

1 **Establishment of an *in vitro* culture system to study the**
2 **developmental biology (growth, mating and nodule formation) of**
3 ***Onchocerca volvulus* with implications for anti-*onchocerca* drug**
4 **discovery and screening**

5 Narcisse Victor T. Gandjui^{1,2}, Abdel Jelil Njouendou^{1,2,3}, Eric Njih Gemeg^{1,2}, Fanny Fri
6 Fombad^{1,2,4}, Manuel Ritter⁵, Chi Anizette Kien^{1,2}, Valerine C. Chunda^{1,2}, Jerome Fru^{1,2},
7 Mathias E. Esum^{1,2}, Marc P. Hübner⁵, Peter A. Enyong^{1,2}, Achim Hoerauf^{5,6}, Samuel
8 Wanji^{1,2,*}

9

10 ¹Research Foundation for Tropical Diseases and the Environment (REFOTDE), Buea,
11 Cameroon.

12 ²Parasite and Vector Research Unit (PAVRU), Department of Microbiology and
13 Parasitology, University of Buea, Buea, Cameroon.

14 ³Department of Biomedical Sciences, Faculty of Health Sciences, University of Buea,
15 Buea, Cameroon.

16 ⁴Department of Zoology and Animal Physiology, Faculty of Science, University of
17 Buea, Buea, Cameroon.

18 ⁵Institute for Medical Microbiology, Immunology and Parasitology (IMMIP), University
19 Hospital Bonn, Bonn, Germany.

20 ⁶German Center for Infection Research (DZIF), Bonn - Cologne partner site, Bonn,
21 Germany.

22

23

24 * Corresponding author. E-mail: swanji@yahoo.fr

25 **Authors E-mails**

26 Narcisse V. T. Gandjui: gvictornarcisse@yahoo.com

27 Abel J. Njouendou: ajnjouendou@gmail.com

28 Eric N. Gemeg: ericnjih@yahoo.co.uk

29 Fanny F. Fombad: ffffombad@gmail.com

30 Manuel Ritter: Manuel.Ritter@ukbonn.de

31 Chi A. Kien: kienchi91.kc@gmail.com

32 [Valerine C. Chunda : chundavalerinne@yahoo.com](mailto:chundavalerinne@yahoo.com)

33 Jerome Fru-Cho : jerome.frucho.1@gmail.com

34 Mathias E. Esum: mathias_mesum@yahoo.fr

35 Marc P. Hübner: huebner@uni-bonn.de

36 Peter A. Enyong: enyongap@gmail.com

37 Achim Hoerauf: achim.hoerauf@ukb.uni-bonn.de

38 Samuel Wanji: swanji@yahoo.fr

39

40 **Keywords:**

41 *O. volvulus*

42 *in vitro* culture

43 growth

44 development

45 motility

46 moulting

47 mating

48 competition

49 cellular aggregation

50 nodulogenesis

51

52 **Abstract**

53 **Background:** Infections with *Onchocerca volvulus* nematodes remain a threat in Sub-
54 Saharan Africa after two decades of ivermectin mass drug administration. Despite this
55 effort, there is still an urgent need for understanding the parasite biology, especially
56 mating behaviour and nodule formation, as well as development of more potent drugs
57 that can clear the developmental (L3, L4, L5) and adult stages of the parasite and
58 inhibit parasite's reproductive and behavioural pattern.

59 **Methodology/Principal Findings:** Prior to culture, freshly harvested *O. volvulus* L3
60 larvae from dissected *Simulium* were purified by centrifugation using a 30% Percoll
61 solution to eliminate fly tissue debris and contaminants. Parasites were cultured in both
62 cell-free and cell-based co-culture systems, and monitored daily by microscopic visual
63 inspection. Exhausted culture medium was replenished every 2–3 days. The cell-free
64 culture system supported the viability and motility of *O. volvulus* larvae for up to 84
65 days (DMEM–10%NCS), while the co-culture system (DMEM–10%FBS–LLC-MK₂)
66 extended the worm survival period to 315 days. Co-culture systems alone promoted
67 the two consecutive parasite moults (L3 to L4 and L4 to L5) with highest moulting rates
68 observed in DMEM–10%FBS–LLC-MK₂ (69.2±30 %), while no moult was observed in
69 DMEM–10%NCS–LEC condition. *O. volvulus* adult worms mating and even mating
70 competitions were observed in DMEM–10% FBS –LLC-MK₂ co-culture system. Early
71 nodulogenesis was observed in both DMEM–10% FBS–LLC-MK₂ and DMEM–
72 10%NCS–LLC-MK₂ systems.

73 **Conclusions/Significance:** The present study describes an *in vitro* system in which
74 *O. volvulus* L3 larvae can be maintained in culture leading to the development of
75 reproductive adult stages. Thus, this platform gives potential for the investigation of

76 mating, mating competition and early stage of nodulogenesis of *O. volvulus* adult
77 worms that can be used as additional targets for onchocercacidal drug screening.

78 **Author summary**

79 River blindness affects people living in mostly remote and underserved rural
80 communities in some of the poorest areas of the world. Although significant efforts
81 have been achieved towards the reduction of disease morbidity, onchocerciasis still
82 affect million of people in Sub-Saharan Africa. The current control strategy is the
83 annual mass administration of ivermectin which have accumulated several drawbacks
84 overtime: as the sole microfilaricidal action of the drug, very long treatment period (15-
85 17 years) and reports of ivermectin losing its efficacy; Therefore, raising the urgent
86 need for new onchocercacidal molecules. Our study has established an *in vitro*
87 platform capable of supporting the growth and development of all developmental
88 stages of *O. volvulus* (L3 infective stage, L4, L5 and adult worms), moreover the
89 platform provided more insight on *O. volvulus* adult worms reproductive and
90 behavioural pattern. Our findings provide more avenues for mass production of
91 different parasite stages, the investigation of parasite developmental biology and the
92 identification of targets for drug discovery against different phases of development of
93 this filaria parasite

94

95 **Introduction**

96 Onchocerciasis is the second major cause of infectious blindness and a major public
97 health problem in many parts of the world [1]. The disease is known as river blindness,
98 because its causative agent *Onchocerca volvulus* is transmitted by *Simulium* (blackfly)
99 vectors, which breed in fast-flowing rivers. Onchocerciasis is endemic in 37 countries

100 in West, East and Central Africa, the Arabian Peninsula and parts of South and Central
101 America. Globally, about 90 million people are at risk of contracting the disease in
102 endemic areas, with 99% of cases occurring in Sub-Saharan Africa, of which more
103 than 17 million are estimated to be infected and 270,000 are permanently blind as
104 complication [2]. In the past decades, two programmes were implemented to control
105 onchocerciasis. Initially, the Onchocerciasis Control Programme (OCP) focused on
106 elimination of the *Simulium* vector using DDT and larvicides. This vector control
107 programme was later supplemented with ivermectin distribution that has helped many
108 millions of persons to live free of disease [3]. Mass drug administration (MDA) of
109 ivermectin through the African programme for Onchocerciasis control (APOC) has
110 been the principal strategy for onchocerciasis control after OCP in West Africa.
111 Despite more than two decades of MDA campaign with ivermectin, the disease still
112 persists mainly in sub-Saharan Africa because of several reasons. Ivermectin is solely
113 microfilaricidal with temporal embryo-static effects on adult worms and thus has to be
114 given once or twice per year for the life-span of the adult worms, which is 16–18 years.
115 In addition, the sub-optimal response to ivermectin in some regions has led to
116 persistent transmission [4-6]. Therefore, there is a need for the development of
117 improved drugs that might not only kill *O. volvulus* microfilariae but also other life-cycle
118 stages (L3, L4, L5 and adult worms). Such new candidates are required to reach the
119 United Nations Sustainable Development Goals to eliminate onchocerciasis by 2030
120 in the majority of endemic countries. However, the advancement of research towards
121 the development of new therapeutics is hindered by the availability of a suitable *in vitro*
122 culture system, where parasite stages such as infective larvae (L3) can be maintained
123 and developed into adults. An artificial system which can mimic the human host micro-

124 environment and support the growth and development of *O. volvulus* parasites from
125 the infective stage larvae (L3) to adults would be ideal.

126 Literally, little is known about the time course of *O. volvulus* parasite developmental
127 process, mating behaviour and nodule formation in the human host. Nevertheless, it is
128 reported that moulting from L3 to L4 occurs within a week (3–7 days) [7], while the L4
129 to L5 moult is estimated to occur after 2 months [8]. Early L5 are considered young
130 adults, and at this stage the worms have partially developed gonads [9]. In the cattle
131 model, it takes 279–532 days post infection for the closely related *O. ochengi* parasite
132 to develop into fully mature and fertile adult worms capable of releasing microfilariae
133 (mf), the worms' offspring [10]. More than 400 days post infection is required for the
134 same achievement in a chimpanzee model for *O. volvulus* [8, 11].

135 Many non-conclusive attempts have been carried out to develop the complete life cycle
136 of *O. volvulus* in artificial *in vitro* systems, though these contributions have been
137 considered as important milestones towards achievement this ultimate goal [7, 9, 12,
138 13, 14, 15, 18, 21, 22, 28, 32, 35, 43]. Serum/cell free systems have been used in
139 several studies to culture filarial parasites and reports have highlighted maintenance
140 of full viable parasites for a week [12–22]. Improvement of the culture conditions have
141 been achieved by supplementing the basic culture media used with serum or other
142 culture ingredients (fatty acids and complex lipids formulation). The serum-based
143 culture systems have been reported to support parasite longevity *in vitro* and cuticle
144 casting [21, 23–32]. Due to the inconsistency of serum composition, serum-free culture
145 systems and co-culture systems using eukaryotic cells as feeder layers have been
146 found successful [33–43]. Moreover, feeder cells were already shown to be crucial for
147 *in vitro* cultivation and growth of *O. volvulus* [44]. From our previous observations on
148 the *in vitro* growth and development of the filarial nematode *Loa loa* [42], among the

149 three most used supplements (feeder layer, serum, and basic culture medium) for
150 filarial parasites *in vitro* culture, feeder cells were classified as top most important
151 requirement followed by the serum type and finally the nature of the basic culture
152 medium. Summarily, the advancement of research towards the development of a
153 suitable *in vitro* culture system for filarial parasites has highlighted the complexity of
154 their requirement in terms of nutritional needs for growth and moulting from one stage
155 to another. This study aimed at identifying suitable *in vitro* culture requirements, which
156 could support the maintenance and promote the growth and development of the human
157 parasite *Onchocerca volvulus* from its infective L3 larvae stage to reproductive adult
158 worms. Such an *in vitro* culture system will contribute to the experimental production
159 of subsequent parasite stages (L4, L5 and adults of *Onchocerca volvulus*), enable
160 investigations on the reproductive behaviour as well as nodule formation that could be
161 used for further understanding of the parasite biology and identification of novel
162 therapeutic drug targets against onchocerciasis.

163

164 **Methods**

165 **Ethical statement**

166 Ethical clearance was obtained from the National Institutional Review board, Yaoundé
167 (N^o 2018/06/1057/CE/CNERSH/SP) after approval of the protocol. Prior to recruitment,
168 the nature and objectives of the study were explained to potential participants and
169 those who agreed to take part in the study signed a consent form. Special
170 consideration was taken to minimize any health risks of the participant. They were
171 followed-up for ivermectin treatment at the end of the study during the normal MDA

172 period. Their participation was strictly voluntary and their documents were given a code
173 for confidentiality.

174

175 **Determination of *O. volvulus* microfilarial load in skin biopsies of volunteers**
176 **prior to *Simulium* engorgement**

177 Participants examined were from the Meme drainage basin (overall Community
178 Microfilarial Loads (CMFL) = 5.2 microfilariae / skin snip) and microfilarial load was
179 determined as described by Wanji *et al.* [45]. Briefly, after the clinical examination, two
180 skin biopsies from the posterior iliac crest were taken using a 2 mm corneo-scleral
181 punch (CT 016 Everhards 2218–15 C, Germany). The skin samples from each
182 participant were placed in two separate wells of a microtitre plate containing 2 drops of
183 sterile normal saline. The corresponding well numbers were reflected on the
184 participant's form. The plates were sealed with parafilm to prevent any spill over or
185 evaporation and incubated at room temperature for 24 hours. All emerged microfilariae
186 were counted using an inverted microscope (Motic AE21) at 10x magnification and
187 expressed per skin snip. Two participants were enrolled in the study that had average
188 microfilariae load of 50 and 65 microfilariae/skin snip respectively.

189 **Collection of engorged *Simulium* flies**

190 Flies were collected along the banks of a fast-flowing river at Mile 16 Bolifamba (South
191 West region – Cameroon). The fly collection team was composed of two trained
192 individuals, one working from 07:00 AM to 12:00 Noon and the other from 12:00 Noon
193 to 18:00 PM for 5 consecutive days. Female blood-seeking *Simulium* flies were allowed
194 to land on exposed legs of the microfilaridemic donor, where they were allowed to

195 blood-feed and then captured using *Simulium* rearing tubes. Captured-engorged
196 *Simulium* were then transported to the laboratory insectarium and maintained for 10
197 days for the development of *O. volvulus* infective L3 larvae.

198 **Laboratory maintenance of engorged *Simulium***

199 Blood-fed *Simulium* were maintained in captivity under controlled experimental
200 conditions as described by [58] for 10 days to allow ingested microfilariae to mature
201 into infective stage larvae (L3). Briefly, captive flies were fed on 15% sucrose solution
202 soaked in cotton wool and maintained at 23–28°C and 79–80% relative humidity.

