

1 *Wolbachia* successfully replicate in a newly established horn fly, *Haematobia*
2 *irritans irritans* (L.) (Diptera: Muscidae) cell line

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4 Short running Title: Transinfection of horn fly cell line with *Wolbachia*

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24 Abstract

25 BACKGROUND: *Haematobia spp.*, horn flies (HF) and buffalo flies (BF), are
26 economically important ectoparasites of dairy and beef cattle. Control of these flies
27 relies mainly on the treatment of cattle with chemical insecticides. However, the
28 development of resistance to commonly used compounds is compromising the
29 effectiveness of these treatments and alternative methods of control are required.
30 *Wolbachia* are maternally transmitted endosymbiotic bacteria of arthropods that
31 cause various reproductive distortions and fitness effects, making them a potential
32 candidate for use in the biological control of pests.

33 RESULTS: Here we report the successful establishment of a continuous HF cell line
34 (HIE-18) from embryonic cells and its stable transinfection with *Wolbachia* strain
35 wAlbB native to mosquitoes, and wMel and wMelPop native to *Drosophila*
36 *melanogaster*. The established HIE-18 cells are typically round and diploid with ten
37 chromosomes ($2n = 10$) or tetraploid with 20 chromosomes ($4n=20$) having a
38 doubling time of 67.2 hours. *Wolbachia* density decreased significantly in the HIE-18
39 cells in the first 48 hours of infection, possibly due to overexpression of antimicrobial
40 peptides through the Imd immune signalling pathway. However, density recovered
41 after this time and stably *Wolbachia*-infected HIE-18 cell lines have now all been
42 subcultured more than 50 times as persistently infected lines.

43 CONCLUSION: The amenability of HF to infection with different strains of *Wolbachia*
44 suggests the potential for use of *Wolbachia* in novel approaches for the control of
45 *Haematobia spp.* Further, the availability of the HIE-18 cell line will provide an
46 important resource for the study of genetics, host-parasite interactions and chemical
47 resistance in *Haematobia* populations.

48 **Keywords:** *Wolbachia*, biocontrol, horn fly, *Haematobia* spp., biopesticide, pest

49 management, veterinary ectoparasite

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94 1. INTRODUCTION

95 *Wolbachia* are maternally inherited endosymbionts of arthropods and filarial
96 nematodes ¹. They are estimated to infect nearly 40% of terrestrial arthropod species
97 ². The nature of *Wolbachia*-host associations varies from parasitic to mutualistic,
98 often depending on the length of the association ^{3, 4}. In longstanding associations,
99 any deleterious effects of *Wolbachia* tend to become attenuated, and *Wolbachia* may
100 eventually form a mutualistic relationship with its host ¹. Some examples of beneficial
101 effects in such associations are in facilitating parasitoid wasp (*Asobara tabida*)
102 oocyte maturation, female development in adzuki bean borer (*Ostrinia scapulalis*)
103 and the supplementation of vitamin B in bedbugs ^{1, 5-7}. The most commonly noted
104 parasitic effects are where *Wolbachia* manipulate the reproductive biology of their
105 hosts to facilitate spread through populations by mechanisms such as cytoplasmic
106 incompatibility (CI), male-killing, feminisation of males and parthenogenesis ⁸.
107 *Wolbachia* may also mediate a range of other effects in their hosts such as the
108 reduction of pathogen transmission, changes in feeding behaviour and locomotion,
109 decrease in offspring survival and reductions in longevity, which can potentially be
110 utilised in the design of programs for the control of arthropods, filarial nematodes and
111 arthropod vectored diseases ^{4, 9-13}.
112 Different *Wolbachia*-host genomic studies have revealed natural horizontal transfer
113 of *Wolbachia* between invertebrate hosts during their evolutionary history ^{9, 14}. The
114 mechanism behind horizontal transfer needs to be further explored, but some
115 proposed mechanisms include co-feeding/ salivary exchange, parasitoid wasp or
116 mite co-infection, and faecal-oral transmission ^{9, 15-18}. Judging from comparisons of
117 phylogenetic associations, natural horizontal transmission of *Wolbachia* must have
118 occurred relatively frequently in past evolutionary history ⁹. However artificial

119 transinfection is usually more complicated and some species such as red flour beetle
120 *Tribolium castaneum*, silkworm (*Bombyx mori*) and the mosquito *Anopheles*
121 *gambiae*, appear relatively resistant to *Wolbachia* infection. Despite many attempts it
122 has not yet been possible to develop sustainably transinfected strains of these
123 insects [4]. Also, newly transinfected hosts tend to have a lower infection frequency
124 than in natural associations and often exhibit unstable maternal inheritance¹⁹⁻²². To
125 overcome these difficulties and facilitate the success of cross-species infection, prior
126 adaption of *Wolbachia* to the target host-context by culturing in a cell line from the
127 target host species has been suggested^{9, 21}. For instance, the adaption of wMelPop
128 *Wolbachia* (isolated from *Drosophila melanogaster*) in *Aedes aegypti* cells aided the
129 development of a *Wolbachia* infected *Ae. aegypti* mosquito line²¹. When the
130 mosquito-adapted *Wolbachia* were reintroduced to *Drosophila*, they showed lower
131 virulence, and lower infection frequency in comparison to *Drosophila*-maintained
132 wMelPop²¹. A recent genomic comparison between wMelPop grown in *D.*
133 *melanogaster* cells, a wMelPop adapted in mosquito cells (wMelPop-CLA) for 3.5
134 years, and wMelPop from *Wolbachia*-infected *Ae. aegypti* mosquitoes (wMelPop-
135 PGPY) four years after transinfection, showed genomic differences between
136 wMelPop, and wMelPop-CLA but no difference in wMelPop-PGPY, further indicating
137 that cell line culture can be an excellent medium for quick adaptation of *Wolbachia* to
138 a new host-context²³. An additional benefit of culturing *Wolbachia* in cell lines is the
139 ready availability of significant amounts of *Wolbachia* for use in transinfection studies
140 ⁴.

141 Insects live in diverse ecological niches where they interact with many different
142 species of microorganisms, yet they successfully evade infections because of strong
143 innate immune responses²⁴. Four major pathways (Bacteria: Toll, and Imd; viruses:

144 JAK/STAT and RNAi) are associated with the innate immune system of insects,
145 which is responsible for defense against pathogens^{19, 24-27}. Usually, a Toll signalling
146 cascade is induced by Gram-positive bacteria and fungi, whereas the immune
147 deficiency (Imd) pathway is induced by Gram-negative bacteria^{26, 27}. These
148 signalling cascades increase the production of antimicrobial peptides (AMPs) to
149 suppress or eliminate the infection²⁴.

150 As the Toll and Imd pathways target bacteria, they can also affect *Wolbachia* density
151 in newly infected hosts¹⁹. *Wolbachia*-host interactions are quite variable, and
152 immune genes from Toll (*Cactus*, *Dorsal*, *MyD88*), Imd (*Caspar*, *Relish*, *dFADD*,
153 *IMD*) and AMPs (*Attacin*, *Cecropin*, *Defensin*) are differentially expressed in different
154 hosts^{19, 28-32}. For instance, researchers have found no effect on the innate immune
155 response with the presence or absence of *Wolbachia* in the native host mosquitoes,
156 *Aedes albopictus* and *Aedes fluviatilis*^{19, 28, 33}. However, innate immune genes were
157 overexpressed when *Ae. albopictus* mosquito cells (Aa23) were infected with *wMel*
158 *Wolbachia*, sand fly cell lines (LL-5) with *wMel* or *wMelPop-CLA* *Wolbachia*, and *An.*
159 *gambiae* cell lines with *wAlbB* *Wolbachia*^{19, 34}.

