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

Narcisse Victor T. Gandjui[1,2], Abdel Jelil Njouendou[1,2,3], Eric Njih Gemeg[1,2], Fanny Fri Fombad[1,2,4], Manuel Ritter[5], Chi Anizette Kien[1,2], Valerine C. Chunda[1,2], Jerome Fru[1,2], Mathias E. Esum[1,2,] , Marc P. Hübner[5], Peter A. Enyong[1,2], Achim Hoerauf[5,6], Samuel Wanji[1,2,*]

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

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

[3]Department of Biomedical Sciences, Faculty of Health Sciences, University of Buea, Buea, Cameroon.

[4]Department of Zoology and Animal Physiology, Faculty of Science, University of Buea, Buea, Cameroon.

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

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

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

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

Abstract

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

Methodology/Principal Findings: Prior to culture, freshly harvested *O. volvulus* L3 larvae from dissected *Simulium* were purified by centrifugation using a 30% Percoll solution to eliminate fly tissue debris and contaminants. Parasites were cultured in both cell-free and cell-based co-culture systems, and monitored daily by microscopic visual inspection. Exhausted culture medium was replenished every 2–3 days. The cell-free culture system supported the viability and motility of *O. volvulus* larvae for up to 84 days (DMEM–10%NCS), while the co-culture system (DMEM–10%FBS–LLC-MK_2) extended the worm survival period to 315 days. Co-culture systems alone promoted the two consecutive parasite moults (L3 to L4 and L4 to L5) with highest moulting rates observed in DMEM–10%FBS–LLC-MK_2 (69.2±30 %), while no moult was observed in DMEM–10%NCS–LEC condition. *O. volvulus* adult worms mating and even mating competitions were observed in DMEM–10% FBS –LLC-MK_2 co-culture system. Early nodulogenesis was observed in both DMEM–10% FBS–LLC-MK_2 and DMEM–10%NCS–LLC-MK_2 systems.

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

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

Author summary

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

Introduction

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

in West, East and Central Africa, the Arabian Peninsula and parts of South and Central America. Globally, about 90 million people are at risk of contracting the disease in endemic areas, with 99% of cases occurring in Sub-Saharan Africa, of which more than 17 million are estimated to be infected and 270,000 are permanently blind as complication [2]. In the past decades, two programmes were implemented to control onchocerciasis. Initially, the Onchocerciasis Control Programme (OCP) focused on elimination of the *Simulium* vector using DDT and larvicides. This vector control programme was later supplemented with ivermectin distribution that has helped many millions of persons to live free of disease [3]. Mass drug administration (MDA) of ivermectin through the African programme for Onchocerciasis control (APOC) has been the principal strategy for onchocerciasis control after OCP in West Africa.

Despite more than two decades of MDA campaign with ivermectin, the disease still persists mainly in sub-Saharan Africa because of several reasons. Ivermectin is solely microfilaricidal with temporal embryo-static effects on adult worms and thus has to be given once or twice per year for the life-span of the adult worms, which is 16–18 years. In addition, the sub-optimal response to ivermectin in some regions has led to persistent transmission [4-6]. Therefore, there is a need for the development of improved drugs that might not only kill *O. volvulus* microfilariae but also other life-cycle stages (L3, L4, L5 and adult worms). Such new candidates are required to reach the United Nations Sustainable Development Goals to eliminate onchocerciasis by 2030 in the majority of endemic countries. However, the advancement of research towards the development of new therapeutics is hindered by the availability of a suitable *in vitro* culture system, where parasite stages such as infective larvae (L3) can be maintained 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

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

Methods

Ethical statement

Ethical clearance was obtained from the National Institutional Review board, Yaoundé (N^0 2018/06/1057/CE/CNERSH/SP) after approval of the protocol. Prior to recruitment, the nature and objectives of the study were explained to potential participants and those who agreed to take part in the study signed a consent form. Special consideration was taken to minimize any health risks of the participant. They were followed-up for ivermectin treatment at the end of the study during the normal MDA

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

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

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

Collection of engorged *Simulium* flies

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

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

Laboratory maintenance of engorged *Simulium*

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

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

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

Preparation of feeder cells and pre-conditioning in culture plates

Monkey kidney cells (LLC-MK_2), mouse lung embryonic cells (LEC), human embryonic kidney cells (HEK-293) and human hepatic cells (HC-04) were provided by the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Each of these feeder cells were cultured in flasks at 37 °C in a humidified CO_2 incubator (Sheldon Mfg. Inch, Cornelius, OR, USA) at 5% CO_2 until the cell layer became fully confluent. For new inoculations and other cell manipulations, cells were dislodged with trypsin solution (25%) containing EDTA and kept at 37 °C for less than 30 minutes. The cell suspension was centrifuged at 239 *x g* for 10 min, the supernatant discarded, and the pellet re-suspended and diluted to 10^5 cells/ml. Aliquots (100 µl) of cell suspensions were plated into each well of a 48-well flat bottom culture plate and kept in the incubator for cells to become fully confluent prior to be used for parasite maintenance in co-culture systems.

