiments using *zpg-cifA* mosquitoes, Colony 1 was used.
289 For all experiments using *zpg-cifA;B* mosquitoes, Colony 2 was used. For all experiments using
290 *vasa-cifA* mosquitoes, Colony 4 was used. Experiments using (*z*)*zpg-cifB* mosquitoes used
291 mosquitoes isolated from either Colony 2 or 5. Experiments using (*v*)*zpg-cifB* males used
292 mosquitoes isolated from Colony 4.

293

294 **Crosses and fertility assays:**

295 To perform crosses between different transgenic lines, individuals were isolated as pupae from
296 these colonies and their transgenes were identified by their respective fluorescent markers. We did
297 not verify whether individuals were homozygous or heterozygous for their transgenes. Pupae were
298 separated by sex under a dissecting microscope, placed in cages with a ratio between 1:1 and 2:1

299 males to females, and allowed to eclose in small BugDorm® cages. Natural mating proceeded and
300 mosquitoes were given *ad libitum* access to 10% glucose solution and water for 5–7 days prior to
301 blood-feeding females and allowing oviposition in individual cups lined with filter paper. Once
302 laid, eggs were stimulated daily by spraying water and allowed to hatch for a minimum of 4 days.
303 We then assessed fertility of females by counting and scoring eggs under a Leica M80 dissecting
304 microscope, and additionally noting the presence or absence of hatched larvae. For any female that
305 showed no fertile embryos, we verified her mating status by checking microscopically for the
306 presence of sperm in the spermatheca. For egg development experiments, egg counts for all
307 females were included regardless of whether they had mated or oviposited, while only those that
308 were mated and oviposited were included in fertility experiments.

309

310 **RNA extraction and qRT PCR:**

311 Male or female reproductive tracts were dissected in pools of 16, collected in TRI reagent (Thermo
312 Fisher Scientific), and stored at -80°C. RNA was extracted according to manufacturing instructions
313 with an additional three ethanol washes of pelleted RNA. Following resuspension, RNA was
314 treated with Turbo DNase (Thermo Fisher Scientific), quantified with a Nanodrop 2000C
315 (Thermo Fisher Scientific), and then 0.75–2µg were used in a 100µL cDNA synthesis reaction,
316 following standard protocols. We designed primers for qRT-PCR (QuantStudio 6 pro, Thermo
317 Fisher Scientific) using NCBI PrimerBLAST³⁵ and after evaluating four different primer sets for
318 *cifB*, we used the following primers for *cifA* and *cifB* at the following concentrations: cifAF: 5'
319 tcgccgagctgatcgtgaa 3' (300nM), cifAR: 5' atcatgtccaggatctccttctctc 3' (300nM), cifBF 5'
320 AGAAGGACCGCCTGATCG 3' (900nM), cifBR 5' AGGCTATCGGCGTAGTAGCC 3'
321 (900nM), RpL19F 5' CCAACTCGCGACAAAACATTC 3' (300nM), RpL19R 5'

322 ACCGGCTTCTTGATGATCAGA 3' (900nM). Relative quantification was determined using the
323 $2^{-(\Delta Ct)}$ equation, using *RpL19* as the standard. For female *cifA* expression (**Fig. 2a**), transcript levels
324 were not found to be different between samples from *cifA* only or *cifA* and *cifB* co-expressing
325 individuals, so these data were pooled.

326

327 **Microscopy and tissue staining:**

328 *Embryo cytology:* Embryos were collected from 10–12 WT females after natural matings with *zpg-*
329 *cifA;B* males. Four hours after oviposition, embryos were bleached, washed, and dechorionated
330 according to methods by Goltsev *et al.*, 2004³⁶, and the endochorion was peeled according to
331 methods by Juhn and James, 2012³⁷. Embryos were then fixed and stained with DAPI and imaged
332 on a Zeiss Inverted Observer Z1 at 100X or 400X magnification.

333 *Brightfield microscopy of embryos:* A sample of oviposited embryos were imaged on filter paper
334 at either 5X or 7.5X on a Leica M80 dissecting scope.

335 *Brightfield and Differential Interference Contrast imaging of ovaries:* Ovaries of 4–7-day-old
336 females were dissected in PBS at either 0h or 24h post-blood-meal and imaged with a Leica M80
337 dissecting scope at 7.5X magnification for general morphology. After initial imaging, ovaries were
338 fixed in 4% PFA and then mounted in Vectashield® mounting media with DAPI counterstain
339 (Vector Laboratories, Burlingame, CA). Ovaries were then imaged using Differential Interference
340 Contrast on a Zeiss Inverted Observer Z1 at 100X magnification.

341

342 **Statistical methods:**

343 In all comparisons of fertility or egg development, Anderson-Darling normality tests showed that
344 all data was not normally distributed, so non-parametric Kruskal-Wallis tests with Dunn's multiple

345 comparisons were used. Distinct samples were used for comparisons. Tests were performed in
346 GraphPad Prism 8. For all fertility or egg development experiments, two to four replicates were
347 performed for all groups. Only one replicate was performed for embryo cytology experiments. For
348 Fig. 4d and 4e, where representative images were selected, one replicate of dissection and imaging
349 of 5–10 individuals from each group was performed. For all qRT-PCR data two to five technical
350 replicates with 16 individuals each were performed for each group, exact *n* given in figure legends.

351

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353

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364 searches, study design, data collection, data analysis, data interpretation and writing, B.C.W
365 contributed to literature searches, study design, data analysis, and data collection, E.K.S.
366 contributed to data analysis and data collection, M.I. contributed to data collection, and F.C.
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370

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372

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