160 To date, most of these studies have been done in a mosquito or *Drosophila* species
161 context. However, different insect species exhibit different responses to *Wolbachia*
162¹⁹. Clarifying the nature of these interactions in *Haematobia* cells may assist the
163 ultimate development of transinfected strains of *Haematobia* and the design of
164 *Wolbachia*-based *Haematobia* control programs.

165 Horn flies (*Haematobia irritans irritans*) (HF) and buffalo flies (*Haematobia irritans*
166 *exigua*) (BF) are closely related obligate hematophagous cattle ectoparasites
167 causing substantial economic losses across the world³⁵. Both species have proven

168 to be highly invasive and they have often been classified as subspecies³⁵. It is hard
169 to morphologically discriminate between them, whereas at the molecular level,
170 ribosomal genes are conserved, and mitochondrial genes have a relatively low
171 divergence of 1.8-1.9%³⁶. The estimated loss associated with the HF alone to North
172 and South America is close to \$US3.56B per annum^{35, 37, 38}. Both HF and BF have
173 developed resistance to commonly used chemical insecticides and new methods are
174 required to reduce reliance on chemical controls in endemic areas and to prevent
175 invasion into new areas^{35, 39, 40}.

176 Here we report the establishment of a HF embryonic cell line (HIE-18) isolated to
177 adapt *Wolbachia* to the *Haematobia* context before BF transinfection. We evaluated
178 the ability of HIE-18 to support growth and development of *wMel* and *wMelPop*
179 *Wolbachia* isolated from *D. melanogaster* cells, and *wAlbB* *Wolbachia* from mosquito
180 (*Ae. albopictus*) cells. Further, we analysed the host immune response by
181 investigating expression of genes from the Toll and Imd pathways and AMPs to
182 understand the early interactions between *Wolbachia* and the new host HIE-18 cells.

183 2. MATERIALS AND METHODS

184 2.1. Establishment of HF primary cell culture and young cell lines

185 Embryonated HF eggs were obtained from a laboratory colony reared in the
186 presence of antibiotics and maintained at the USDA/ARS lab in Kerrville, TX, USA⁴¹.
187 Eggs were sent to the University of Minnesota and processed following the protocol
188 described in Goblirsch et al. (2013) used for the successful establishment of an
189 embryonic cell line from *Apis mellifera*⁴². Briefly, HF eggs were surface disinfected
190 by sequentially rinsing in a 0.5% sodium hypochlorite and Tween 80 mix, 0.5 %
191 benzalkonium chloride, 70% ethanol and several times in sterile water to remove
192 residual chemicals. The eggs were finally rinsed three times with modified Leibovitz

193 L-15 cell culture medium ⁴³ (hereafter referred to as L15C) and gently crushed with a
194 pestle (Kimble Chase, Vineland, NJ) in 1.5 ml sterile Eppendorf tubes to release
195 internal embryonic tissue ⁴⁴. The cellular homogenate was centrifuged at 100 x g for
196 2 min to remove yolk particles and inoculated in 12.5 cm² non-ventilating screw cap
197 culturing flasks (Falcon, Corning Inc. Tewsbury, MA) in 2 mL of culture medium
198 supplemented with FBS (10%), tryptose phosphate broth (5%), lipoprotein
199 concentrate (0.1%; MP Biomedicals), antibiotics (100 µg/mL penicillin, 100 µg/mL
200 streptomycin, and 0.25 µg/mL amphotericin) (Life Technologies, Grand Island, NY).
201 Flasks were incubated in a non-humidified incubator at 32°C, and media were
202 changed weekly. Confluent primary cultures were dispersed by gentle pipetting and
203 one half of the suspension transferred to a new flask. Young lines (passage 10 or
204 less) were subcultured at a ratio of 1:2 every 2 - 4 weeks and maintained in 25 cm²
205 culture flasks (Cellstar; Greiner Bio-One, Monroe NC) at 32°C. Cell lines in passage
206 2-5 were frozen by suspending cells in freezing media (L15C supplemented with
207 20% FBS and 10% DMSO) at the rate of -1°C / min using CoolCell alcohol-free
208 freezing container (BioCision, LLC, Mill Valley, CA) and stored in liquid nitrogen.

209 2.2. Maintenance of *Haematobia irritans* embryonic (HIE) line HIE18

210 We selected the fastest growing line, HIE18, for continuation. At passage 20, when
211 the line could be split 1:5 every 3-4 weeks, the cells were adapted to Schneider's
212 medium by gradually increasing the proportion of Schneider's medium to L15C from
213 20% to 100% in 20% intervals. Since passage 20, the HIE-18 line has been
214 maintained at 28°C in Schneider's medium supplemented with 10% FBS.

215 2.3. Other cell lines

216 *Ae. aegypti* mosquito cell lines (Aag2) infected with *wMel* and *wMelPop*, provided by
217 the Eliminate Dengue laboratory at Monash University, Australia, and the *Ae.*
218 *albopictus* mosquito cell line (Aa23T) infected with the *wAlbB Wolbachia* strain were
219 cultured in 75 cm² culturing flask with 12 ml of Mitsuhashi and Maramorosch medium
220 (M&M) (Sigma Aldrich, NSW, Australia) and Schneider's medium (Sigma Aldrich,
221 NSW, Australia) mixed in a ratio of 1:1 at 26°C. The medium was supplemented with
222 10% fetal bovine serum (Gibco, MD, USA). All cell lines were subcultured once every
223 6-7 days, and no antibiotics were used during culturing.

224 2.4. Molecular identification of HIE-18 cells

225 DNA was isolated from whole adult BF and HIE-18 cells using an Isolate II Genomic
226 DNA kit (Bioline, NSW, Australia) following the manufacturer's protocol. Forward
227 primer HCOI_F 5'- TGAATTAGGACATCCTGGAGCTT-3' and reverse primer
228 HCOI_R 5'-CACCAGTTAATCCTCCAACCTG-3' were used to target the mitochondrial
229 COI gene of the *Haematobia* species⁴⁵. Each PCR tube contained 1.5 µl DNA, 1µm
230 of each primer, 1µl of 10x PCR buffer (Qiagen, Melbourne, Australia), 1mM dNTP,
231 0.1 µl Taq DNA polymerase (Qiagen, Melbourne, Australia) and nuclease-free water
232 to make a total volume of 10 µl. Thermocycling conditions for the reactions were as
233 follows: an initial incubation at 94°C for 3 min, followed by 35 cycles of denaturation
234 at 94°C for 30 sec, annealing at 50°C for 30 sec, and elongation at 72°C for 45 sec
235 with a final extension at 72°C for 10 min. PCR product was amplified in a DNA
236 Engine Thermal Cycler (Bio-Rad, Sydney, Australia) and electrophoresed on a 1%
237 agarose gel. The DNA band was visualised by staining with GelRed (Biotium, NSW,
238 Australia).

239 2.5. Karyotyping

240 Cell lines in log phase growth were incubated in fresh modified Leibovitz's L-15
241 medium containing 12.5 μM colchicine overnight at 32°C. After 12 hours the cells
242 were pelleted at 300 x g for 10 min, resuspended in hypotonic KCl (75mM), and
243 further incubated for 60 min at 34°C. Post incubation HIE-18 cells were fixed in a
244 mixture of methanol and acetic acid (3:1) for 30 min at room temperature. HIE-18
245 cells were then pelleted and resuspended in fresh fixative. A droplet containing the
246 resuspended cells was placed on a pre-chilled (-20°C) slide to air dry, and stained
247 with 3.2 % Giemsa stain prepared in Sørensen's buffer (pH=6.8) for an hour at 34°C
248 ⁴². Metaphase chromosomes were counted under an Eclipse E400 phase-contrast
249 microscope (Nikon, NY, USA).