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

Harvested L3 from different batches of dissected flies were mixed and pooled to obtain parasites culture material. Two sera supplements were used separately at 10 % concentrations each: fetal bovine serum (Sigma- Aldrich, St Louis, USA) and newborn calf serum (Sigma-Aldrich, Berlin, Germany). Five basic media were used: RPMI-1640, IMDM, NCTC-135, MEM (Sigma-Aldrich, St Louis, USA), and DMEM (Gibco Life Technologies, Cergy-Pontoise, France). Penicillin-Streptomycin-Neomycin (PSN, 2 %) was used as antibiotic and Amphotericin B (2.5 µg/ml) as antifungal. Flat bottom culture plates (48-well) with lids (Corning, Kennebunk, ME, USA) were loaded as follows: For the co-culture systems, parasites (range 8–13 L3) in 1200 µL of the studied medium (basic culture medium + 10 % serum) were loaded into a feeder-cell type pre-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 ceded. 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 µL 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-MK_2) 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:

$$\text{Motility (\%)} = \frac{\sum \text{SiNi}}{3.\sum \text{Ni}} \times 100$$

where Si is the score of point scale i and Ni is the total number of worms at a point scale i [47].

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

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

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

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

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

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

Results

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

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

Since larvae did not become adults and stopped their motility by day 84 at the latest in the cell-free culture systems at stage 4, we next evaluated the combination of the best cell – free system (DMEM - NCS/FBS) with each of the four mammalian cell lines (LLC-MK_2, HC-04, HEK-293 and LEC) in order to improve parasite motility and viability as well as moulting.

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

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

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

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

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

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

Fig 6. Length of *O. volvulus* L4 and L5 larvae observed from the *in vitro* cell-based co-culture systems with respect to days. (Bar representing the median). **A** DMEM – LLCMK$_2$ – 10% FBS. **B** DMEM – LLCMK$_2$ – 10% NCS. **C** DMEM – HC04 – 10% NCS. **D** DMEM – HEK293 – 10% NCS. **E** DMEM – LEC – 10% NCS.

Fig 7. Width of *O. volvulus* L4 and L5 larvae observed from the *in vitro* cell-based co-culture systems with respect to days (Bar representing the median). A DMEM – LLCMK$_2$ – 10% FBS. **B** DMEM – LLCMK$_2$ – 10% NCS. **C** DMEM – HC04 – 10% 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 – LLCMK$_2$ – 10% NCS, DMEM – LLCMK$_2$ – 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 - $LLCMK_2$ and DMEM – 10 % NCS - $LLCMK_2$. 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 - $LLCMK_2$ 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 femle). **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 competion for mating, C3: Sollicited 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 - LLCMK$_2$) 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-MK$_2$ 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).

Fig 10. Cellular aggregation around *O. volvulus* young adults *in vitro*. A&B (A1 and B1: Early recruitment of cell-derived insoluble particles along the worm external wall, A2 and B2: Young adult worm trapped in the cellular aggregate). **C&D** (C2 and D2: Gathering of cell-derived insoluble particles into a globular/oval mass shape engulfing the young adult worms, C1 and D1: Young adult worm being engulfed).

This process lasted for 138 days after which parasites perished. The proportion of adult 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)

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

The contribution of the various culture media and supplements used in the improvement of worm viability were identified based on their standardized coefficient (S5 Table). Among these factors, the presence of feeder cells pre-eminently influenced *O. volvulus* larvae viability, HC-04 feeder cells were classified as topmost factor (β = 0.299) followed by LLC-MK$_2$ feeder cells (β = 0.269), HEK-293 feeder cells (β = 0.129), LEC feeder cells (β = 0.060). Although the basic culture media types also promoted *O. volvulus* larvae viability, their impact was less important than that of feeder cells but higher as compared to that of sera supplements. DMEM basic medium had the leading effect (β = 0.042) and the least effect was observed with IMDM (β = - 0.068 10^{-3}). Both sera supplements, FBS (β = - 0.074) and NCS (β = - 0.052) had unfavourable effect