203 **Dissection of flies, isolation and purification of *O. volvulus* L3**

204 After 10 days of rearing, the flies were dissected in Petri dishes (CytoOne, UK)
205 containing RPMI 1640 medium (Sigma-Aldrich, St Louis, USA). The head, thorax and
206 abdomen were separated and teased apart in three different dishes. Fly tissues were
207 incubated for 20 min to allow L3 larvae to migrate out of the tissue. A sterile pipette
208 was used to pick the larvae and pooled in a shallow convex glass dish [46]. The worms
209 were transferred into 15 ml centrifuge tubes (Corning, Kennebunk-ME, USA) for
210 purification. Only L3 harvested from the head (where more mature larvae are expected
211 to be found) were used in this study. The L3 were washed using a Percoll® (GE
212 Healthcare, Pharmacia, Uppsala, Sweden) centrifugation technique as described by
213 Zofou *et al.* [42]. In summary, the L3 suspension concentrated in less than 1 ml RPMI
214 was slowly layered on the surface of a 15 ml tube containing stock iso-osmotic Percoll®
215 and centrifuged (Humax 14k human, Germany) at 68 x g for 10 min. The process was
216 repeated to remove microbial contaminants. At the end, the L3 were washed twice with
217 RPMI-1640 by centrifugation at 239 x g for 10 min to remove Percoll® remnant.

218 **Preparation of feeder cells and pre-conditioning in culture plates**

219 Monkey kidney cells (LLC-MK₂), mouse lung embryonic cells (LEC), human embryonic
220 kidney cells (HEK-293) and human hepatic cells (HC-04) were provided by the
221 American Type Culture Collection (ATCC, Manassas, Virginia, USA). Each of these
222 feeder cells were cultured in flasks at 37 °C in a humidified CO₂ incubator (Sheldon
223 Mfg. Inch, Cornelius, OR, USA) at 5% CO₂ until the cell layer became fully confluent.
224 For new inoculations and other cell manipulations, cells were dislodged with trypsin
225 solution (25%) containing EDTA and kept at 37 °C for less than 30 minutes. The cell
226 suspension was centrifuged at 239 x g for 10 min, the supernatant discarded, and the
227 pellet re-suspended and diluted to 10⁵ cells/ml. Aliquots (100 µl) of cell suspensions
228 were plated into each well of a 48-well flat bottom culture plate and kept in the incubator
229 for cells to become fully confluent prior to be used for parasite maintenance in co-
230 culture systems.

231 ***In vitro* culture of *O. volvulus* larvae**

232 Harvested L3 from different batches of dissected flies were mixed and pooled to obtain
233 parasites culture material. Two sera supplements were used separately at 10 %
234 concentrations each: fetal bovine serum (Sigma- Aldrich, St Louis, USA) and newborn
235 calf serum (Sigma-Aldrich, Berlin, Germany). Five basic media were used: RPMI-1640,
236 IMDM, NCTC-135, MEM (Sigma-Aldrich, St Louis, USA), and DMEM (Gibco Life
237 Technologies, Cergy-Pontoise, France). Penicillin-Streptomycin-Neomycin (PSN, 2 %)
238 was used as antibiotic and Amphotericin B (2.5 µg/ml) as antifungal. Flat bottom culture
239 plates (48-well) with lids (Corning, Kennebunk, ME, USA) were loaded as follows: For
240 the co-culture systems, parasites (range 8–13 L3) in 1200 µL of the studied medium
241 (basic culture medium + 10 % serum) were loaded into a feeder-cell type pre-
242 conditioned plate while in cell-free systems, they were loaded into empty wells. Five

243 batches of infective L3 larvae were used throughout this study and each experimental
244 culture system was carried out in quadruplet wells.

245 **Assessment of parasite viability**

246 The viability of the parasites was assessed daily, by visual inspection (by two
247 individuals) under an inverted microscope until movement ceased. Their motility was
248 scored on a 4- point scale as described in [47]. Briefly, score 0, no movement or
249 immotile, score 1, intermittent shaking of head and tail, score 2, sluggish (shaking of
250 the whole worm on a spot), score 3, vigorous movement (shaking of the whole worm
251 and migration from one spot to another) was considered.

252 **Parasite long term *in vitro* maintenance strategy**

253 To achieve long term maintenance, 800 μ L exhausted culture medium was removed
254 of each well and replaced with the same volume of fresh culture medium every 2–3
255 days. Additionally, cultured parasites were transferred from one culture plate (old) to
256 another (new) either when feeder cell growth overshadowed parasites motility scoring
257 (parasite entanglement within overgrown cell) with the following cell lines (HC-04, LEC
258 and HEK-293) every 2 weeks or when feeder cells (LLC-MK₂) underwent apoptosis
259 every 7 weeks.

260 **Data processing and analysis**

261 Three variables were used to assess the viability, growth and development of the
262 parasites (mean motility, moulting rate and parasite stage morphometry). Raw data
263 were saved in a spreadsheet and using the above described 4-point scale, the
264 percentage (%) of motility was calculated according to the following formula:

265

$$\text{Motility (\%)} = \frac{\sum S_i N_i}{3 \cdot \sum N_i} \times 100$$

266 where S_i is the score of point scale i and N_i is the total number of worms at a point
267 scale i [47].

268 Filarial parasite moulting is one of the key phenomena providing clear evidence of
269 worm growth and development. Moulded worms display casted cuticles and
270 morphological changes. The moulting rate was calculated as previously described [41,
271 42]:

272

$$\text{Moulting rate (\%)} = \frac{\text{Number of moulted worms (in well } i)}{\text{Total number of worms (in well } i)} \times 100$$

273 With respect to parasite morphometry, photographs of *O. volvulus* at different stages
274 were recorded under an inverted microscope (OPTIKA, Ponteranica, Italy) and their
275 length was determined using the OPTIKA IS view software Version. 2.0 (Ponteranica,
276 Italy). ImageJ 1.52 software (NIH, USA) was used to generate scale bars of displayed
277 photographs.

278 GraphPad Prism 8 software (GraphPad, San Diego, USA) was used to generate mean
279 motility, moulting rate and morphometry graphs. Results of replicates were expressed
280 as mean \pm standard deviation (SD) for the following variable (motility and moulting)
281 while median was used to summarise morphometric parameters of the parasite. The
282 Kruskal-Wallis one-way analysis test was used to assess differences in motility,
283 moulting rate and worm's morphometry between sets of studied culture systems.
284 Dunn's *post-hoc* test was applied for pairwise multiple comparisons of the ranked data.

285 Factors that promoted parasite survival were identified using the multiple linear
286 regression. The general linear model (GLM) was built using the hierarchical stepwise
287 method. A total of 4 blocks were achieved with the 5 factors (incubation time, presence
288 or absence of feeder cells, basic medium, serum) and those that contributed
289 significantly to the improvement of the model were identified based on the F-statistics
290 and the adjusted R-square. The incubation time was treated as a metric factor.
291 Dichotomous variables such as the presence of monkey kidney cells were coded using
292 binary figures. For each nominal factor (Basic culture media, serum), sets of dummy
293 variables were created and compared to one of the categories defined as reference.
294 While RPMI-1640 was used as a reference against DMEM, IMDM, MEM and NCTC.
295 FBS serum was used as a reference against NCS.

296 The passage of *O. volvulus* larvae from the third (L3) to the fourth (L4) stages then to
297 the fifth (L5) stage was further considered the second target product profile in
298 assessing the suitability of the culture systems tested. Finally, the model was used to
299 predict T_{20} and T_{10} values (Days), defined as the duration (incubation time) at which
300 20 and 10 % of the worms were still active respectively (score 3). For all statistical
301 comparisons, the p -values below 5% were evidence for rejecting null hypotheses.

302

303 **Results**

304 Purified *O. volvulus* infective larvae were cultured in two distinct systems: the cell-free
305 culture system and the cell-based co-culture system. The first step consisted at
306 evaluating the potential of the cell – free systems (combination of each of the five basic
307 culture media and a single concentration of either of the two sera) on the viability,
308 growth and development of *O. volvulus* larvae. Secondly, to subject the best cell – free

309 culture setting on four different mammalian cell lines in order to evaluate the beneficial
310 effect of co – culture with feeder layers (co – culture systems).

311 **Evaluation of cell-free culture systems on the growth and development of *O.***
312 ***volvulus* larvae**

313 The cell-free system was made of the combination of each of the five basic culture
314 media supplemented with 10 % of any of the two sera (NCS and FBS).

315 With respect to the cell-free system, the various study culture settings sustained *O.*
316 *volvulus* larvae viability for a maximum of 84 days. Complete inactivity of all larvae was
317 recorded in culture combinations IMDM – 10 % FBS and NCTC135 – 10 % FBS after
318 54 days and in DMEM – 10 % NCS and IMDM – 10 % NCS after 84 days. Generally,
319 freshly dissected and cultured *O. volvulus* infective L3 larvae were not vigorously active
320 (motility score = 2, sluggish). Their motility significantly increased from day 3 to day 5
321 (motility score = 3, vigorously active) when the L3 stage larvae casted their cuticles to
322 become L4. The parasite motility waned in all culture conditions tested after around 45
323 days of culture and according to the *in vitro* culture combination (Medium-Serum) all
324 parasites were immobile at day 54 to 84 (Fig 1).

325 **Fig 1. Motility pattern of *O. volvulus* (from L3 to L4) in the cell-free culture**
326 **systems. A DMEM - 10 % FBS/NCS. B IMDM - 10 % FBS/NCS. C MEM - 10 %**
327 **FBS/NCS. D NCTC 135 - 10 % FBS/NCS. E RPMI1640 - 10 % FBS/NCS. (Results**
328 **were pooled from different independent experiments, n =3 and each experimental**
329 **setting conducted in quadruplets).**

330

331 *Onchocerca volvulus* moult was also used as an indicator to assess larvae
332 development *in vitro*. The moult profile of *O. volvulus* larvae *in vitro* was culture system
333 dependent and with respect to cell-free systems, only the first parasite molt (L3 to L4
334 = M1) was observed. The cuticle of L3 larvae was casted within 3–5 days of culture
335 and the moulting rate ranged from 0 % (MEM – 10 % NCS) to 92 % (DMEM – 10 %
336 NCS). The culture system that best supported *O. volvulus* L3 moult was DMEM – 10
337 % NCS, although no statistically significant difference was observed as compared to
338 DMEM – 10 % FBS, RPMI – 10 % NCS, RPMI – 10 % FBS, NCTC 135 – 10 % FBS
339 and IMDM – 10 % FBS (Fig 2).

340 **Fig 2. Influence of cell-free culture systems on *O. volvulus* larvae moults *in vitro*.**

341 (Results were pooled from different independent experiments [n =3] and each
342 experimental setting conducted in quadruplets).

343

344 In cell – free systems, 3 – 5 days were required for *O. volvulus* infective larvae to moult
345 from L3 to L4. Preliminary values of L3 and L4 length and width showed highly
346 overlapping data with no significance, therefore focus was drifted towards comparing
347 L4 and L5 values. In addition, no proof of parasite maturity was recorded since all
348 parasites that succeeded to achieve their first moults (from L3 to L4) failed to undergo
349 the second moult (L4 to L5). The absence of mammalian cells in this system hindered
350 complete parasite growth and development. In summary, the cell – free system
351 supported parasite moult into L4 larvae and viability for up to 84 days, but was below
352 the timeframe necessary to allow full parasite development. Thus, also no cellular
353 mass forming around *O. volvulus* worms was observed in cell – free systems.

354 **Evaluation of cell-based co-cultured systems on the growth and development**
355 **of *O. volvulus* larvae**

356 Since larvae did not become adults and stopped their motility by day 84 at the latest
357 in the cell-free culture systems at stage 4, we next evaluated the combination of the
358 best cell – free system (DMEM - NCS/FBS) with each of the four mammalian cell
359 lines (LLC-MK₂, HC-04, HEK-293 and LEC) in order to improve parasite motility and
360 viability as well as moulting.

361 Interestingly, *O. volvulus* larvae survived for a longer period of time (up to 315 days) in
362 cell-based co-culture systems (Fig 3). Thus, the culture of *O. volvulus* larvae on feeder
363 cells increased their longevity by 3.75 folds as compare to those cultured in cell-free
364 systems. As similarly observed in cell-free systems, cultured *O. volvulus* infective L3
365 larvae from freshly dissected *Simulium* flies did not displayed vigorous activity (motility
366 score = 2, sluggish). The infective larvae (L3) motility significantly increased as from
367 day 3 to 7 (motility score = 3, vigorously active) when L3 stages casted out their cuticles
368 to become L4 larvae. The L4 larvae motility remained rectilinear till day 48 when the
369 first L4 to L5 moult was observed. As from day 48, except for larvae cultured in DMEM
370 – 10 % NCS - HC-04, L5 larvae motility started dropping and movement completely
371 ceded in larvae cultured in DMEM LEC 10% NCS after 103 days and in larvae cultured
372 in DMEM HEK 10% NCS after 162 days. The sole L5 larvae motility amplification was
373 observed in DMEM – 10 % NCS - HC-04 culture system with more than 90 % of
374 parasite being very active until 125 days of culture. The motility of this group started
375 dropping afterwards. At day 233 larvae cultured in DMEM – 10 % NCS - HC-04, DMEM
376 – 10 % NCS LLCMK2 and DMEM – 10 % FBS LLCMK2 still showed a motility of 10-
377 20% (Fig 3).

378 **Fig 3. Motility pattern of *O. volvulus* (from L3 - L4 - L5) in the DMEM cell-based**
379 **co-culture systems supplemented with 10 % FBS and 10 % NCS.** (Results were
380 pooled from different independent experiments, n =3 and each experimental setting
381 conducted in quadruplets).

382

383 In contrast to cell-free systems, *O. volvulus* larvae underwent two consecutive moults
384 in the cell-based co-culture systems. The first moult (L3 to L4 = M1) was observed
385 within 3–7 days of culture while the second moult (L4 to L5 = M2) was observed
386 between day 48 and day 78, in which L4 larvae cuticle ecdysis to become L5 larvae
387 (Fig 4). The use of cell lines as feeder layer triggered parasites second moult and it
388 was consistently observed that amongst the *O. volvulus* larvae that achieved the first
389 moult, if not all, a great proportion underwent a successful second moult. *O. volvulus*
390 moulting rates ranged from 0 % (DMEM – 10 % NCS - LEC) to 99.2 % (DMEM – 10 %
391 FBS - LLCMK2). The cell-based co-culture system that was the best to support parasite
392 moulting from L3 to L5 was DMEM – 10 % FBS - LLCMK2 (M1 = 69.2±30 % and M2
393 = 69.2±30 %), though no statistically significant difference was observed as compared
394 to DMEM – 10 % NCS - LLCMK2 (M1 = 57.8±30.7 % and M2 = 49.2±41%), DMEM –
395 10 % NCS - HC04 (M1 = 52.8±21.5 % and M2 = 52.8±21.5 %) and DMEM – 10 %
396 NCS - HEK293 (M1 = 58.8±18.2 % and M2 = 57.0±15.3 %) except to DMEM – 10 %
397 NCS – LEC (M1 = 1.7±4.7 % and M2 = 0.0±0.0 %) as shown (Fig 5).