250 2.6. Identification of optimal culturing temperature and media for HIE-18 cells

251 HIE-18 cells were seeded into 25 cm² flasks with 3.5 ml L-15C at a cell density of
252 approximately 1.1 x 10⁶ cells/ml. Flasks were incubated at 30°C overnight to
253 establish a baseline cell density as a control. The next day flasks were randomly
254 assigned to test temperatures 26-32°C and incubated for six days. On day seven,
255 cells were dislodged by gentle pipetting, pelleted by centrifugation at 300 x g, and
256 incubated in 0.5 ml of NaOH (0.5 N) overnight at 27°C to solubilise cellular protein.
257 The solubilised protein (10 μl) was mixed with 290 μl of Quick Bradford reagent
258 (Sigma Aldrich, NSW, Australia) and incubated at room temperature for 5 min ⁴².
259 Optical density readings for samples were taken at 595 nm using a BMG LABTECH
260 microplate reader (BMG LABTECH, Ortenberg, Germany). Bovine serum albumin
261 (Sigma-Aldrich, NSW, Australia) stock (10mg/ml) in Milli-Q water was used to
262 prepare a protein standard curve. All the experiments were carried out in triplicate.

263 Similar to the above assay, HIE-18 cells were inoculated into 25 cm² flasks at a
264 density of 0.8 x10⁶ cells/ml and incubated overnight to assign a baseline value. The
265 next morning medium from each flask was replaced with one of the test media
266 [modified Leibovitz's L-15 (Sigma Aldrich, NSW, Australia), Schneider's (Sigma
267 Aldrich, NSW, Australia), 1:1 mix of Leibovitz's and Schneider's, and Shield's (Sigma
268 Aldrich, NSW, Australia)], and cells were left to grow for five days. Protein estimation
269 was carried out according to the previously described protocol.

270 2.7. Transfecting HIE-18 cells with *Wolbachia*

271 HIE-18 cell transinfection was carried out using the protocol described by Herbert et
272 al. (2017) ¹⁹. Briefly, wAlbB, wMel, and wMelPop infected mosquito cell lines were
273 grown in 75 cm² flasks, each containing 15 ml of M&M media (Sigma Aldrich, NSW,
274 Australia) having 10% FBS. Cells were dislodged from the flask after seven days by
275 vigorous pipetting and spun at 2000 x g. The resulting pellet was washed three times
276 with 5 ml SPG buffer (218 mM sucrose, 3.8 mM KH₂PO₄, 7.2 mM MK₂HPO₄, 4.9 mM
277 L-glutamate, pH 7.5), and sonicated on ice at 24% power using a Q125 sonicator
278 (Qsonica, CT, USA). Cellular debris was pelleted by spinning at 1000 x g for 10 min
279 at 4°C, and the supernatant was filtered through 50 µm and 2.7 µm acrodisc syringe
280 filters (Sigma Aldrich, NSW, Australia). The filtered supernatant was centrifuged at
281 12000 x g, and the *Wolbachia* pellet was resuspended in 100 µl of SPG buffer. The
282 pellet suspension was immediately added drop by drop into the cell line flasks when
283 the HIE-18 cells were 80% confluent. The infected cell lines were cultured for 5-6
284 days and then split in a ratio of 1:2 into the new flasks.

285 2.8. Detection and quantification of *Wolbachia* in HIE-18 cells

286 DNA was isolated from cells using the Isolate II Genomic DNA kit (Bioline, NSW,
287 Australia) following the manufacturer's protocol. Detection of *Wolbachia* was carried
288 out by real-time PCR on a Rotor-Gene Q machine (Qiagen, Australia) using strain-
289 specific primers and probes. Primers for *wAlbB* amplified the *wsp* gene (forward
290 primer: 5'- GGT TTT GCT GGT CAA GTA-3', reverse primer: 5'-
291 GCT GTA AAGA ACG TTG ATC-3', probe: 5'- FAM-TGTTAGTTATGATGT
292 AACTCCAGAA-TAMRA-3')⁴⁶, whereas the primers used for *wMel* amplified the
293 *WD0513* gene (forward primer: 5'- CAA ATT GCT CTT GTC CTG TGG-3', reverse
294 primer: 5'- GGG TGT TAA GCA GAG TTACGG-3', probe: 5'-
295 CyTGAAATGGAAAATTGGCGAGGTGTAGG-BHQ-3') and the primers for
296 *wMelPop* targeted the IS5 element in *WD1310* gene (forward primer: 5'-CTC ATC
297 TTT ACC CCG TAC TAA AAT TTC-3', reverse primer: 5'-TCT TCC TCA TTA AGA
298 ACC TCT ATC TTG-3', probe: 5'- Joe-TAG CCT TTT ACT TGT TTC CGG ACA
299 ACCT-TAMRA -3')⁴⁷. The total volume of each reaction mix was 10 µl, containing 5
300 µl of the PrimeTime ® Gene Expression Master Mix (IDT, NSW, Australia), 0.5 µl
301 each of 10 µM forward and reverse primer, 0.25 µl of 5 µM probe and 3 µl of
302 genomic DNA. The optimised amplification conditions for *wMel* and *wMelPop* were 3
303 min at 95°C followed by 45 cycles of 10 s at 95°C, 15 s at 51°C, and 15 s at 68°C,
304 whereas for *wAlbB*, amplification was done for 3 min at 95°C followed by 45 cycles
305 of 20 s at 94°C, 20 s at 50°C, and 30 s at 60°C.

306 For relative quantification of the *Wolbachia* single-copy *wsp* gene (forward primer: 5-
307 'TGG TCC AAT AAG TGA TGA AGA AAC-3', reverse primer: 5'- AAA AAT TAA
308 ACG CTA CTC CA-3') and the host *GAPDH* gene (forward primer: 5'-378_F_CCG
309 GTG GAG GCA GGAATGATGT-3', reverse primer: 5'- 445_R

310 CCACCCAAAAGACCGTTGACG-3') were amplified ³³. Each sample was run in
311 triplicate on a Rotor-gene Q Instrument (Qiagen, Australia) with total reaction volume
312 of 10 µl containing 5 µl Rotor-Gene SYBR[®] Green PCR Kit (Bioline, Australia), 0.3 µl
313 each of 10 µM forward and reverse primer and 2 µl of genomic DNA. Negative and
314 positive controls were run with all samples. The optimised thermocycling conditions
315 were initial incubation for 5 min at 95°C followed by 45 cycles of 10 s at 95°C, 15 s at
316 55°C, and 15 s at 69°C, acquiring green at the end of the step. The relative density
317 of *Wolbachia* was calculated using the delta-delta CT method ⁴⁸.

318 2.9. Effect of *Wolbachia* on population doubling time

319 *Haematobia* cells infected with *wAlbB* (*wAlbB*-HIE-18), *wMel* (*wMel*-HIE-18),
320 *wMelPop* (*wMelPop*-HIE-18) and non-infected cells (HIE-18) were dislodged by
321 pipetting and seeded at a density of 1.5×10^6 cells/mL into three different 25 cm²
322 culture flasks. The flasks were incubated at 28°C in Schneider's medium (Gibco Life
323 Technologies, Australia) supplemented with 10% FBS. The media were changed
324 every seven days. Cells were visualised under a Motic AE 30 inverted microscope
325 at 40X magnification with a Nikon model DS-L3 camera. A grid (2 cm X 2 cm) was
326 set up on the camera screen (Nikon, Sydney, Australia) for cell counting. Ten
327 randomly selected fields were counted for each flask at 24 h intervals from seeding
328 until they reached confluence >80% ⁴². The experiment was replicated three times,
329 and data for cell density was plotted as the number of cells per cm² (\pm SD) per day.