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

Discussion

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

studies did not provide a consensus on filarial parasite nutritional needs. Zofou *et al.* 2018 [42] established a hierarchical profile of most used *in vitro* culture supplements for filarial parasites, in which the presence of feeder cells was ranked as most top important followed by culture supplementation with serum and finally the culture media type. Based on these observations, four feeder layer cell line types (LLC-MK_2, HC-04, HEK-293 and LEC), two serum types (FBS and NCS) and five basic culture media (RPMI-1640, DMEM, MEM, NCTC-135 and IMDM) were evaluated either in combination in the cell-free systems or in the co-culture systems for the growth and development of *O. volvulus* larvae *in vitro*.

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

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

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

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

Conclusions

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

Abbreviations

APOC: African Programme for Onchocerciasis Control, OCP: Onchocerciasis Control Programme, PTS: Post-Treatment Surveillance, HC-04: Human Hepatocyte cells, HEK-293: embryonic human kidneys cells, LLC-MK2: Monkey Kidney cells, LEC: 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.

691 **Acknowledgements**

692 We thank Mr. Oben Bruno for his assistance in obtaining the necessary vector material
693 for this work.

694 ## Availability of data and materials

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.

699 ## Funding

700 This work was funded through grants awarded to AH, MR, MPH and SW from the
701 German Research Foundation (DFG) (Grant DFG HO 2009/10-1, HO 2009/14-1, HU
702 2144/3-1) within the "German-African Cooperation Projects in Infectiology". In addition,
703 SW and AH are financially supported by the Federal Ministry of Education and
704 Research (BMBF, initiative Research Networks for Health Innovations in sub-Saharan
705 Africa: TAKeOFF) as well as SW, MPH and AH by the Horizon 2020 Framework

706 Programme: HELP. AH is a member of the Excellence Cluster Immunosensation
707 (DFG, EXC 1023) and the German Center of Infectious Disease (DZIF).

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

720 References

721 1. WHO. Onchocerciasis. Available from https://www. who. i nt/news-room/fact-
722 sheets/detail/onchocerciasis. Accessed on 28 January 2020.
723 2. WHO. Progress report on the elimination of human onchocerciasis, 2017-2018.
724 Wkly Epidemiol Rec 2018, 47:633-648.
725 3. Tsalikis G. The onchocerciasis control programme (OCP) in West Africa: a review
726 of progress. Health Policy Plan. 1993; 8(4):349-359.
727 4. Doyle SR, Bourguinat C, Nana-Djeunga HC, Kengne-Ouafo JA, Pion SD, Bopda
728 J, Kamgno J, Wanji S, Che H, Kuesel AC. Genome-wide analysis of ivermectin
729 response by *Onchocerca volvulus* reveals that genetic drift and soft selective

730 sweeps contribute to loss of drug sensitivity. PLoS Negl Trop Dis. 2017;
731 11(7):e0005816.
732 5. Churcher TS, Pion SD, Osei-Atweneboana MY, Prichard RK, Awadzi K,
733 Boussinesq M, Collins RC, Whitworth JA, Basáñez M-G. Identifying sub-optimal
734 responses to ivermectin in the treatment of River Blindness. Proc. Natl. Acad. Sci.
735 2009; 106(39):16716-16721.
736 6. Frempong KK, Walker M, Cheke RA, Tetevi EJ, Gyan ET, Owusu EO, Wilson
737 MD, Boakye DA, Taylor MJ, Biritwum N-K. Does increasing treatment frequency
738 address suboptimal responses to ivermectin for the control and elimination of
739 river blindness? Clin Infect Dis. 2016; 62(11):1338-1347.
740 7. Lustigman S, Huima T, Brotman B, Miller K, Prince AM. *Onchocerca volvulus*:
741 biochemical and morphological characteristics of the surface of third-and fourth-
742 stage larvae. Exp Parasitol. 1990; 71(4):489-495.
743 8. Morris CP, Evans H, Larsen SE, Mitre E. A comprehensive, model-based review
744 of vaccine and repeat infection trials for filariasis. Clin Microbiol Rev. 2013;
745 26(3):381-421.
746 9. Abraham D, Lange AM, Yutanawiboonchai W, Trpis M, Dickerson JW, Swenson
747 B, Eberhard ML. Survival and development of larval *Onchocerca volvulus* in
748 diffusion chambers implanted in primate and rodent hosts. J Parasitol. 1993; 571-
749 582.
750 10. Trees A, Graham S, Renz A, Bianco A, Tanya V. Onchocerca ochengi infections
751 in cattle as a model for human onchocerciasis: recent developments. Parasitol.
752 2000; 120(7):133-142.
753 11. Eberhard ML, Dickerson JW, Tsang VC, Walker EM, Ottesen EA, Chandrashekar
754 R, Weil GJ, Trpis M, Strobert E, Constantinidis I. *Onchocerca volvulus*:

Parasitological and serologic responses in experimentally infected chimpanzees and mangabey monkeys. Exp parasitol. 1995; 80(3):454-462.

12. Ngu J, Neba G, Leke N, Titanji V, Asonganyi T, Ndumbe P. Selective recovery of living microfilariae from *Onchocerca volvulus* nodules. Determination of optimal conditions for their culture *in vitro* for excretory/secretory products. Acta trop. 1981; 38(3):261-266.
13. Walter R. *In vitro* maintenance of *Onchocerca volvulus* for harvest of excretory and secretory products. Trop. med: official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit (GTZ). 1988; 39:448-449.
14. Strotel G, Bokhof A, Comley J. Viability of *Onchocerca volvulus in vitro*. Parasitol. 1993; 107(2):175-182.
15. Smith HL, Paciorkowski N, Babu S, Rajan T. Development of a serum-free system for the *in vitro* cultivation of *Brugia malayi* infective-stage larvae. Exp parasitol. 2000; 95(4):253-264.
16. Gunawardena NK, Fujimaki Y, Aoki Y, Mishima N, Ezaki T, Uni S, Kimura E. Differential effects of diethylcarbamazine, tetracycline and the combination on *Brugia pahangi* adult females *in vitro*. Parasitol. Int. 2005; 54(4):253-259.
17. Kwan–Lim G, Gregory W, Selkirk M, Partono F, Maizels R. Secreted antigens of filarial nematodes: a survey and characterization of *in vitro* excreted/secreted products of adult Brugia malayi. Parasite immunol. 1989; 11(6):629-654.
18. Sakwe A, Evehe M-S, Titanji V. *In vitro* production and characterization of excretory/secretory products of *Onchocerca volvulus*. Parasite. 1997; 4(4):351-358.

779 19. Waa EAV, Foster LA, DeRuiter J, Guderian RH, Williams JF, Geary TG.
780 Glutamine-supported motility of adult filarial parasites *in vitro* and the effect of
781 glutamine antimetabolites. J Parasitol. 1993; 173-180.
782 20. O'Neill M, Mansour A, DiCosty U, Geary J, Dzimianski M, McCall SD, McCall JW,
783 Mackenzie CD, Geary TG. An *in vitro/in vivo* model to analyze the effects of
784 flubendazole exposure on adult female *Brugia malayi*. PLoS Negl Trop Dis. 2016;
785 10(5).
786 21. Hagen H, Grunewald J, Ham P. The *in vitro* culture of *Onchocerca* spp.: II. The
787 excretory/secretory products of the first and third stage larvae of *Onchocerca*
788 *lienalis* and *O. ochengi*. Appl. Parasitol. 1995; 36(2):155-159.
789 22. Townson S, Tagboto SK. *In vitro* cultivation and development of *Onchocerca*
790 *volvulus* and *Onchocerca lienalis* microfilariae. Am J Trop Med Hyg. 1996;
791 54(1):32-37.
792 23. Hussein H, El Sammani S. *Onchocerca raillieti:* Release from skin snips,
793 maintenance *in vitro* and periodicity of microfilariae. VET RES COMMUN. 1990;
794 14(1):31-39.
795 24. McDiarmid SS, Matthews BE. Survival and behavior of *Onchocerca gutturosa*
796 microfilariae *in vitro*. J parasitol. 1982; 470-477.
797 25. Comley J, Szopa T, Strote G, Buttner M, Darge K, Buttner W. A preliminary
798 assessment of the feasibility of evaluating promising antifilarials *in vitro* against
799 adult *Onchocerca volvulus*. Parasitology. 1989; 99(3):417-425.
800 26. Njouendou AJ, Fombad FF, O'Neill M, Zofou D, Nutting C, Ndongmo PC,
801 Kengne-Ouafo AJ, Geary TG, Mackenzie CD, Wanji S. Heterogeneity in the *in*
802 *vitro* susceptibility of *Loa loa* microfilariae to drugs commonly used in
803 parasitological infections. Parasites & vectors. 2018; 11(1):223.