398

399 **Fig 4. *O. volvulus* L4 larvae cuticle ecdysis to become L5 (1 = casted cuticles, 2**
400 **= cuticle being casted and 3 = newly moulted L5).**

401 **Fig 5. Influence of cell-based co-culture systems on *O. volvulus* larvae moults *in***
402 ***vitro*.** (Results were pooled from different independent experiments, n =3 and each
403 experimental setting conducted in quadruplets).

404

405

406 The length and width of *O. volvulus* larvae increased significantly throughout the
407 duration of this study (233 days) and these changes were stage dependent. *O. volvulus*
408 larvae morphometry increased as larvae switched from one stage to another. The L4
409 larvae length varied from 1450.17 μm to 2121.63 μm and 44.73 μm – 68.82 μm width,
410 while L5 larvae length varied from 1478.09 μm to 3349.74 μm and 38.82 μm – 114.54
411 μm width. Although the length and width of both stages overlapped since male worms
412 are shorter female worms, significant difference was observed between median length
413 and width of L4 and L5 stages. Highest L5 larvae lengths were observed in NCS/LLC-
414 MK2 (length = 3327.48 μm / width = 81.51 μm) and FBS/LLC-MK2 (length = 3349.74
415 μm / width = 114.54 μm) as shown in Fig 6 and Fig 7. Outlier points from either
416 NCS/LLC-MK2 and FBS/LLC-MKS co-culture systems proved to be young female
417 adult worms as worm mating was later observed.

418 **Fig 6. Length of *O. volvulus* L4 and L5 larvae observed from the *in vitro* cell-**
419 **based co-culture systems with respect to days.** (Bar representing the median). **A**
420 **DMEM – LLCMK₂ – 10% FBS. B DMEM – LLCMK₂ – 10% NCS. C DMEM – HC04 –**
421 **10% NCS. D DMEM – HEK293 – 10% NCS. E DMEM – LEC – 10% NCS.**

422 **Fig 7. Width of *O. volvulus* L4 and L5 larvae observed from the *in vitro* cell-based**
423 **co-culture systems with respect to days (Bar representing the median). A DMEM**
424 **– LLCMK₂ – 10% FBS. B DMEM – LLCMK₂ – 10% NCS. C DMEM – HC04 – 10%**
425 **NCS. D DMEM – HEK293 – 10% NCS. E DMEM – LEC – 10% NCS.**

426

427

428 **Divergence in morphometry pattern of *O. volvulus* male and female worm's**

429 The length of female and male adult worms varied according to the cell-based co-
430 culture system where they were cultured. Among all cell-based co-culture systems
431 used, only DMEM – LLCMK₂ – 10% NCS, DMEM – LLCMK₂ – 10% FBS and DMEM –
432 HC04 – 10% NCS displayed the best appreciable differences. Globally, no sex related
433 *O. volvulus* larvae differentiation was possible between day 0 and day 104. Within this
434 interval (0 – 104 days), the length of larvae recorded showed a wide variation of values
435 but with no definitive conclusion on parasite sex. Above day 104, the discrepancy
436 earlier observed in worm's length values started dropping, therefore, leading to better
437 categorization of worms based on their length. At most, *O. volvulus* adult male worms
438 did not exceed 2900 µm while the female adult worms could reach up to 3300 µm (Fig
439 8). In addition to the differences in worm length, the early development of gonads of
440 gonads was also used as discriminatory indicator between female and male adult
441 worms.

442 **Fig 8. Disparity in sex-dependent worm morphometry changes in different cell-**
443 **based co-culture systems. A** DMEM – LLCMK₂ – 10% NCS. **B** DMEM – LLCMK₂
444 – 10% FBS. **C** DMEM – HEK293 – 10% NCS. **D** DMEM – HC04 – 10% NCS.

445

446 **Evidence of *O. volvulus* adult worms' maturity: Mating and mating competition**
447 ***in vitro***

448 Generally, experiments involving co-cultured systems were monitored for up to 233
449 days except for DMEM – 10 % FBS - LLCMK₂ and DMEM – 10 % NCS - LLCMK₂. The
450 latter were the sole co-culture systems which could support the growth, development
451 and mating of *O. volvulus* larvae from their infective larvae stage to adult worms and
452 parasites were monitored in both systems for up to 315 days. Early L4 moults into L5
453 larvae were observed as from day 48 and lately by day 78. Newly moulted L5 larvae
454 required an additional 4 months and 2 weeks (134 days) *in vitro* maintenance before
455 the first parasite mating could be observed. Parasites copulation was observed only in
456 the DMEM – 10 % FBS - LLCMK₂ cell-based co-culture system. By day 212 of culture,
457 the first *O. volvulus* adult worms mating was recorded (Figs 9A and B, S1 Media) and
458 12 days later a scene of mating competition was also recorded. The mating competition
459 involved 2 adult males battling to copulate with an adult female worm (Fig 9C, S2
460 Media). The mating competition lasted for 11 days after which only one single adult
461 male succeeded to mate with the female. The victorious adult male worm continued
462 mating with the female worm for up to day 294. In summary, the whole mating process
463 had a duration of 82 days and these copulated parasites survived for another 3 weeks
464 (Figs 9D and F).

465 **Fig 9. *O. volvulus* adult worms mating and mating competition.**

466 **A** Copulation topography during single mating. (A1: Posterior region of an adult female
467 worm, A2: Anchored adult male worm mating with an adult female). **B** Copulation
468 topography during single mating. (B1: Mating adult female worm, B2: Mating adult male
469 worm, B3: Copulation region entailing male specules insertion into female worm vulva,
470 B4: Male and female worms anchor region). **C** Copulation topography during mating
471 competition (C1: First adult male worm in competition for mating, C2: Second male

472 worm in competition for mating, C3: Solicited adult female worm for mating). **D** Adult
473 female *O. volvulus* worm. **E** Adult male *O. volvulus* worm. **F** Mated adult female worm.
474

475 Overall, the co-culture system (DMEM – 10 % FBS - LLCMK₂) could sustain and
476 support *O. volvulus* larvae for up to 315 days and exhibiting parasite mating and mating
477 competition. The proportion of adult worms involved in the mating and mating
478 competition phenomenon is shown **Table 1**.

479

Table 1. Proportion of adult worms involved in the mating process

Cell-based co – culture systems	Sex of worms involved	Proportion (%)
DMEM – 10 % FBS - LLCMK ₂	2 females and 3 males	5/131 (3.81)
DMEM – 10 % NCS - LLCMK ₂	0	0/59 (0)

480

481

482 **Cellular aggregation around *O. volvulus* adult worms in the *in vitro* cell-based** 483 **co-culture system**

484 From day 177, we observed changes in worm environment, notably cellular
485 aggregation around the *O. volvulus* L5. This feature was displayed only in co-culture
486 systems, mainly LLC-MK₂ in combination either with DMEM – 10 % NCS or DMEM –
487 10 % FBS. The process started with the recruitment of thin transparent and insoluble
488 cell-derived particles along worm cuticle (**Figs 10A and B, S3 Media**). These cell-
489 derived transparent and insoluble particles lately came together to form a globular or
490 oval shape aggregation around the worms that remained trapped in this mass (**Figs**
491 **10C and D, S4 Media**).

492 **Fig 10. Cellular aggregation around *O. volvulus* young adults *in vitro*. A&B (A1**
493 **and B1: Early recruitment of cell-derived insoluble particles along the worm external**
494 **wall, A2 and B2: Young adult worm trapped in the cellular aggregate). C&D (C2 and**
495 **D2: Gathering of cell-derived insoluble particles into a globular/oval mass shape**
496 **engulfing the young adult worms, C1 and D1: Young adult worm being engulfed).**
497 This process lasted for 138 days after which parasites perished. The proportion of adult
498 worms involved in nodulogenesis is shown **Table 2**.

Table 2. Proportion of adult worms involved in nodulogenesis

Co – culture systems	Number of adult worms involved	Proportion (%)
DMEM – 10 % FBS - LLCMK ₂	26	26/129 (20.15)
DMEM – 10 % NCS - LLCMK ₂	10	10/59 (16.949)

499

500 **Linear regression analysis: factors influencing the growth and development of**
501 ***Onchocerca volvulus* larvae *in vitro***

502 The contribution of the various culture media and supplements used in the
503 improvement of worm viability were identified based on their standardized coefficient
504 (**S5 Table**). Among these factors, the presence of feeder cells pre-eminently influenced
505 *O. volvulus* larvae viability, HC-04 feeder cells were classified as topmost factor ($\beta =$
506 0.299) followed by LLC-MK₂ feeder cells ($\beta = 0.269$), HEK-293 feeder cells ($\beta = 0.129$),
507 LEC feeder cells ($\beta = 0.060$). Although the basic culture media types also promoted *O.*
508 *volvulus* larvae viability, their impact was less important than that of feeder cells but
509 higher as compared to that of sera supplements. DMEM basic medium had the leading
510 effect ($\beta = 0.042$) and the least effect was observed with IMDM ($\beta = - 0.068 \cdot 10^{-3}$). Both
511 sera supplements, FBS ($\beta = - 0.074$) and NCS ($\beta = - 0.052$) had unfavourable effect

512 as compared to RPMI. The model was diagnosed by assessing the assumptions of
513 normal distribution and homoscedasticity. The histogram of the residuals (errors) in the
514 model was used to check if they are normally distributed (S6 Figure). Although not
515 perfect, the frequency distribution of the residuals displayed a shape close to that of
516 the normal Gauss curve, indicating evidence of normal distribution. Additionally, Q-Q
517 plot was used for further check (S7 Figure). Here, the theoretical and observed
518 quantiles were closed suggesting that the assumption of normal distribution of the
519 residual was far to be not violated. The model was used to predict T_{20} and T_{10} values
520 (Days) which correspond to the duration at which 20 and 10 % of the worms were still
521 active (score 3) as shown in (S8 Figure). Co-culture with feeder cells in DMEM medium
522 represented the systems that could extend survival of parasites for longer periods.

523 Discussion

524 This study was conducted to establish an *in vitro* platform which could support and
525 promote the growth and development of *O. volvulus* larvae from the *Similium* derived
526 L3 larvae to the adult stages. Several attempts have been carried out in the perspective
527 of achieving the complete life cycle of *O. volvulus* human stages in *in vitro* systems.
528 Early studies used serum/cell free systems to culture filarial parasites and reported the
529 maintenance of their full viability for up to a week [12-22]. Our previous reports and
530 those from other investigators highlighted improvement of the culture conditions by
531 supplementing the basic culture media used with serum or other synthetic additives.
532 The serum-based culture systems achieved better parasite longevity and cuticle
533 casting [21, 23-30, 32]. Due to challenges of inconsistency of serum composition, other
534 researches opted to develop serum-free culture systems and co-culture systems using
535 eukaryotic cells as feeder layers which yield best results obtained so far [33-43]. These

536 studies did not provide a consensus on filarial parasite nutritional needs. Zofou *et al.*
537 2018 [42] established a hierarchical profile of most used *in vitro* culture supplements
538 for filarial parasites, in which the presence of feeder cells was ranked as most top
539 important followed by culture supplementation with serum and finally the culture media
540 type. Based on these observations, four feeder layer cell line types (LLC-MK₂, HC-04,
541 HEK-293 and LEC), two serum types (FBS and NCS) and five basic culture media
542 (RPMI-1640, DMEM, MEM, NCTC-135 and IMDM) were evaluated either in
543 combination in the cell-free systems or in the co-culture systems for the growth and
544 development of *O. volvulus* larvae *in vitro*.

545 In order to assess *O. volvulus* larvae *in vitro* growth and development five variables
546 were monitored: mean motility, moulting rate, parasite stage specific morphometry,
547 mating and nodule formation. With respect to *O. volvulus* larvae viability, parasite mean
548 motility was used as first indicators among others (mean motility, moulting rate, worm
549 morphometry). Regardless the culture system, *O. volvulus* larvae motility displayed a
550 “saw teeth” evolution pattern. For both systems (cell-free and co-culture systems),
551 cultured L3 larvae motility started reducing as from day 0 and persisted still day 3 when
552 the first L3 moults were observed. The switch from one stage to another was in first
553 instance marked by a significant drop in motility followed by an abrupt increase in
554 motility (shift from score 2 to score 3), and this same phenomenon was also later on
555 observed when larvae moulted from L4 to L5. The progressive drop in motility till
556 moulting followed by a drastic increase could be an evidence that the greatest fraction
557 of energy produced by the larvae is stilted towards cuticle ecdysis which is prioritized
558 at this time point and less energy assigned to worm twitching. Page and Johnstone
559 [48] reported that the moulting process in *C. elegans* nematodes is preceded by a
560 period of decreased general activity and feeding, known as lethargus, when the old

561 cuticle begins to disconnect from the underlying hypodermis. Singh and Sulston [49],
562 stated that during apolysis, the old cuticle is separated, allowing the newly synthesized
563 one to be secreted in the space between the two layers. The moulting cycle is
564 completed with ecdysis, when the old cuticle is completely shed and the worm emerges
565 to the next stage with a new cuticle. Moreover, it was observed that, only co-culture
566 systems could support *O. volvulus* larvae moulting from L4 to L5. These are further
567 indications ascertaining that some nutrients secreted/excreted by feeder cells were key
568 factors for parasite development as it was clearly demonstrated that no L4 to L5 larvae
569 moults were observed in cell-free culture systems. Toback *et al.* [50] reported that
570 kidney epithelial cells release growth factors such as epidermal growth factor (EGF),
571 transforming growth factor-type - alpha (TGF- α), insulin-like growth factor I (IGF-I),
572 platelet-derived growth factor (PDGF), and insulin which are being exploited by the
573 parasite for their growth and development. McConnell *et al.* [51] also showed that non-
574 transfected HEK-293 release nerve growth factor which were beneficial to *O. volvulus*
575 larvae growth and development. Without feeder cells, *O. volvulus* larvae stayed viable
576 *in vitro* for up to 84 days, their longevity rose by 3.7 folds in the co-culture system (315
577 days). The co-culture system developed in this study is superior to the one reported by
578 Voronin *et al.* [44] in the sense that they used two distinct culture settings to achieve
579 L5 stage while our system made use of a single cell – based co-culture system (DMEM
580 – LLC-MK2 – 10% NCS/FBS) and the maximum attainable longevity they reported for
581 their system was 117 days versus 315 days for this new system. Additionally, our
582 system achieved higher L3 and L4 moulting rate (69.2 \pm 30 %) as compared to theirs
583 (Max. of 60 %). Moreover, our single cell-based co-culture system supported adult *O.*
584 *volvulus* worms mating, mating competition and early nodulogenesis that wasn't

585 achieved with their system. Our findings open up new avenues for drug screening and
586 in-depth investigation of *O. volvulus* biology.