330 2.10. Upregulation of immune response genes in *Haematobia* cells

331 HIE-18 cells were sampled without infection and then at 12, 24, 36 and 48 h post-
332 infection with *wAlbB*, *wMel*, and *wMelPop*. Total RNA extraction was performed
333 using the Isolate II RNA Mini Kit (Bioline, NSW, Australia) following the

334 manufacturer's protocol. Primers were designed as detailed below to target the *H.*
335 *irritans* innate immune genes *cactus*, *relish*, *attacin*, *cecropin*, and *defensin*.
336 Targeted gene sequences of *D. melanogaster* were collected from the Flybase web
337 server (<https://flybase.org/>) and searched on the NCBI database to find similar
338 sequences in *Musca domestica*, the closest species to *Haematobia* for which gene
339 sequences were available. Gene sequences from *M. domestica* were searched for
340 sequence similarity in the recently sequenced *H. irritans* genome (Accession
341 number: GCA_003123925.1) to find similar contigs. Finally, primers were designed
342 based on those contigs using Genious Prime software (See Table 1). A SensiFAST
343 Probe No-ROX One-Step Kit (Bioline, NSW, Australia) was used to carry out the
344 gene expression study following the manufacture's protocol with a Rotor-gene Q
345 Instrument (Qiagen, Australia). Melt curve analyses were performed in the range 55–
346 90°C to examine reaction specificity of the designed primers. All of the assays were
347 performed in triplicate. Gene expression was normalised to the host *GAPDH* gene,
348 and change in expression level was calculated using the delta-delta CT method⁴⁸.

349 3. RESULTS

350 3.1. Features of HIE-18 cells isolated from HF embryos

351 Twenty-two primary cultures were made to initiate primary cell lines from HF eggs. In
352 some instances, a slight variation was made to cell establishment protocols, either
353 by changing the seeding quantity of the inoculant (50 - 200 embryos) or centrifuging
354 cellular homogenate to remove the excess of yolk material. We expected that having
355 more cells from embryos would help in increasing cell-cell interaction, and extra yolk
356 material would provide the necessary nutritional support for cellular growth.
357 However, an excess of yolk material was found to interfere with cellular adherence in
358 the flasks. One culture of HIE-18 cells from HF embryos grew in aggregates of 20-30

359 cells where cellular morphology was hard to discriminate in the first two-three weeks
360 (Fig. 1A). Nevertheless, within eight weeks cells reached confluence and a distinct
361 morphologically heterogeneous population of round epitheliocytes, neuronal-, and
362 fibroblast-like cells were seen. Later, round cells (5-10 μm) became predominant.
363 These were isolated and cryopreserved (Fig. 1B). Currently, HIE-18 cells have been
364 successfully passaged over 200 times (Fig. 1C). Ten of the remaining 22 primary
365 cultures developed into lines (passage 7 or less) and are currently stored in liquid
366 nitrogen.

367 3.2. Species confirmation using PCR and chromosomal analysis

368 HIE-18 cell identity was confirmed by amplifying the *COI* gene of approximately 920
369 bp size with *Haematobia*-specific primers. Both the HIE-18 cells and positive control
370 BF were amplified (Fig. 2A). HIE-18 cells were comprised predominantly of round
371 diploid cells with ten mitotic chromosomes or tetraploid cells with 20 chromosomes
372 (Fig. 2B-C). Diploid cells had five pairs of chromosomes, two of which were sub-
373 metacentric and three metacentric. There were no heteromorphic (sex)
374 chromosomes.

375 3.3. Identification of optimal culturing temperature and media for HIE-18 cells

376 Protein concentration was used as a measure of growth of the newly established
377 HIE-18 cell line incubated at four different temperatures (26, 28, 30 and 32°C) (Fig.
378 3A). There was a significant difference in cellular replication at different temperatures
379 (one-way ANOVA: $F_{4,10} = 237.1$, $p < 0.0001$). Highest protein concentration was seen
380 at 30°C (2.6+0.72 mg), followed by 28°C (2.33+0.79 mg) indicating the best growth
381 at these two temperatures. There was also a significant difference in the
382 concentration of protein in different culturing media (one-way ANOVA: $F_{4,10}=118.3$,

383 $p < 0.0001$) (Fig. 3B) with the HIE-18 cells growing best in Schneider's medium (2.74
384 + 0.11 mg) and 1:1 mix of Schneider's and L-15 culture media (2.49 + 0.13 mg).

385 3.4. *Wolbachia* replication in HIE-18 cell lines

386 It took multiple rounds of *Wolbachia* introduction into the HIE-18 cell lines to
387 establish persistent infections of *wAlbB* (two rounds), *wMel* (three rounds) and
388 *wMelPop* (two rounds). We saw a significant decrease in the density of *Wolbachia*
389 within 48 hours of infection: *wAlbB* (one-way ANOVA: $F_{3,8} = 10.87$, $p = 0.0034$), *wMel*
390 (one-way ANOVA: $F_{3,8} = 27.30$, $p = 0.0001$), and *wMelPop* (one-way ANOVA: $F_{3,8} =$
391 56.34, $p < 0.0001$; Fig. 4A-C). To date, HIE-18 cells infected with *wAlbB*, *wMel*, and
392 *wMelPop* have been successfully maintained for 70, 60 and 50 passages,
393 respectively, with stable infection and no significant change in *Wolbachia* density
394 over these passages (Fig. 4D-F). Interestingly, *wAlbB* appeared to grow at lower
395 density than the other two strains, *wMel* and *wMelPop*, in HIE-18 cells (Fig. 4A-F).

396 3.5. Effect of *Wolbachia* infection on the population doubling time of HIE-18 **397** cells

398 Population doubling time was calculated after stably infecting HIE-18 with *wAlbB*,
399 *wMel*, and *wMelPop* strains. The population doubling time for the uninfected HIE-18
400 cells was approximately 67.0 hours and the slope of the cell number by timeline plot
401 consequently higher than the infected lines ($y = 7.2032x + 12.088$; $r^2 = 0.998$) (Fig.
402 5). For the *wMel*-HIE-18 (77.0 hours) and *wAlbB*-HIE-18 (79.2 hours) doubling time
403 was longer and the slope of the population growth lines consequently not as steep
404 ($6.479x + 11.716$; $r^2 = 0.997$; $5.7843x + 11.756$, $r^2 = 0.994$). The *wMelPop*-HIE-18
405 grew more slowly than the others with a doubling time of approximately 86.4 hours (y
406 = $4.363x + 13.036$; $r^2 = 0.992$). The newly introduced *Wolbachia* infections appear to

407 exert varying levels of stress on the new host cells, increasing their doubling time
408 and this effect was most marked in the case of the *wMelPop* infection.

409 **3.6. Effect of culture media and temperature on *Wolbachia* growth in HIE-18**
410 **cells**

411 *Wolbachia* infected HIE-18 cells in three different standard media (Schneider's
412 medium, Leibovitz's L-15 medium, and Shield's medium) and a mixture of Leibovitz's
413 L-15 and Schneider's media (1:1) were grown for *Wolbachia* quantification after
414 seven days of incubation. There was a significant effect of the Schneider's medium
415 on *wAlbB* strain density (Tukey test: $p=0.02$) and Schneider's (Tukey test: $p=0.01$)
416 and 1:1 mix of Schneider's and L-15 media (Tukey test: $p=0.03$) on *wMelPop* density
417 (Fig. 6) when compared to baseline. However, no effect of the media was seen on
418 *wMel* density.