27. Tippawangkosol P, Choochote W, Riyong D, Jitpakdi A, Pitasawat B. A simple technique for the *in vitro* cultivation of nocturnally subperiodic *Brugia malayi* infective larvae. Southeast Asian J. Trop. Med. Public Health. 2003; 33:16-22.

28. Pollack R, Lok J, Donnelly J. Analysis of glutathione-enhanced differentiation by microfilariae of *Onchocerca lienalis* (Filarioidea: Onchocercidae) *in vitro*. J parasitol. 1988; 353-359.

29. Strote G. *In vitro* study on the effect of the new chemotherapeutic agents CGP 6140 and CGP 20376 on the microfilariae of *Onchocerca volvulus*. Trop. med: official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit (GTZ) 1987; 38(3):211-213.

30. Schulz-Key H, Karam M. Quantitative assessment of microfilariae and adults of *Onchocerca volvulus* in ethanol-fixed biopsies and nodules. Trans R Soc Trop Med Hyg. 1984; 78(2):157-159.

31. Engelbrecht F, Schulz-Key H. Observations on adult *Onchocerca volvulus* maintained *in vitro*. Trans R Soc Trop Med Hyg. 1984; 78(2):212-215.

32. Lok J, Pollack R, Cupp E, Bernardo M, Donnelly J, Albiez E. Development of *Onchocerca lienalis* and *O. volvulus* from the third to fourth larval stage *in vitro*. Tropenmed Parasitol. 1984; 35(4):209-211.

33. Townson S, Connelly C, Muller R. Optimization of culture conditions for the maintenance of Onchocerca gutturosa adult worms *in vitro*. J HELMINTHOL. 1986; 60(4):323-330.

34. Pudney M, Litchfield T, Bianco A, Mackenzie C. Moulting and exsheathment of the infective larvae of *Onchocerca lienalis* (Filarioidea) *in vitro*. Acta trop. 1988; 45(1):67-76.

828 35. Litchfield T, Mackenzie C, Varma M. The conditions required for the maintenance
829 of *Onchocerca lienalis* microfilariae *in vitro*. Acta trop. 1991; 48(4):293-304.
830 36. Cupp M, Cupp E, Poulopoulou C. The use of cell-conditioned medium for the *in*
831 *vitro* culture of Onchocerca spp. larvae (Nematoda: Filarioidea). Trop. Med:
832 official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche
833 Gesellschaft fur Technische Zusammenarbeit (GTZ) 1990; 41(1):20-24.
834 37. Townson S, Connelly C, Dobinson A, Muller R. Drug activity against Onchocerca
835 gutturosa males *in vitro*: a model for chemotherapeutic research on
836 onchocerciasis. J HELMINTHOL. 1987; 61(4):271-281.
837 38. Njouendou AJ, Kien CA, Esum ME, Ritter M, Ndongmo WPC, Fombad FF,
838 Gandjui NVT, Njiokou F, Enyong P, Pfarr K. *In vitro* maintenance of *Mansonella*
839 *perstans* microfilariae and its relevance for drug screening. Exp Parasitol. 2019;
840 107769.
841 39. Falcone F, Zahner H, Schlaak M, Haas H. *In vitro* cultivation of third-stage larvae
842 of Brugia malayi to the young adult stage. Trop Med Parasitol. 1995; 46(4):230-
843 234.
844 40. Fombad FF, Njouendou AJ, Ndongmo PC, Ritter M, Chunda VC, Metuge HM,
845 Gandjui NVT, Enyong P, Njiokou F, Hoerauf A. Effect of flubendazole on
846 developing stages of *Loa loa in vitro* and *in vivo*: a new approach for screening
847 filaricidal agents. Parasites & vectors. 2019; 12(1):14.
848 41. Njouendou AJ, Ritter M, Ndongmo WPC, Kien CA, Narcisse GTV, Fombad FF,
849 Tayong DB, Pfarr K, Layland LE, Hoerauf A. Successful long-term maintenance
850 of *Mansonella perstans* in an *in vitro* culture system. Parasites & vectors. 2017;
851 10(1):563.