587 Moulting entails synthesis of the new skin and shedding of the old, and represents an
588 important phenomenon for the growth and maturation of filarial parasites. The moulting
589 process is critical for filarial parasites and disruption of moult can have serious
590 consequences for survival and reproductive success. It is vital for filarial parasites to
591 undergo two consecutive moults and metamorphosis in order to become fully mature.
592 The cell-free culture system could only support the first moult (M1) of *O. volvulus*
593 infective larvae leading to L4 stage larvae ranging from 0 % (MEM – 10 % NCS) to
594 78.8 ± 13.2 % (DMEM – 10 % NCS), the basic culture medium type combined with
595 animal serum used provided the necessary nutrients (proteins, electrolytes and
596 hormones) required by *O. volvulus* infective larvae to moult to L4. The second parasite
597 moult (M2) was only observed in the co-culture system. It was therefore clearly
598 established that the feeder cells play a crucial role in the development and maturation
599 of *O. volvulus* parasite. Previous studies to culture other filarial parasites also
600 demonstrated the pre-eminent role of feeder cells in their successful *in vitro*
601 maintenance [26, 41, 42, 44]. Except from (DMEM – 10 % NCS – HEK293), (DMEM –
602 10 % BCS – LLC-MK2) and (DMEM – 10 % NCS – LLC-MK2) co-culture systems, all
603 *O. volvulus* L3 larvae that successfully undertook the M1 moults equally performed the
604 M2 moults. Highest M1 and M2 moulting rate were reported in (DMEM – 10 % FBS –
605 LLC-MK2), which were 69.2 ± 30.0 % and 69.2 ± 30.0 %, respectively. This could account
606 to the fact that FBS has served as an additional source of essential nutrients, growth
607 factors and hormones as the monkey kidney cells. Moreover, FBS could bind and
608 protect essential nutrients that are otherwise unstable. It could also function to

609 neutralize toxic substances in the medium or supply necessary transport factors or
610 enzymes [52].

611 *O. volvulus* morphometry was also used as an indicator to assess parasite growth,
612 although *O. volvulus* L4 stage morphometry overlapped with the L5 stages. The length
613 of L4 stages ranged from 1450 μm to 2122 μm (Length median = 1794 μm) and width
614 from 45 μm to 69 μm (Width median = 57 μm) and L5 length ranged from 1478 μm to
615 3350 μm (Length median = 1892 μm) and width from 39 μm – 115 μm (Width median
616 = 60 μm). There was no significant difference between L4 larvae measured from cell-
617 free systems and L4 larvae from co-culture systems that failed to moult to L5 stages,
618 whereas L4 larvae lengths significantly differ from those of L5. Highest L5 larvae length
619 was recorded in (DMEM - 10 % NCS – NCS) and (DMEM – 10 % FBS – LLC-MK2)
620 co-culture systems, moreover L5 larvae length from both systems differed significantly
621 ($P = 0.0418$). We also noticed that outlier lengths recorded in (DMEM - 10 % NCS –
622 NCS), (DMEM – 10 % FBS – LLC-MK2) and (DMEM - 10 % NCS – HC-04) co-culture
623 systems were female *O. volvulus* larvae, which were later involved in mating and
624 mating competition.

625 During this study, evidences of adult worm maturity were given. In filarial parasite
626 biology, mating can only occur when parasites are mature with well-developed gonads.
627 Moreover, these parasites have to be mature enough to be able to produce and
628 respond to mating related signals for the process to be carried on. For the first time
629 ever, *O. volvulus* adult parasites mating behaviour was documented and
630 photographed. The first mating scene was observed as from day 212 as *O. volvulus*
631 larvae were co-cultured in (DMEM – 10 % FBS – LLC-MK2). Within 12 days, two adult
632 male worms battled/competed to copulate with a female. At the end of the process,
633 only the victorious male worm succeeded to copulate with the female worm. As earlier

634 reported with *C. elegans in vitro* [53-55], the initiation of *O. volvulus* female worm
635 copulation with a mature male worm may have been triggered by attractants (hormones)
636 excreted/secreted by the ready-to-mate female worm. The presence of this
637 chemotactic substance may have at first instance attracted the first adult male worm
638 then the second which resulted to a fierce struggle for mating. The nature and
639 composition of this attractant is unknown and requires extensive investigations. In total,
640 the mating process took 82 days after which these parasites remain viable for 3 more
641 weeks (21 days). Trees *et al.* 2000 reported that it takes 279–532 days post infection
642 (dpi) for the closely related *O. ochengi* parasite of cattle to develop into fully mature
643 and fertile adult worms capable of releasing microfilariae and more than 400 days post
644 infection (dpi) for *O. volvulus* to do the same in a chimpanzee model [8, 11]. Since the
645 female worms survived only 3 weeks after copulation, we did not observe release of
646 microfilariae in this system. It's possible that at this time point, the female worms
647 needed a particular stimulus either from the environment or self-produced to trigger
648 the embryogenesis and later on the release of microfilariae. This calls for further
649 investigation to generate the complete reproduction cycle of *O. volvulus in vitro*.

650 *Onchocerca volvulus* adult worms that were involved in both the mating process
651 followed by formation of nodules survived for a longer period of time (315 days) as
652 compared to those that failed to undergo these events (234 days). In the chronology
653 of biological events, *O. volvulus* adult worms recruited of cell derived insoluble particles
654 as early as from day 177 which was later exhibited by a linear aggregation of these
655 particles along the parasite. The next event entailed parasites mating which occurred
656 as from day 212 and finally these cell-derived insoluble particles gathered into a
657 globular/oval shape mass engulfing the concerned parasites as from day 224. This
658 event could indicate early nodulogenesis, however further studies are required to

659 clarify the composition of these aggregates as well as its role in worm development
660 (an attempt for the parasite to produce a shelter to protect itself so that it can complete
661 its developmental cycle as observed with nodule formation in the mammalian host).
662 According to Collins *et al.* [56] nodule forms only around female worms and mating
663 probably occurs before or early during nodule formation. The production of
664 microfilariae by the female *O. volvulus* is not essential for nodule formation since many
665 nodules contained non-fecund, living females. Al-Qaoud *et al.* [57] reported that filarial
666 worm encapsulation in the murine model was IL-5 dependent. These observations on
667 the incomplete nodule formation *in vitro* deserves further investigations by providing to
668 the *in vitro* system some immunologic effectors that exist *in vivo*.

669 **Conclusions**

670 This study has successfully established an *in vitro* platform for *O. volvulus* growth and
671 development that mimic the parasite biology in the human host. The platform enables
672 us to culture *O. volvulus* for 315 days and observe for the first-time moulting (L3-adult
673 worms/L5) and mating behaviour as well as mating competition and early phase of
674 nodule formation. The establishment of this platform therefore stands as an important
675 achievement in *O. volvulus* developmental biology and has potential for the
676 identification of targets for drug discovery against different phases of development of
677 this filaria parasite.

678 **Abbreviations**

679 APOC: African Programme for Onchocerciasis Control, OCP: Onchocerciasis Control
680 Programme, PTS: Post-Treatment Surveillance, HC-04: Human Hepatocyte cells,
681 HEK-293: embryonic human kidneys cells, LLC-MK2: Monkey Kidney cells, LEC:
682 Mouse embryonic lung cells, L3: Infective Larvae, L4: Stage 4 larvae, L5: Stage 5

683 larvae, RPMI: Roswell Park Memorial Institute, DMEM: Dulbecco's Minimum Essential
684 Medium, MEM: Minimum Essential Medium, IMDM: Iscove's Modified Dulbecco
685 Medium, NCTC: New jersey Cell Type Collection, NCS: New-born Calf Serum, FBS:
686 Foetal Bovine Serum, BCS: Bovine Calf Serum, MDA: Mass Drug Administration,
687 WHO: World Health Organisation, CMFL: Community Microfilariae Loads, EGF:
688 Epidermal Growth Factor, TGF- α : Transforming Growth Factor - alpha, IGF - I: Insulin-
689 like Growth Factor - I, PDGF: Palettes-Derived Growth Factor, DDT:
690 Dichlorodiphenyltrichloroethane.

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695 The datasets used and/or analysed during the current study are available from the
696 corresponding author upon reasonable request.

697 **Competing interests**

698 The authors declare that they have no competing interests.

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708 **Author's contributions**

709 Conceptualization: AH, PAE, MPH, SW

710 Data curation: AJN, NVTG, SW

711 Formal analysis: AJN, NVTG, SW

712 Funding acquisition: AH, MR, MPH, SW

713 Investigation: NVTG, AJN, EJG, FFF, CAK, WPCN

714 Methodology: AJN, NVTG, MR, MPH, AH, SW

715 Resource provision: AH, MR, MPH, AH

716 Writing original draft: NVTG, AJN

717 Writing review and editing: AJN, NVTG, MR, MPH, MEE, SW

718

719

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905

906 **Supporting information**

907 **S1 Media.** *O. volvulus* adult parasites mating *in vitro*.

908 **S2 Media.** *O. volvulus* adult parasites mating competition *in vitro*.

909 **S3 Media.** Early recruitment of cell-derived insoluble particles *in vitro*.

910 **S4 Media.** *O. volvulus* adult parasites early nodulogenesis *in vitro*.

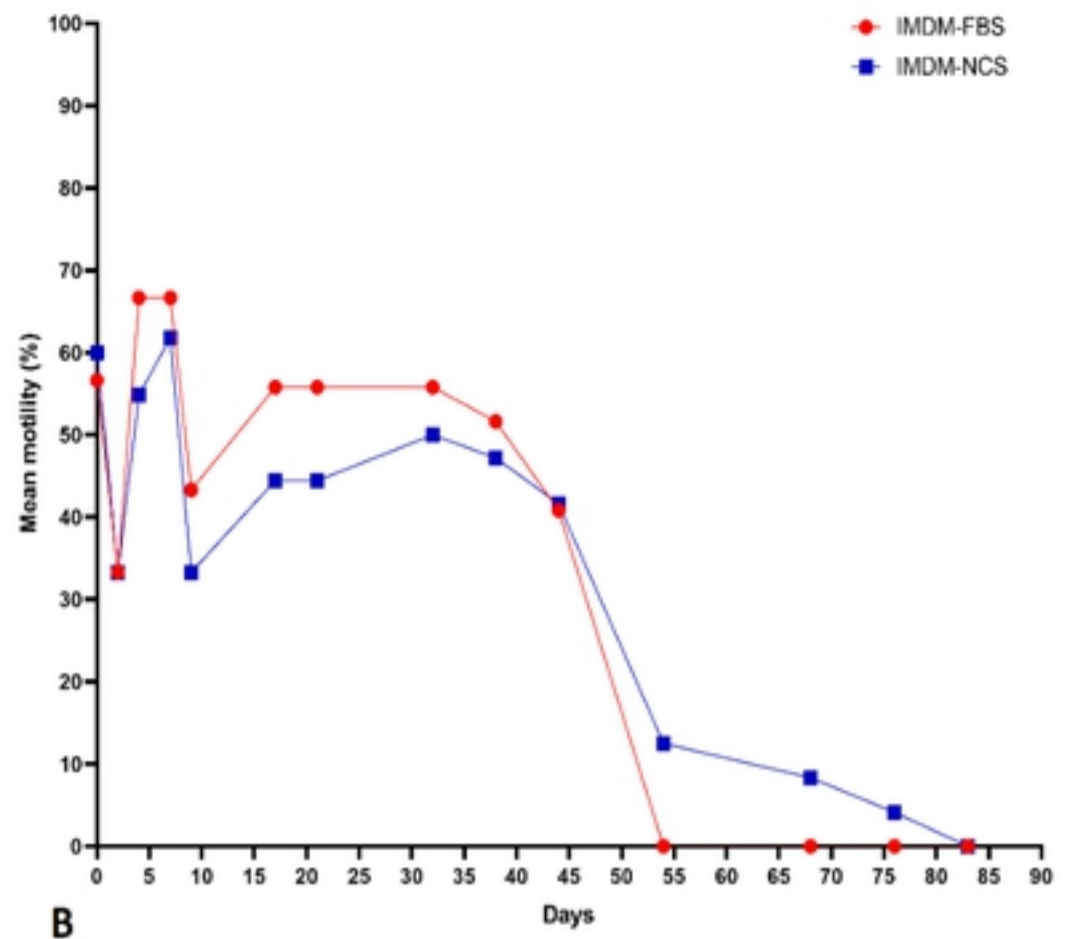
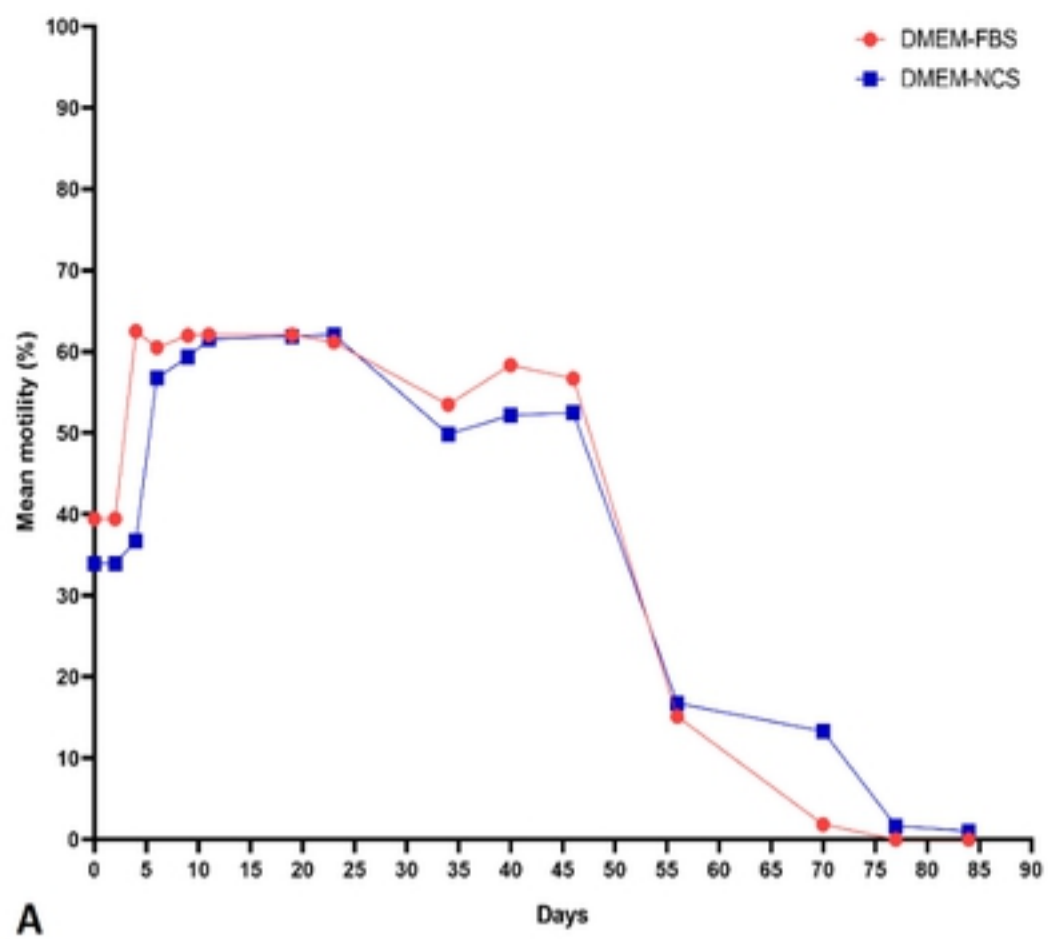
911 **S5 Table.** Experimental factors introduced in the model influencing *O. volvulus* larvae
912 viability and their standardized coefficients.