419 Infected cells were grown at four different temperatures (26, 28, 30, and 32°C) and
420 *Wolbachia* was quantified using a similar protocol to that described above (Fig. 7).
421 Overall, seven days of incubation at assigned temperatures resulted in significant
422 differences between temperatures in *Wolbachia* density of *wAlbB*-HIE-18 (one-way
423 ANOVA: $F_{4,10}=22.97$, $p<0.0001$), *wMel*-HIE-18 (one-way ANOVA: $F_{4,10}=27.24$,
424 $p<0.0001$), and *wMelPop*-HIE-18 (one-way ANOVA: $F_{4,10}=39.99$, $p<0.0001$).
425 However, there was a strain-specific response to temperature. In *wAlbB*-HIE-18
426 cells, *Wolbachia* density was highest at 28°C and decreased with increase in
427 temperature above this (Fig. 7A). *wMel* maintained highest density at 26°C (Fig. 7B),
428 whereas *wMelPop* density increased with the temperature, highest at 30°C, but
429 decreased at 32°C (Fig. 7C).

430 3.7. Innate immune response of HIE-18 cell line against *Wolbachia*

431 Changes in *H. irritans* expression levels were investigated for the Imd (Immune
432 deficiency) pathway transcription factor Relish (Fig. 8A-C), Toll pathway repressor
433 Cactus (Fig. 8D-F), and three AMPs, Attacin, Cecropin, and Defensin (Fig. 8G-O)
434 within 48 hours of initial infection with *Wolbachia* to better understand the *H. irritans*
435 innate immune response towards the endosymbiont. There was a significant
436 increase in Relish expression within 12 hours for *wAlbB* (Tukey test: $p=0.005$), *wMel*
437 (12 hours; Tukey test: $p<0.0001$), and *wMelPop* (12 hours; Tukey test: $p<0.0001$),
438 which remained overexpressed for *wMel* and *wMelPop* until 24 hours suggesting
439 activation of the Imd signalling cascade, followed by deactivation. Cactus was
440 significantly upregulated in comparison with the baseline at 12, 24 and 36 hours with
441 a maximum at 24 hours (Tukey test: $p<0.0001$) but decreased after this time. AMPs
442 are effector molecules for immune response and in HIE-18 cells all three AMPs
443 tested were significantly upregulated within 36 hours of infection with few exceptions
444 at 12 hours (see Fig. 8I, L, N). Overall, expression of AMPs was highest at 24 hours
445 and decreased after this time.

446 4. DISCUSSION

447 Insect cell lines play a critical role in understanding vital biological processes and
448 insect-microbe interactions. Most mosquito cell lines were initiated with the intention
449 of studying mosquito-arboviruses interactions⁴⁹. They have since been utilised in
450 studying molecular interactions between hosts and endosymbionts that are unable to
451 grow in the absence of host cells or tissues⁵⁰⁻⁵². Here we report the establishment of
452 an *H. irritans* cell line (HIE-18) from embryos and studies of the interaction of HIE-18
453 cells with the endosymbiont *Wolbachia*.

454 Donor tissue plays a critical role in establishing insect cell lines. In the past, many
455 attempts using specific adult tissues have resulted in limited success with relatively
456 few significant exceptions ⁵³. Currently, the use of whole embryonic and neonate
457 larvae as an inoculant is a common approach as cells directly derived from this
458 tissue often grow and proliferate better ⁵³. We tried three different tissue sources as
459 inoculum to initiate the primary cell line from HF: whole embryos (egg), neonate
460 larvae and pupae. A major challenge to the establishment of the *Haematobia* cell
461 culture from larvae and pupae was avoiding microbial contamination. Despite the
462 use of multiple different disinfection protocols, fresh cattle manure used to maintain
463 the source *Haematobia* colony was speculated to be a primary source of
464 contaminant for the cell lines ⁵⁴. In embryonic culture, one of the flasks HIE-18 with
465 heterotypic cellular morphologies resulted in better cellular interaction leading to the
466 successful establishment of a cell culture system. In the HIE-18 cell line, cell types
467 with an approximately circular morphology have become predominant.

468 Frequently, primary cells have limited differentiation capabilities and undergo
469 senescence within a short period (40-50 generations for mammalian primary cells) ⁴²,
470 ⁵⁵. Sometimes, differentiation capabilities can be altered either naturally or through
471 mutation resulting in a continuously proliferating cell system ⁴². The developed HIE-
472 18 cell line has been subcultured through more than 200 passages, which equates to
473 more than 400 generations suggesting that the HIE-18 cells have formed a
474 continuous cell line. We have also thawed cryopreserved cells and in most instances
475 the thawed cells have been found to be mitotically active and viable, indicating that
476 HIE-18 cell line can be maintained for long term storage.

477 Horn flies are karyotypically diverse ⁵⁶. Of three chromosomal investigations in HF
478 two sourced insects from the USDA Livestock Insect Research Laboratory, Kerrville

479 TX, one from a wild population from Argentina and one from a population of HF from
480 Uruguay⁵⁶. Horn flies from the USDA Lab colony had five pairs of chromosomes,
481 without a sex chromosome^{57, 58}. However, the wild HF populations from Argentina
482 and Uruguay had an extra B-chromosome and the resultant karyotype of $2n=11$ ($2n$
483 = $10+ B$) in nearly 50% of the tested flies⁵⁶. Our cell line was established from
484 embryos sourced from the USDA laboratory HF colony and had a similar
485 chromosomal karyotype to that previously reported from the USDA flies⁵⁸.

486 Identification of appropriate media, and temperature for optimal cell growth, is a
487 critical step in the establishment of a cell line. Growth conditions for early cell lines
488 were usually determined by tedious experimentation and testing of different
489 permutations of media components and growth conditions⁵³. The current preferred
490 approach is to first test different commercially available media⁵⁹. Initially, the HIE-18
491 cell lines were established and cultured in modified Leibovitz's L-15 medium
492 designed for culturing a tick cell line by adding cholesterol, vitamins, glucose and
493 amino acids to the L-15 medium⁴⁴. Based on our findings, we have recently moved
494 to using Schneider's medium for improved HIE-18 growth. Schneider's medium is
495 widely used for culturing *D. melanogaster* cell lines and differs in composition (mainly
496 inorganic salts, vitamins, amino acids and cholesterol) from modified L-15 medium
497⁶⁰. Mostly, insect cells are cultured between 25 and 30°C and have been found to
498 have reduced cell replications at lower temperatures⁶¹. However, as a result of the
499 study described here we are now maintaining the HIE-18 cells at 30°C for better
500 proliferation.

501 Most available knowledge of insect cellular immune responses has been derived
502 from studies with either *Drosophila* or mosquito cells, but the immune response in
503 *Haematobia* species is less well understood. Cellular transinfection of insect cells

504 with *Wolbachia* from different host backgrounds has shown mixed results in
505 mosquitoes, and other dipteran species^{19, 34, 49}. It took a number of attempts to
506 stably infect HIE-18 cell lines with *Wolbachia* as density in the infected HIE-18 lines
507 decreased significantly in the first 48 hours after introduction. To better understand
508 the initial interaction between the HIE-18 cells and *Wolbachia*, several gene
509 expression studies were carried out with the Imd pathway transcription factor Relish,
510 Toll pathway repressor gene Cactus, and three AMPs, Attacin, Cecropin, and
511 Defensin. The immune gene expression study suggested that *wAlbB*, *wMel*,
512 *wMelPop* infections in the HIE-18 cell line activated the Imd pathway, resulting in the
513 production of AMPs and reduced survival of *Wolbachia* until the infection became
514 established in the HIE-18 lines. These results are consistent with the upregulation of
515 the *Relish*, *Attacin*, *Cecropin*, and *Defensin* genes seen within 72 hours of infection
516 of the LL-5 sand fly cell line with *wMel* and *wMelPop-CLA* *Wolbachia*, and *Cactus* in
517 the *Spodoptera frugiperda* Sf9 cell line infected with *wMel* *Wolbachia*^{19, 34}. Despite
518 the initial upregulation of immune response genes, HIE-18 cell lines have become
519 stably infected with *wAlbB*, *wMel*, and *wMelPop* which suggests a level of
520 permissiveness of *Haematobia* species to *Wolbachia* or that *Wolbachia* is able to
521 suppress or overcome the initial *H. irritans* innate response.