913 **S6 Figure.** Histogram of the residuals (errors) in the model for normal distribution
914 and homoscedasticity.

915 **S7 Figure.** Q-Q plot suggesting normal distribution of the residual.

916 **S8 Figure.** Model predicted T_{20} and T_{10} values (Days).

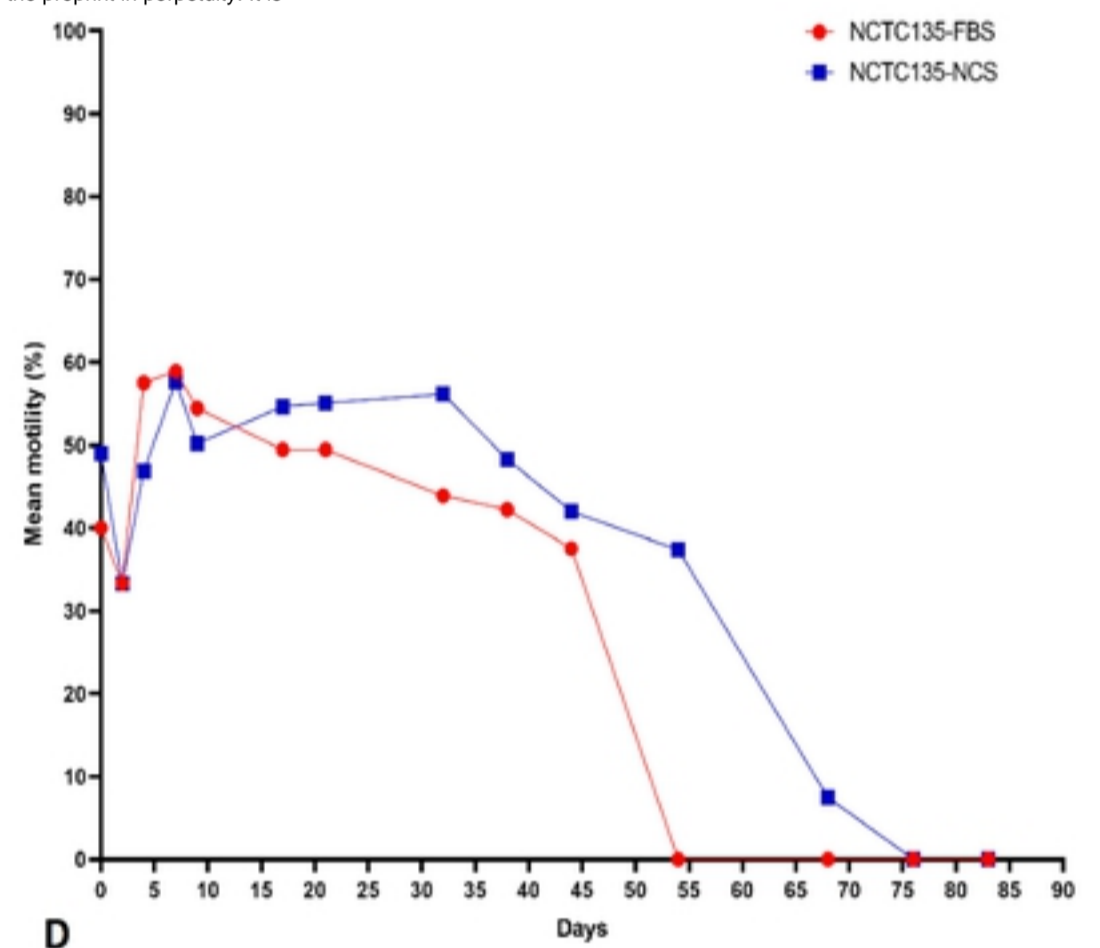
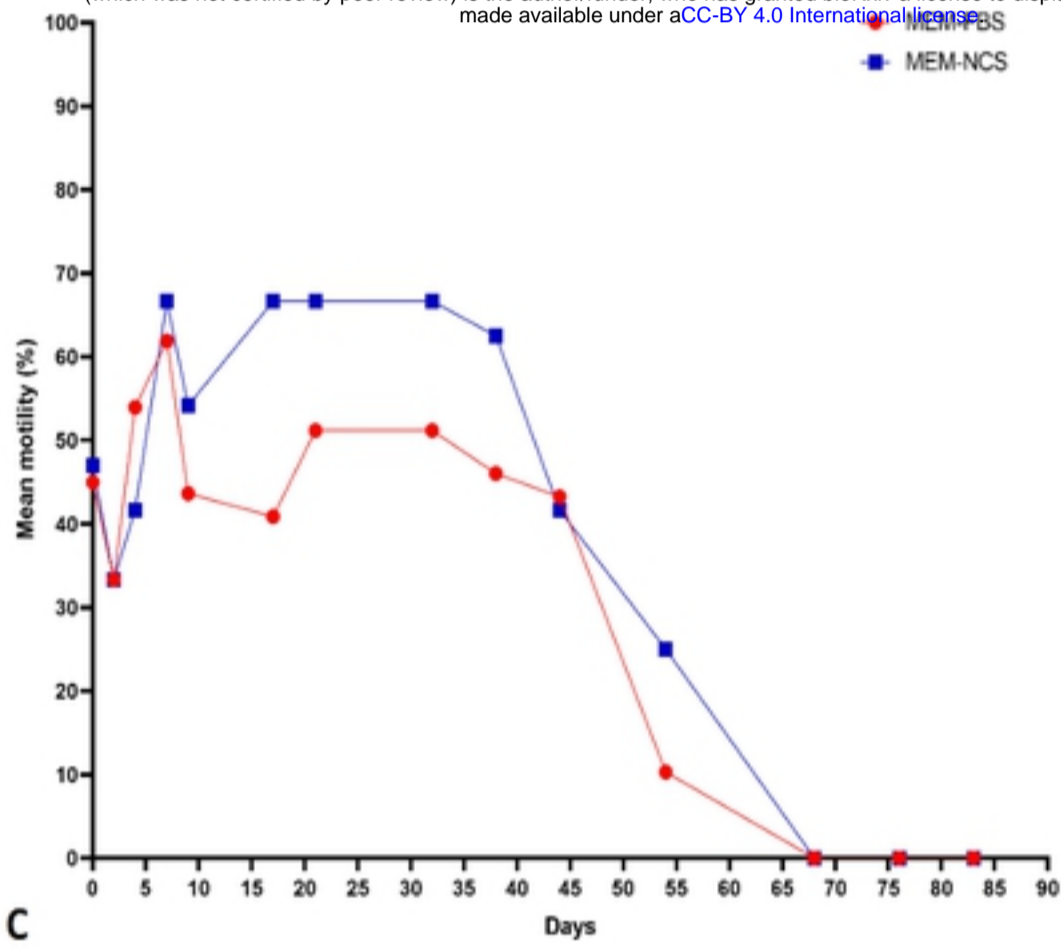
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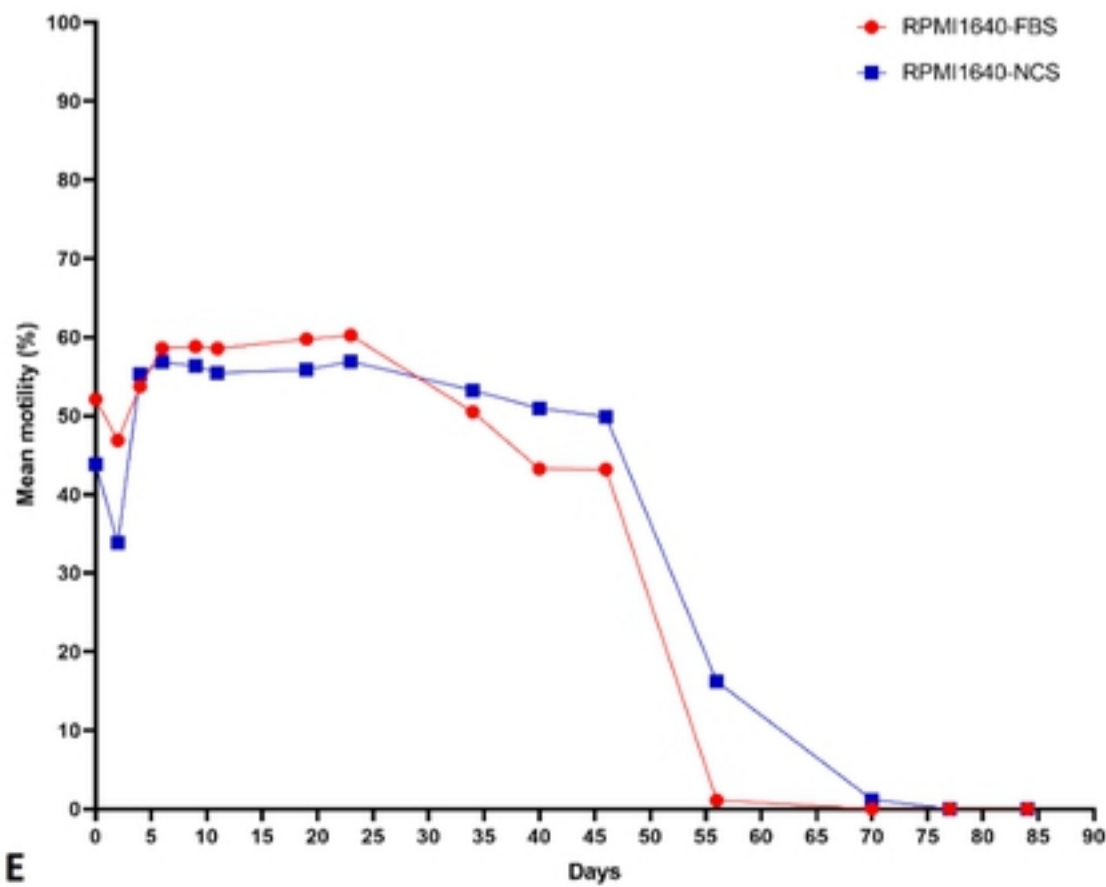