522 After the establishment of stable infections of *Wolbachia* in the HIE-18 cell line the
523 optimal medium and temperature to achieve maximum densities of *Wolbachia* were
524 determined. Microbial endosymbionts play an essential role in host metabolism by
525 nutrition provisioning, and vice versa⁶². Host nutrition and metabolism also influence
526 endosymbiont dynamics⁶². For example, supplementation with thiamine
527 monophosphate decreased endosymbiont *Sodalis* and *Wigglesworthia* density in
528 tsetse flies. Another similar example is supplementation of a *Drosophila* diet with

529 sucrose which led to a higher density of *Wolbachia* in comparison to a yeast
530 enriched diet ⁶²⁻⁶⁴. Temperature is another critical factor that can modulate host-
531 endosymbiont interactions as it directly affects the rate of biochemical reactions,
532 which can alter the performance and survival of organisms ⁶⁵. Most temperature
533 related studies have been done in either *Drosophila* or mosquito species and
534 reported effects on *Wolbachia* density inside hosts can be complex and quite
535 variable ⁶⁶⁻⁷⁰. Very little is known about the influence of culture medium and
536 temperature in the context of different *Wolbachia* strain infected host cell lines.
537 Interestingly, culture medium did not have any effect on wMel density inside HIE-18
538 cells, but wAlbB and wMelPop had higher density in HIE-18 cells cultured in
539 Schneider's medium. *Wolbachia* strains differ in temperature preference as
540 previously seen with other insect species ^{68, 70}. This study has found that incubating
541 *Wolbachia*-infected cell lines at 28°C will result in higher densities of *Wolbachia*,
542 which will be required for future transinfection of *Haematobia* spp.

543 5. CONCLUSION

544 We have successfully developed a continuous cell line from *H. irritans* (HIE-18) and
545 identified Schneider's cell culture medium and 30°C incubation temperature as
546 optimal for growth. The density of *Wolbachia* (wMelPop, wMel, and wAlbB)
547 decreased in the HIE-18 cells within 36 hours of infection, associated with the
548 production of AMPs via the Imd immune pathway, but densities subsequently
549 recovered. Currently, HIE-18 cells stably infected with these three strains of
550 *Wolbachia* have been subcultured over 50 passages suggesting permissiveness of
551 the HIE-18 cells for growth of this bacterium. The *Wolbachia* described here,
552 adapted to and growing in high density in the *Haematobia* cell lines, will provide an

553 important resource toward the development of novel control approaches for buffalo
554 flies and horn flies.

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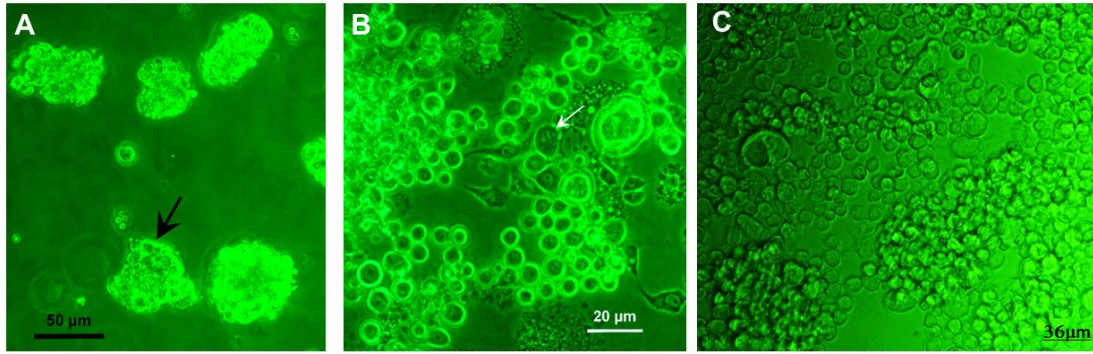
738 **Table. 1.** List of primers designed to study immune response to infection with
739 *Wolbachia* in *H. irritans*.

740

Gene	Primer (5'-3')	Size (bp)
Relish	60_F_TGTCGAATAACCACTGGTGCCAGA 159_R_TCAATCGCCTGTTGACAAGGACA	100
Cactus	461_F_AAGGAAGCAAGCCGAACATGACGA 560_R_AGTCTTGTTGCGGAATGTCCCAGA	100
Attacin	163_F_AGCTCCATGACCATTGGCATGAT 262_R_TGCCACAGCCTTCCATAGTCGT	100
Cercopin	253_F_CGAATGCCCACTGATGTCACAACC 351_R_TCGCTGATCATCACCATAATGCTCA	100
Defensin	164_F_ACTTGTTGAGCGGTACTGGTGT 263_R_ACACAAACCGCTTTGGAGTTGCA	100
GAPDH	378_F_CCGGTGGAGGCAGGAATGATGT 445_R_CCACCCAAAAGACCGTTGACG	88

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742



743

744 Fig. 1. Cellular morphology and features of *H. irritans* cell cultures. **A.**

745 Embryonic fragments seeded into primary cultures, one-day post-inoculation; arrow

746 indicates a cluster of 20-30 cells. **B.** Round cells predominate in young HIE18

747 cultures, third transfer, two months in culture. Arrow indicates a cell in metaphase. **C.**

748 Current continuous HIE-18 isolate cells (June 5, 2019) maintained in Schneider's

749 medium. The cells are predominantly round, 5 - 10 μm , loosely adherent and form

750 multicellular clusters.