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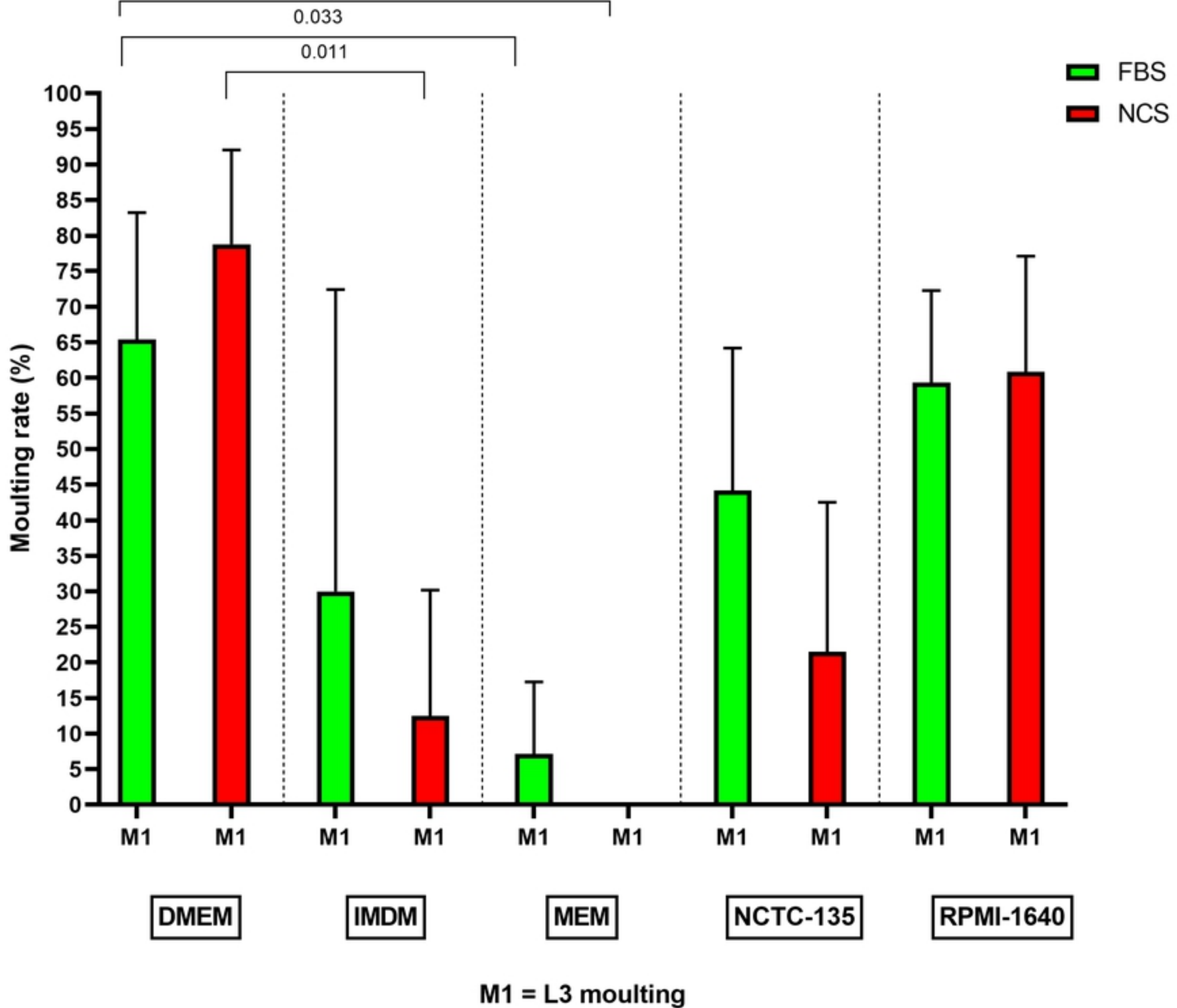
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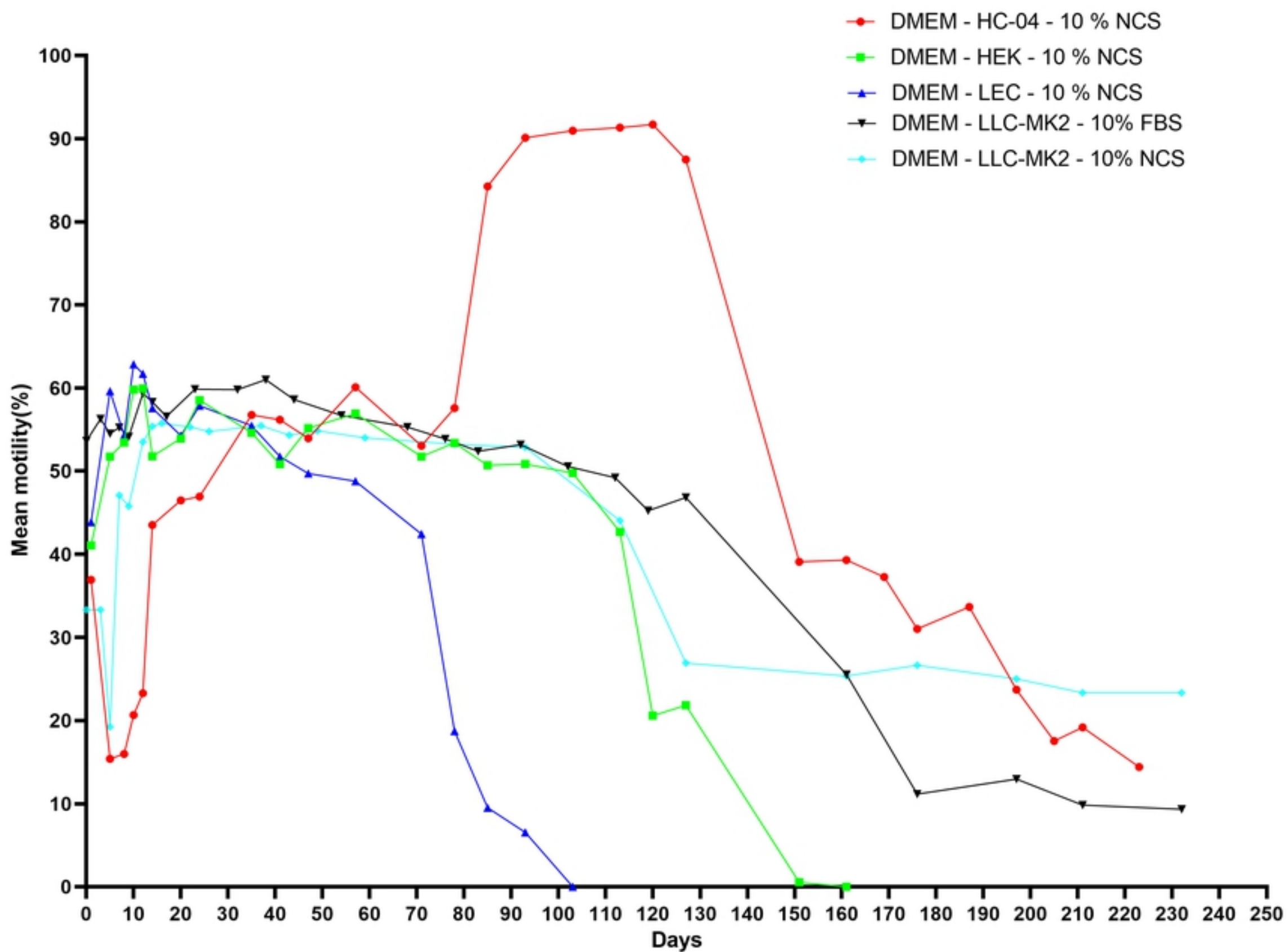
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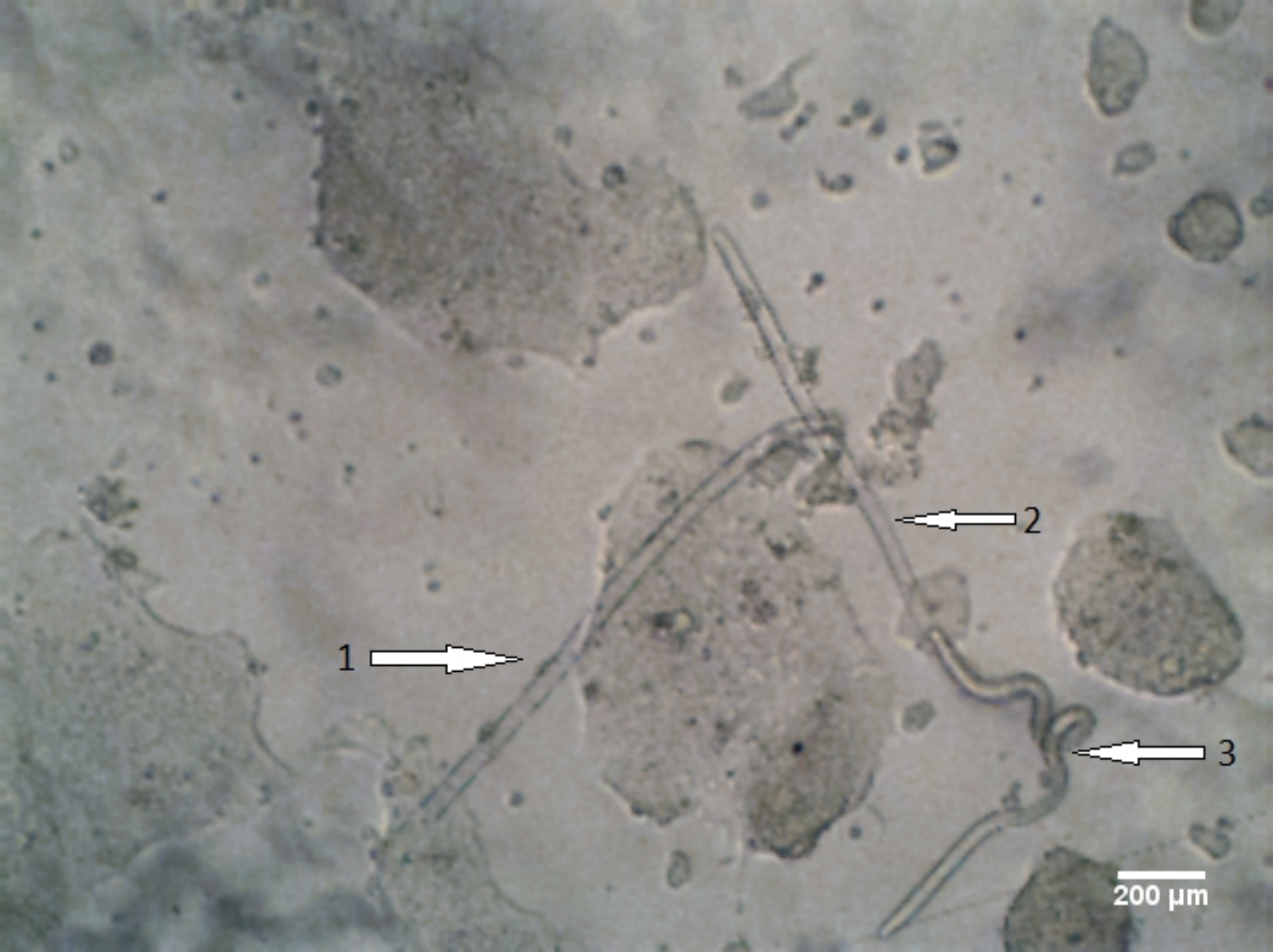
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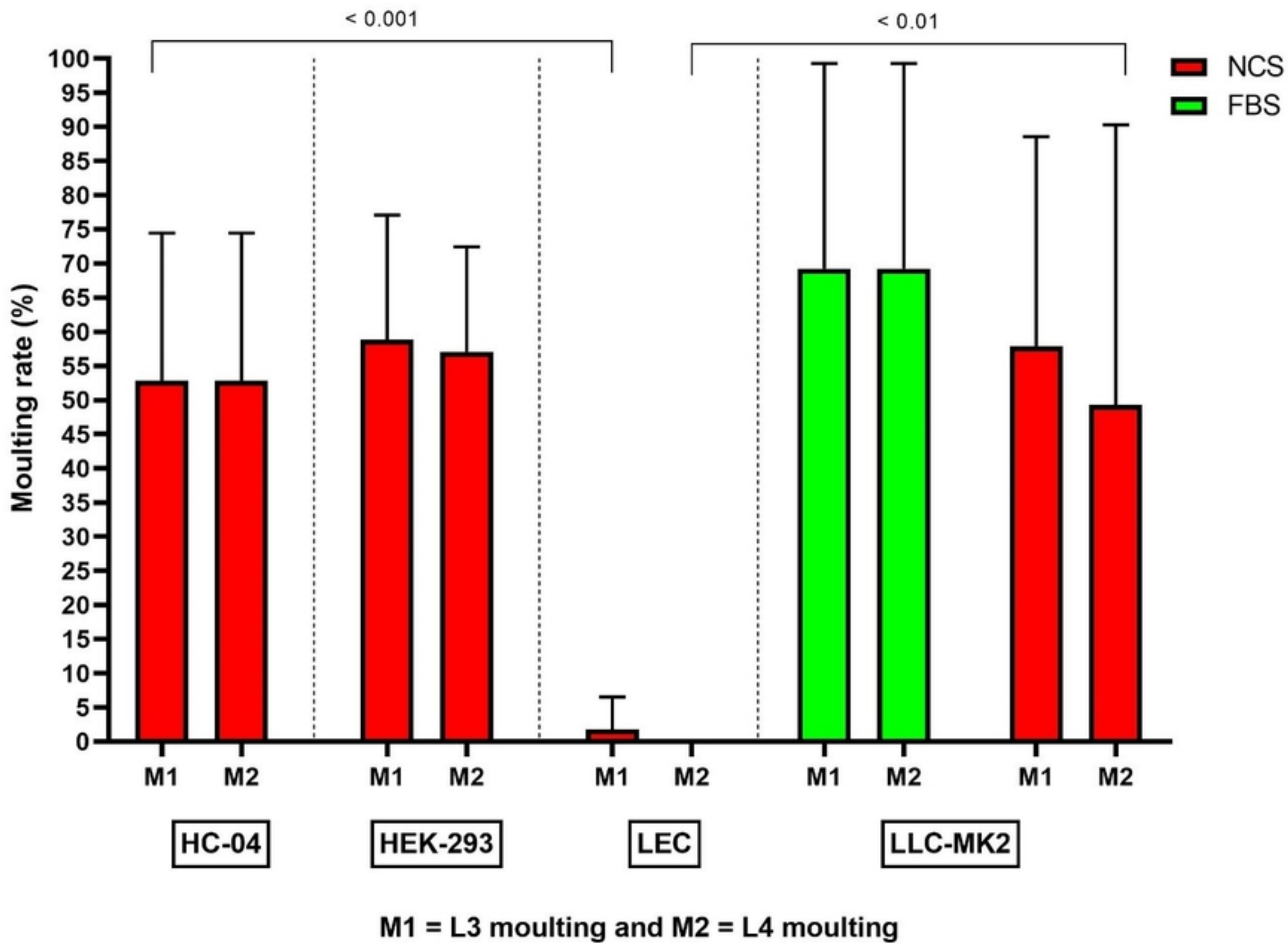