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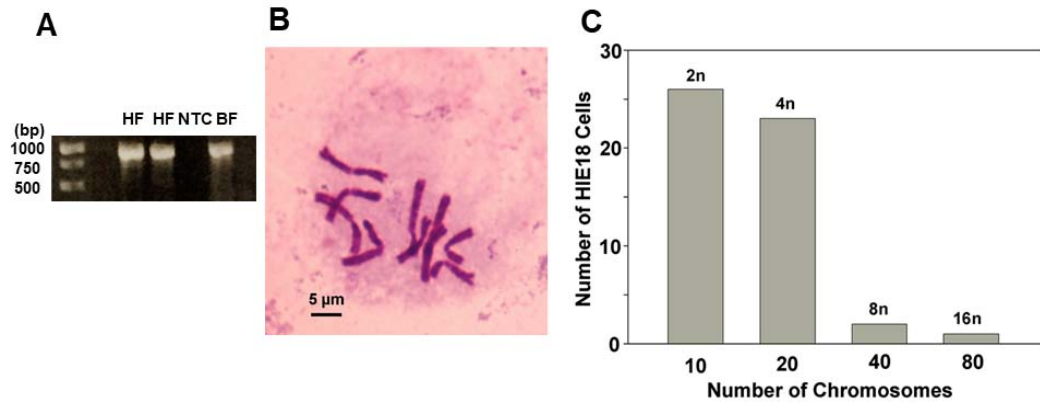
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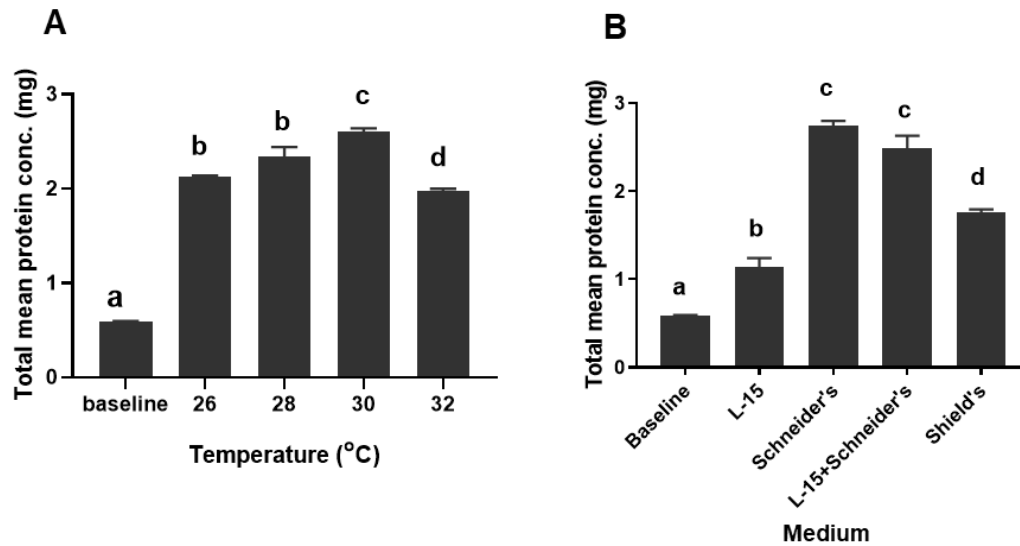


757

758 **Fig. 2. PCR and chromosome profile confirmation of HIE18.** 759 **A.** Approximate 920-
760 bp product amplified using mitochondrial-encoded COI primer specific to
761 *Haematobia* spp. HF: *H. irritans* HIE-18 cell line, NTC: Negative control, BF: *H.*
762 *exigua*. **B.** Metaphase chromosome spread in Giemsa stained diploid HIE-18 cells.

763 **C.** Graph shows a number of metaphase chromosomes in *H. irritans* cells.

763



764

765 Fig. 3. Protein production in HIE-18 cells grown in different temperatures and

766 culturing mediums. Left axis shows protein concentration used as a proxy for the

767 HIE-18 cellular growth, determined using a Bradford assay. Here, baseline data is

768 protein production from the HIE-18 cells grown in modified L-15 medium for 24 hours

769 at 30°C before assigning test temperature and test medium. **A.** Total protein

770 production was significantly different between the cultures incubated at different

771 temperatures ($F_{4, 10} = 237.1, p < 0.0001$). **B.** Total protein production was significantly

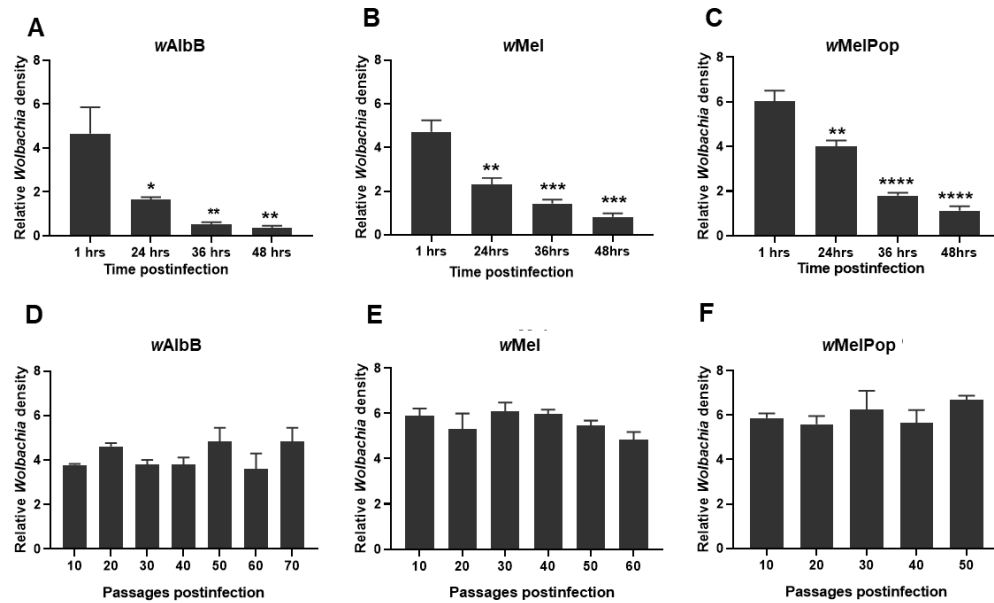
772 different between the cultures grown in different culturing media ($F_{4, 10} = 118.3,$

773 $p < 0.0001$). Error bars are SEM calculated from three replicates of culture flasks.

774 Columns with different letters are significantly different (Tukey's multiple comparison

775 test; $p = 0.05$).

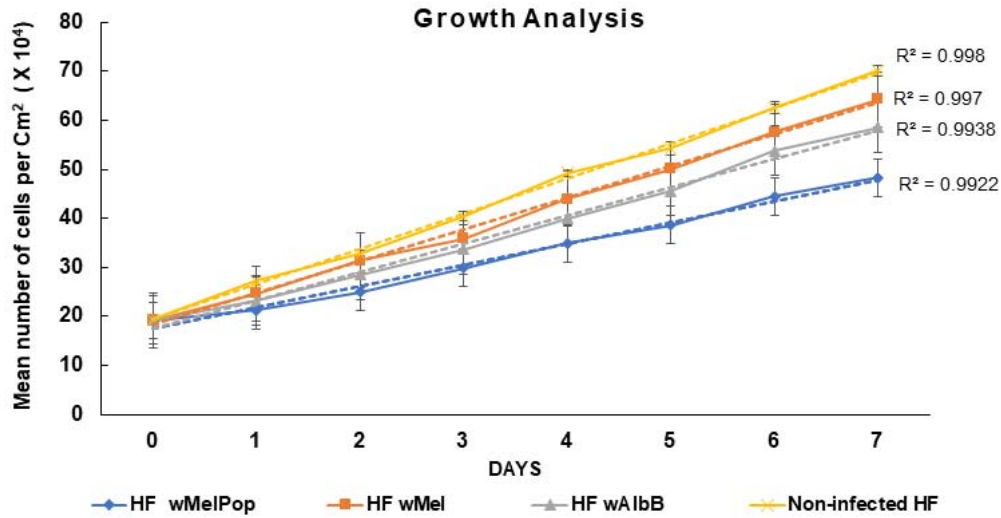
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778 Fig. 4. Quantification of *Wolbachia* (*wsp*) relative to host (*GAPDH*) using real-
779 time PCR in HIE-18 cell post-infection. (A-C). Significant decreases in wAlbB,
780 wMel and wMelPop were observed within 48 hours post-infection. Bars with
781 asterisks represent significant difference compared to one hour post-infection using
782 Dunn's test; * $p < 0.02$, ** $p < 0.003$, *** $p < 0.0003$, **** $p < 0.0001$. D. wAlbB dynamics in
783 HIE-18 cell line over 70 passages. E. wMel dynamics in HIE-18 cell line over 60
784 passages. F. wMelPop strain dynamics in HIE-18 cell line over 50 passages. Tukey's
785 test ($p = 0.05$) showed no significant variation in *Wolbachia* infection over passages
786 (D-E). Error bars indicate SEM from triplicate culturing flasks.