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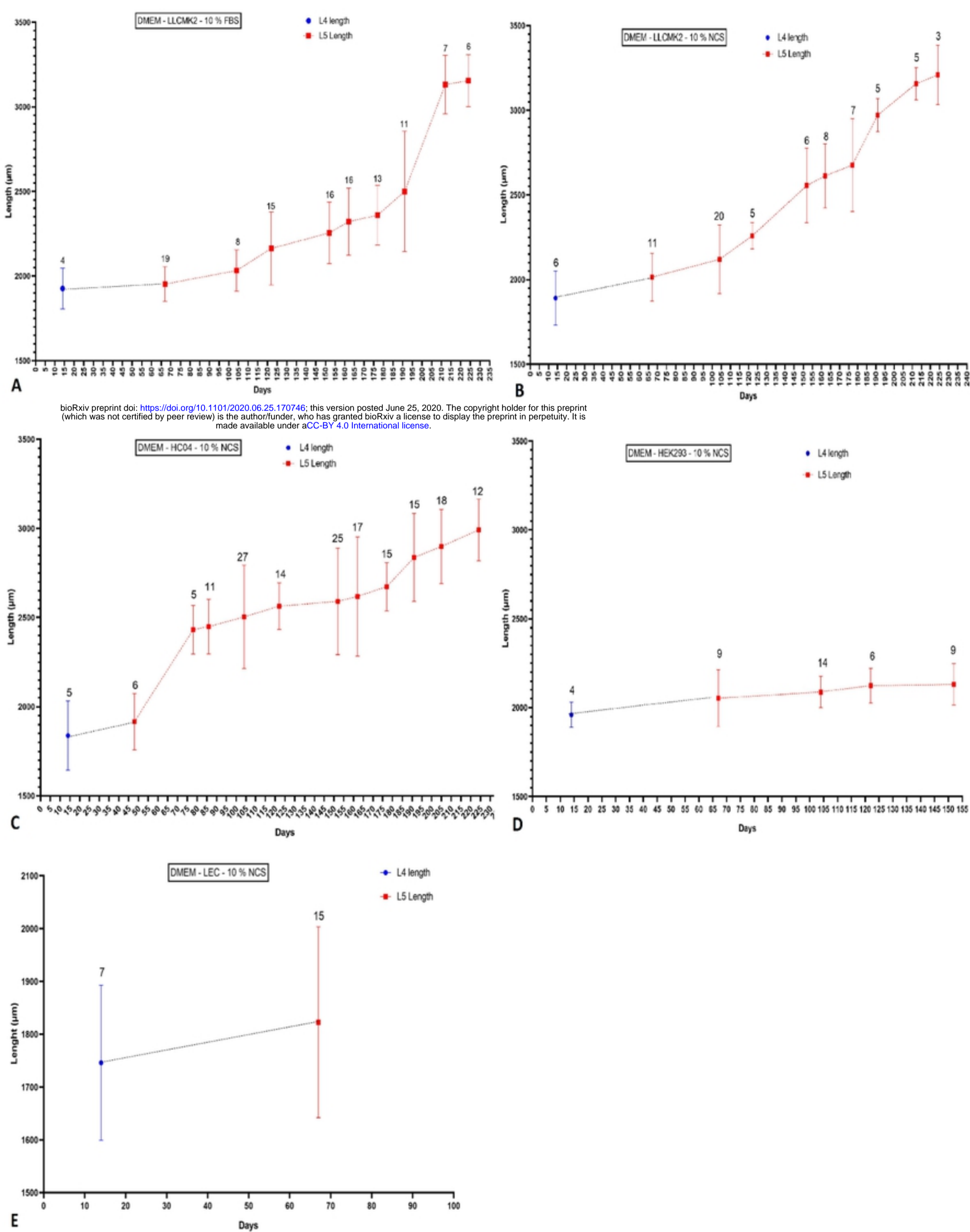
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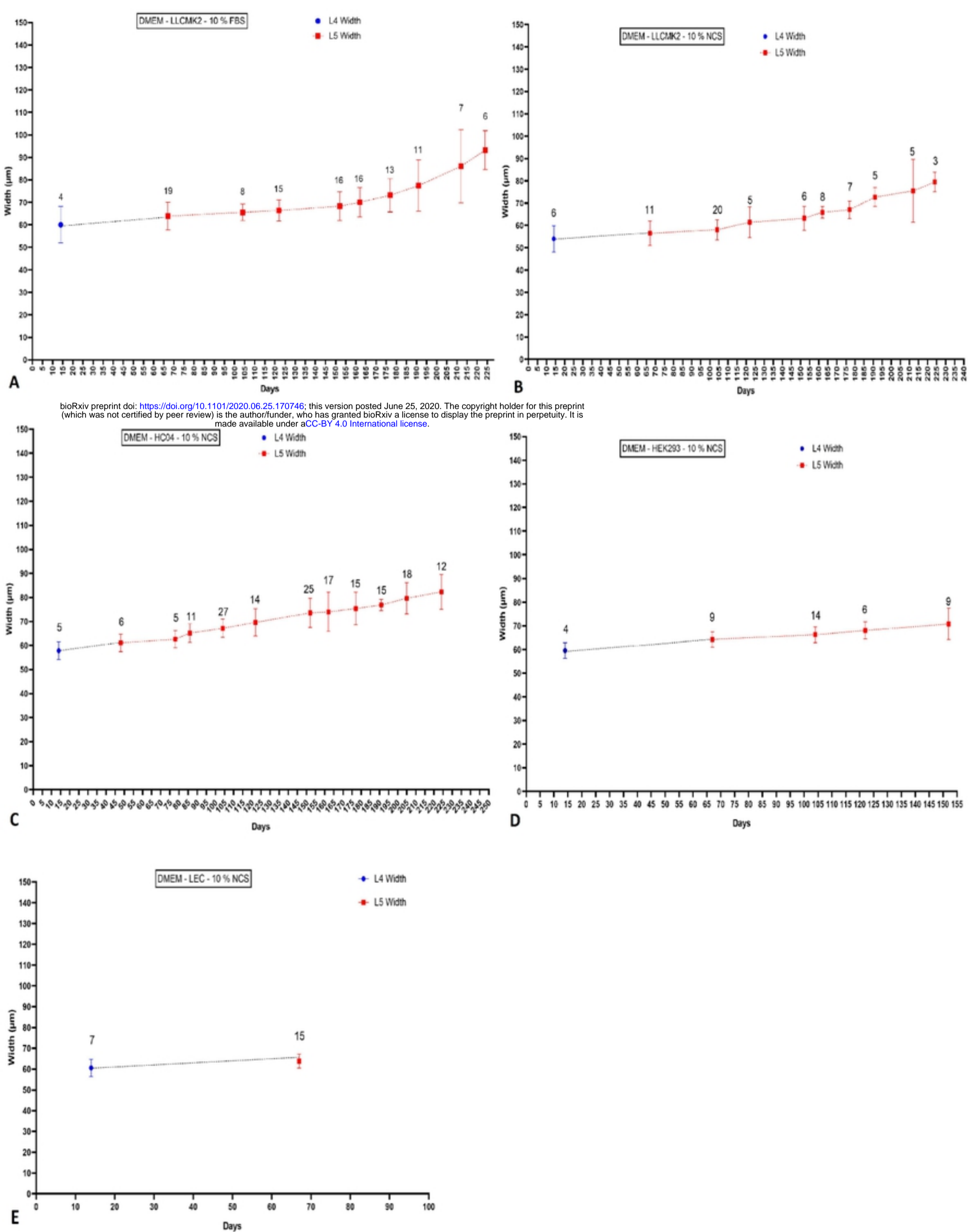
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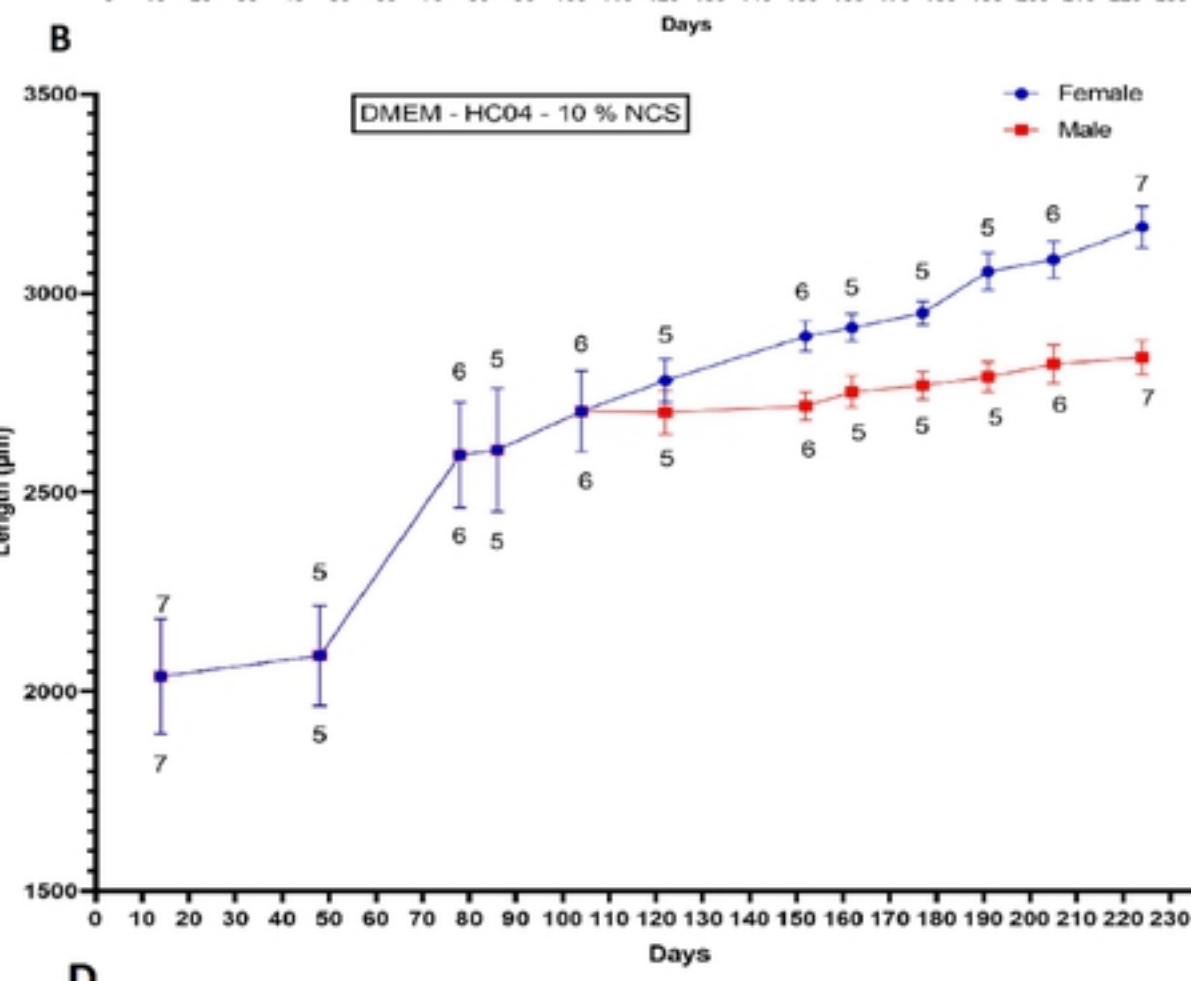
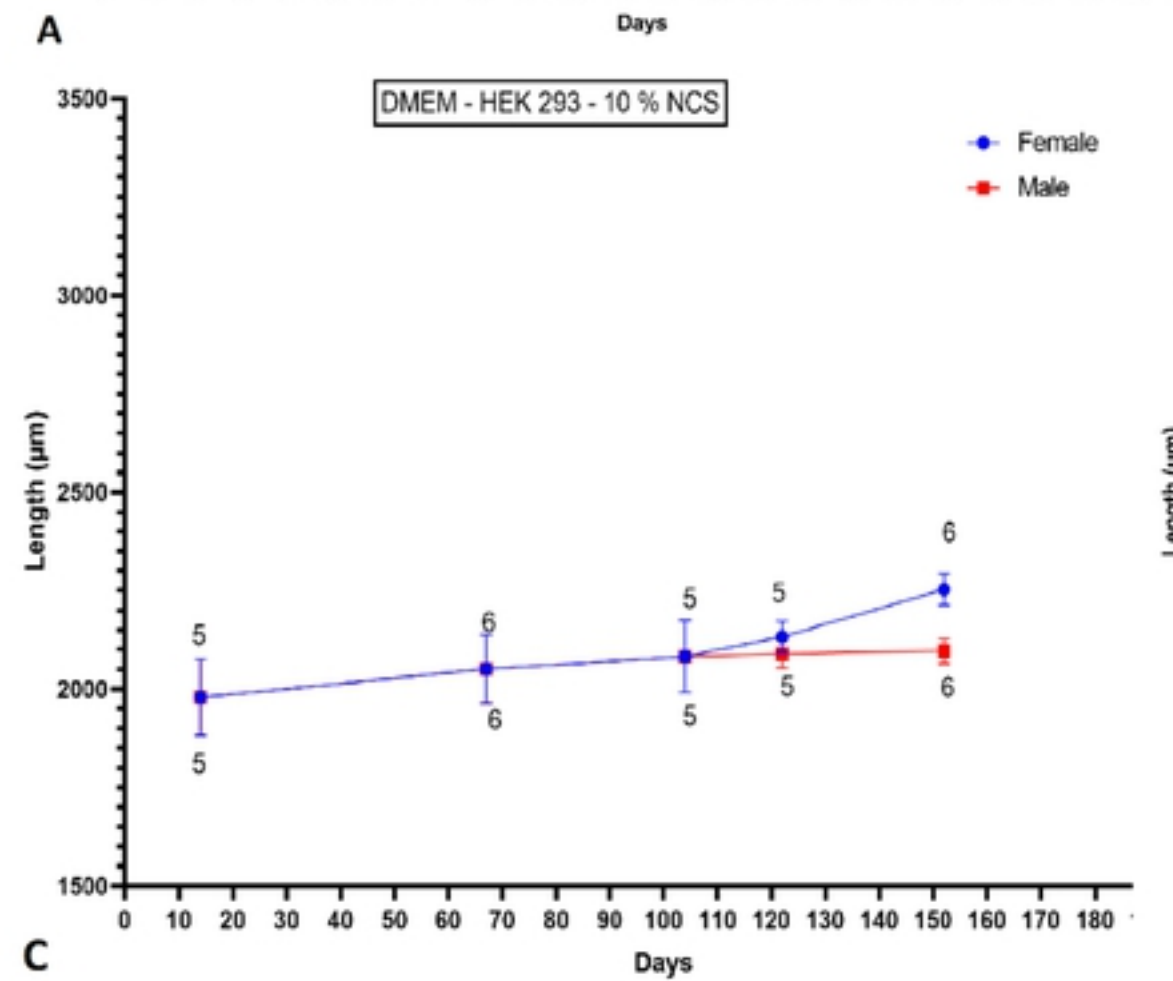
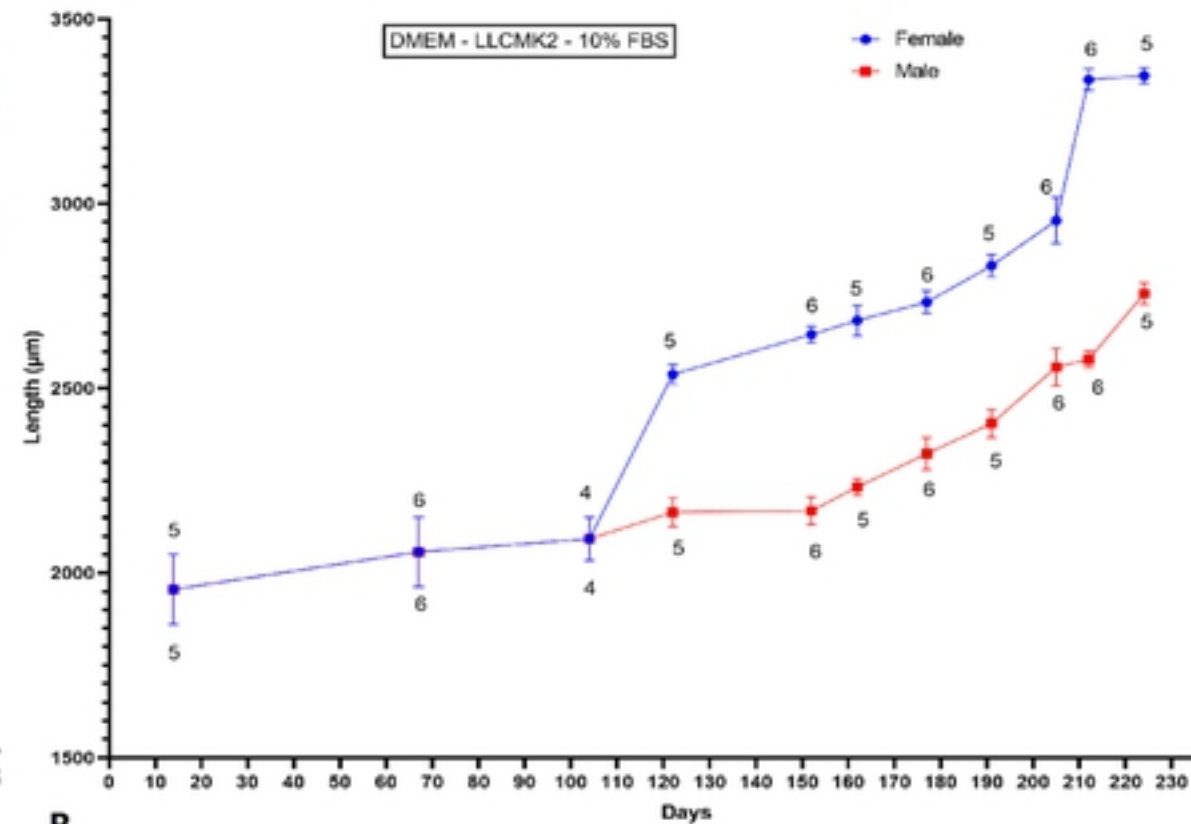
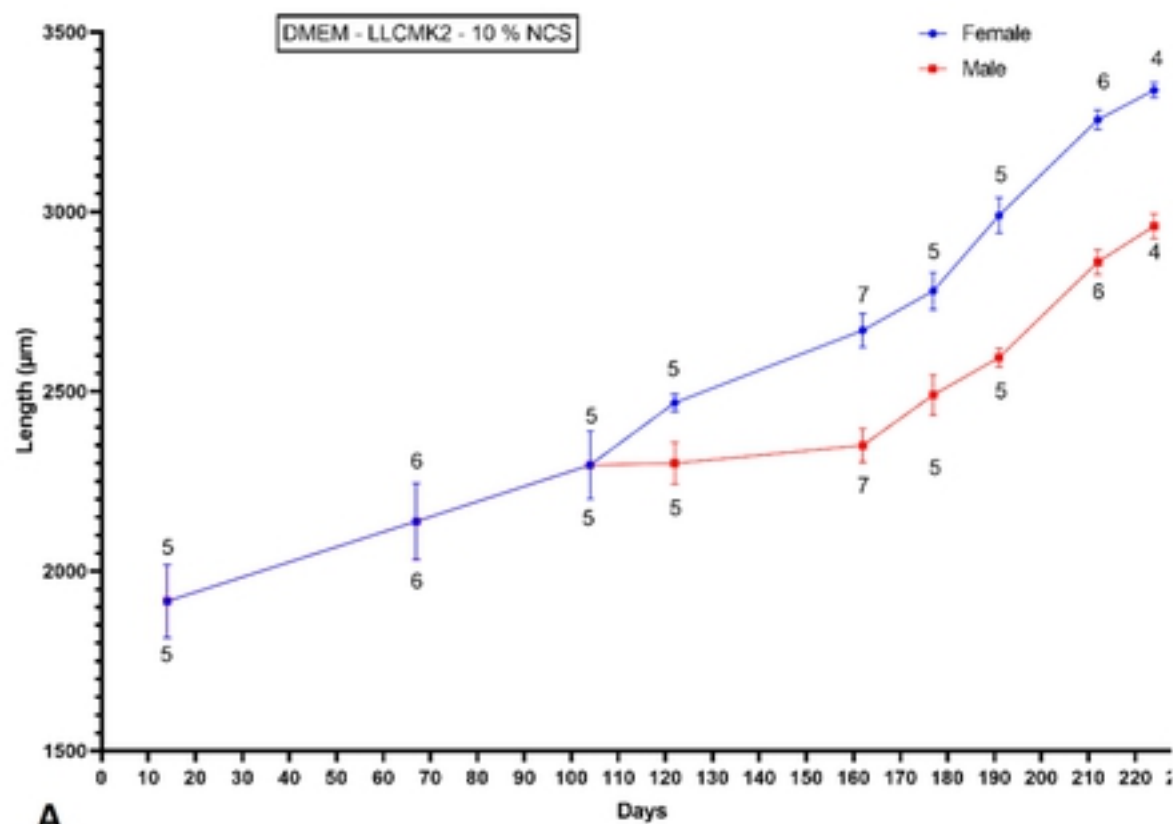