787



788

789 Fig. 5. *In vitro* proliferation of the non-infected and differentially *Wolbachia*

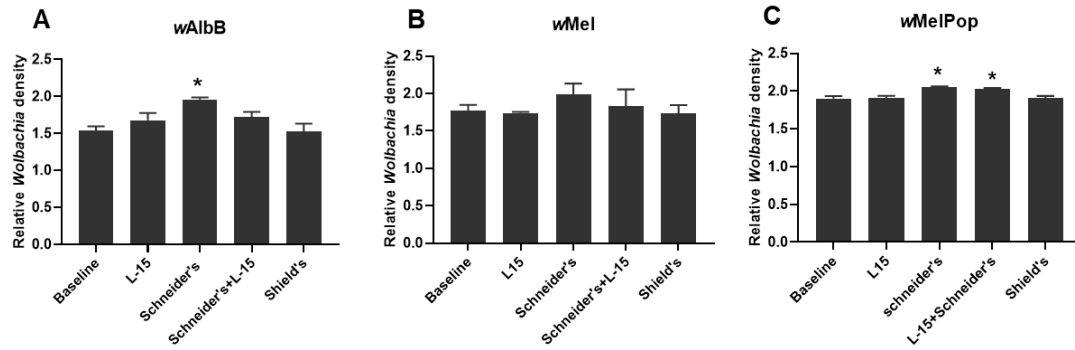
790 infected HIE-18 cell lines. Cells infected with *Wolbachia* (*wAlbB*, *wMel*, and

791 *wMelPop*) proliferated slowly, resulting in increased doubling time. Horizontal axis

792 represents the mean number of cells per cm^2 area + SD of three replicate culturing

793 flasks.

794



795

796 Fig. 6. *Wolbachia* density in HIE-18 cells grown in different media. *Wolbachia*

797 density was calculated relative to host *GAPDH* after growing infected HIE-18 cells in

798 test medium for seven days. Here, baseline is *Wolbachia* density in HIE-18 cells

799 grown in modified L-15 medium for 24 hours before assigning the test medium. **A.**

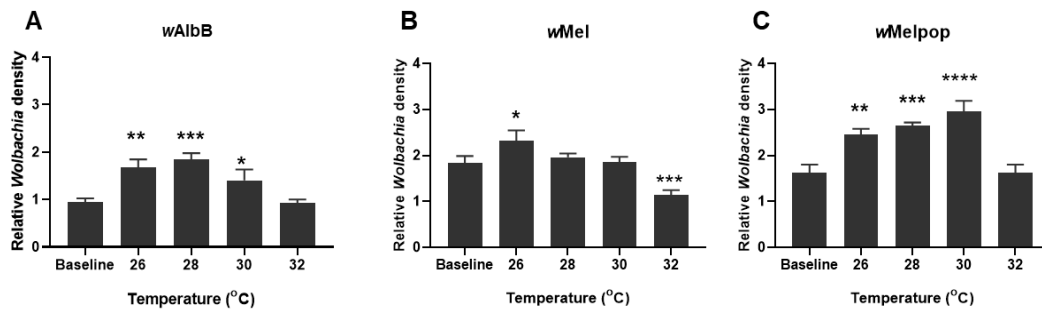
800 wAlbB infected HIE-18 cell line. **B.** wMel infected HIE-18 cell line. **C.** wMelPop

801 infected HIE-18 cell line. Error bars are SEM calculated from three replicates of

802 culturing flasks. Asterisks represent significance compared to baseline growth using

803 Tukey's test (* $p < 0.05$).

804



805

806 Fig. 7. *Wolbachia* density in HIE-18 cells incubated at different temperatures.

807 *Wolbachia* density is calculated relative to host GAPDH after growing infected HIE-

808 18 cells at test temperatures for seven days. Here, baseline data is *Wolbachia*

809 density in HIE-18 cells grown in modified L-15 medium for 24 hours at 30°C before

810 assigning test temperature. **A.** wAlbB-infected HIE-18 cell line. **B.** wMel-infected HIE-

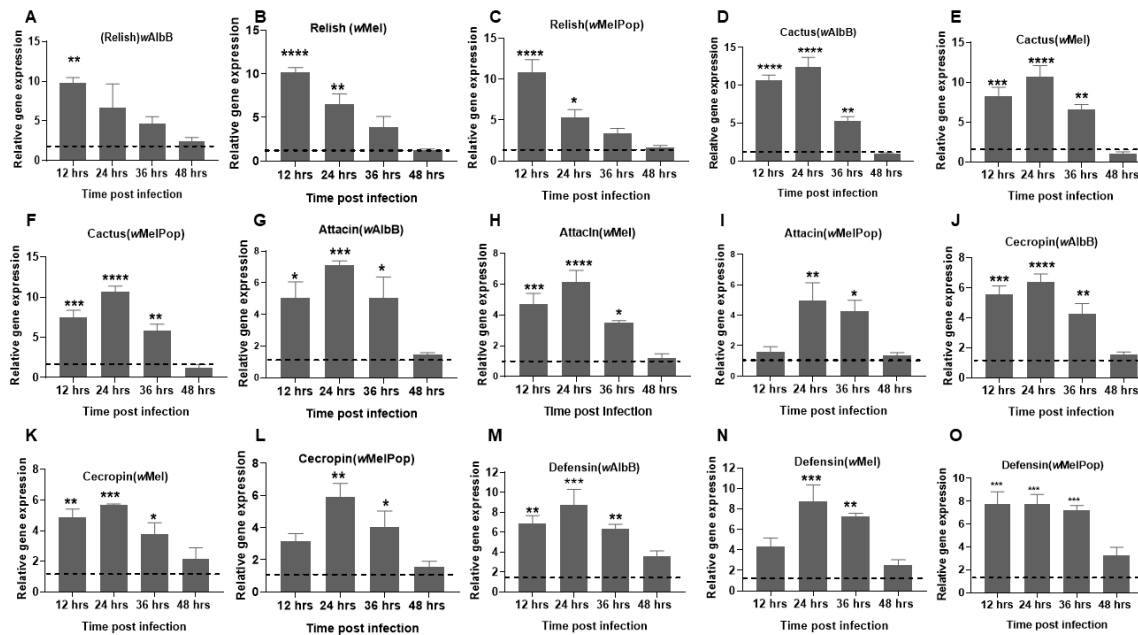
811 18 cell line. **C.** wMelPop-infected HIE-18 cell line. Error bars are SEM calculated

812 from three replicate culturing flasks. Asterisks represent significance compared to

813 baseline using Tukey's test (* $p < 0.03$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$).

814

815



816

817 Fig. 8. Relative gene expression of the immune pathways IMD, Toll, and AMPs
 818 in *H. irritans* determined by real-time PCR within 48 hours of infection with
 819 *Wolbachia* (wAlbB, wMel, wMelPop). (A-C) Relative gene expression of *relish*, a
 820 regulator of the Imd pathways. (D-F) Relative expression of the *cactus* gene
 821 regulator of the Toll pathway. Relative gene expression of AMPs (G-I) *attacin* (J-L)
 822 *cecropin* (M-O) *defensin*. Error bar is SEM calculated from three independent
 823 experimental replicates. Quantification was normalised to the housekeeping gene
 824 *GAPDH*, and the dotted line here represents the relative gene expression of the non-
 825 infected control. Asterisks represent significance compared to control using a
 826 Tukey's test (* $p < 0.03$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$).