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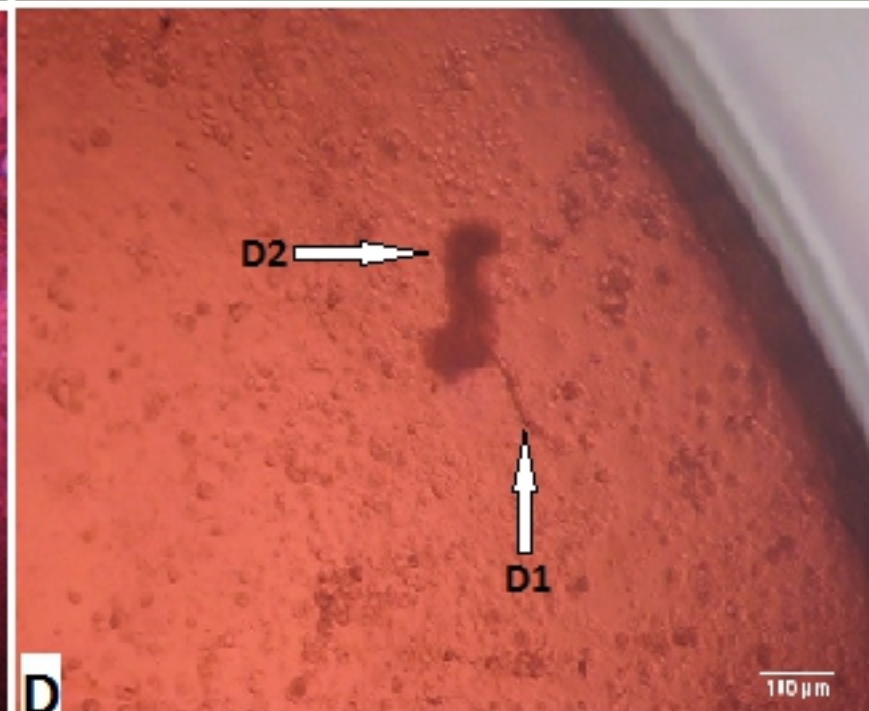
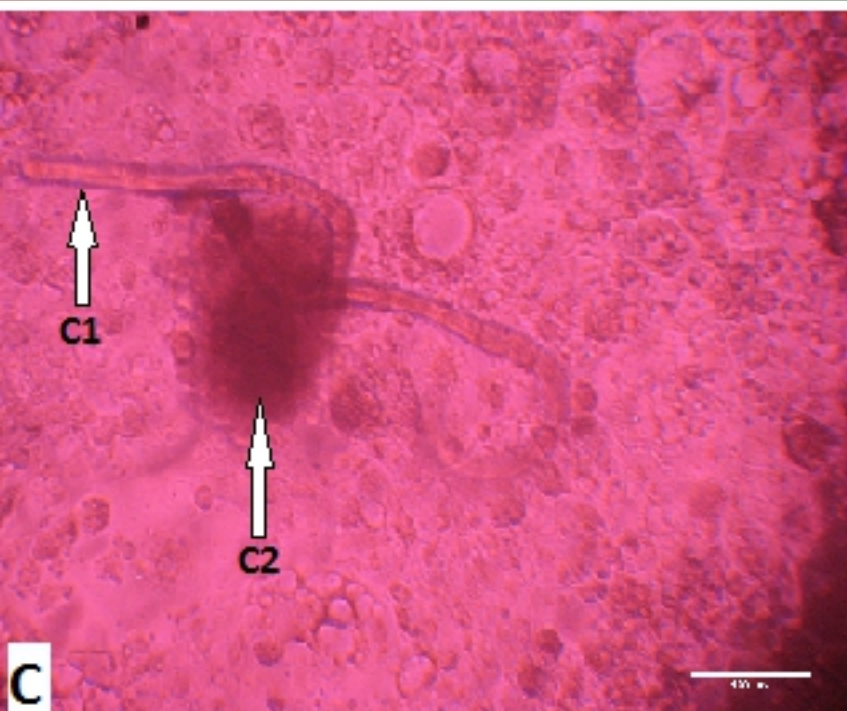
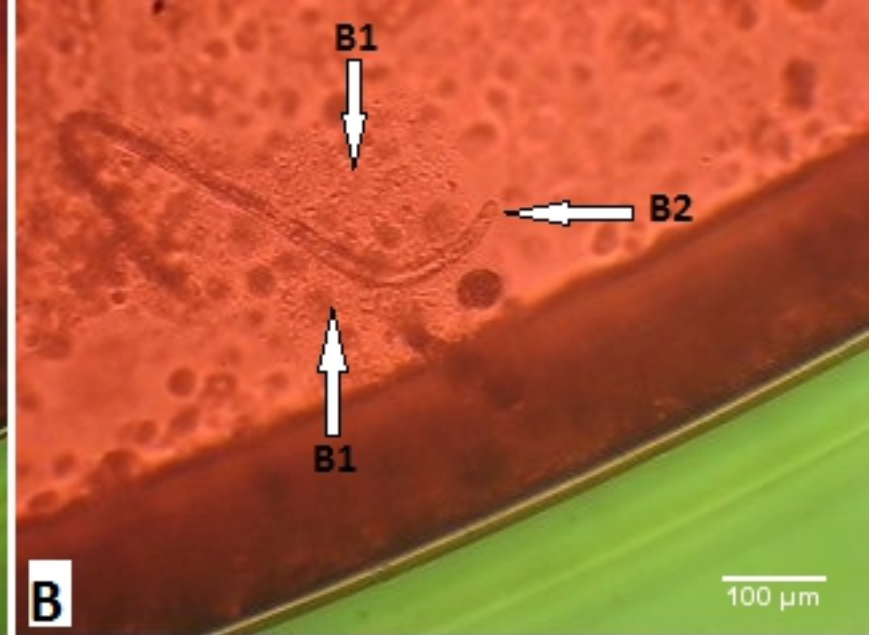
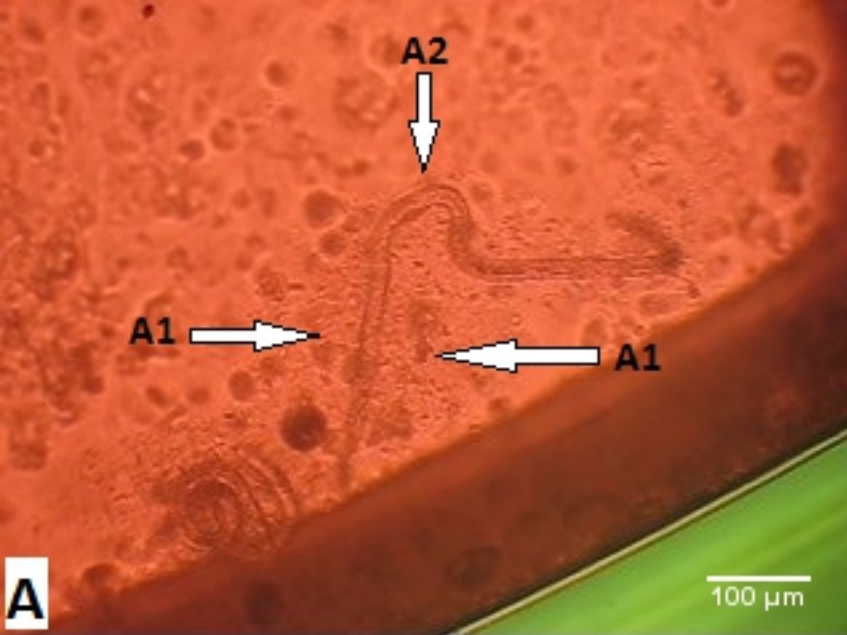
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