852 42. Zofou D, Fombad FF, Gandjui NV, Njouendou AJ, Kengne-Ouafo AJ, Ndongmo
853 PWC, Datchoua-Poutcheu FR, Enyong PA, Bita DT, Taylor MJ. Evaluation of *in*
854 *vitro* culture systems for the maintenance of microfilariae and infective larvae of
855 *Loa loa*. Parasites & vectors. 2018; 11(1):275.
856 43. Townson S, Tagboto S, Castro J, Lujan A, Awadzi K, Titanji V. Comparison of the
857 sensitivity of different geographical races of *Onchocerca volvulus* microfilariae to
858 ivermectin: studies *in vitro*. Trans R Soc Trop Med Hyg. 1994; 88(1):101-106.
859 44. Voronin D, Tricoche N, Jawahar S, Shlossman M, Bulman CA, Fischer C,
860 Suderman MT, Sakanari JA, Lustigman S. Development of a preliminary *in vitro*
861 drug screening assay based on a newly established culturing system for pre-adult
862 fifth-stage *Onchocerca volvulus* worms. PLoS Negl Trop Dis. 2019;
863 13(1):e0007108.
864 45. Wanji S, Kengne-Ouafo JA, Esum ME, Chounna PW, Tendongfor N, Adzemye
865 BF, Eyong JE, Jato I, Datchoua-Poutcheu FR, Kah E. Situation analysis of
866 parasitological and entomological indices of onchocerciasis transmission in three
867 drainage basins of the rain forest of South West Cameroon after a decade of
868 ivermectin treatment. Parasites & vectors. 2015; 8(1):202.
869 46. Tendongfor N, Wanji S, Ngwa JC, Esum ME, Specht S, Enyong P, Matthaei KI,
870 Hoerauf A. The human parasite *Loa loa* in cytokine and cytokine receptor gene
871 knock out BALB/c mice: survival, development and localization. Parasites &
872 vectors. 2012; 5(1):43.
873 47. Njouendou AJ, Ritter M, Kien CA, Esum ME, Chounna Ndongmo WP, Fombad
874 FF, Gandjui NVT, Njiokou F, Enyong P, Pfarr K *et al*. Dataset on *in vitro*
875 maintenance of *Mansonella perstans* microfilariae and drug testing. Data Brief.
876 2019; 104930.

877 48. Page AP, Johnstone I. The cuticle. In: WormBook. The C. elegans Research
878 Community .2007. Available from http://www.wormbook.org.
879 49. Singh R, Sulston J. Some observations on moulting in Caenorhabditis elegans.
880 Nematologica 1978, 24(1):63-71.
881 50. Toback FG, Walsh-Reitz MM, Mendley SR, Kartha S. Kidney epithelial cells
882 release growth factors in response to extracellular signals. Pediatr Nephrol. 1990;
883 4(4):363-371.
884 51. McConnell MP, Dhar S, Naran S, Nguyen T, Bradshaw RA, Evans GR. *In vivo*
885 induction and delivery of nerve growth factor, using HEK-293 cells. Tissue Eng.
886 2004; 10(9-10):1492-1501.
887 52. Wisnewski N, Weinstein PP. Growth and development of *Brugia pahangi* larvae
888 under various *in vitro* conditions. J parasitol. 1993; 390-398.
889 53. Barker DM. Copulatory plugs and paternity assurance in the nematode
890 Caenorhabditis elegans. Anim. Behav. 1994; 48(1):147-156.
891 54. Garcia LR, Mehta P, Sternberg PW. Regulation of distinct muscle behaviors
892 controls the C. elegans male's copulatory spicules during mating. Cell. 2001;
893 107(6):777-788.
894 55. Shan G, Walthall W. Copulation in C. elegans males requires a nuclear hormone
895 receptor. Dev. Biol. 2008; 322(1):11-20.
896 56. Collins R, Lujan L, Figueroa M, Campbell C. Early formation of the nodule in
897 Guatemalan onchocerciasis. Am J Trop Med Hyg. 1982; 31(2):267-269.
898 57. Al-Qaoud KM, Pearlman E, Hartung T, Klukowski J, Fleischer B, Hoerauf A. A
899 new mechanism for IL-5-dependent helminth control: neutrophil accumulation
900 and neutrophil-mediated worm encapsulation in murine filariasis are abolished in
901 the absence of IL-5. Int. Immunol. 2000; 12(6):899-908.

902 58. Boakye AD, Raybould NJ. The Effect of Different Methods of Oviposition
903 Inducement on Egg Fertility Rates in a Simulium Damnosum Theobald Complex
904 Species (Diptera: Simuliidae). J AM MOSQUITO CONTR. 1985; 1(4):535-537.

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.*

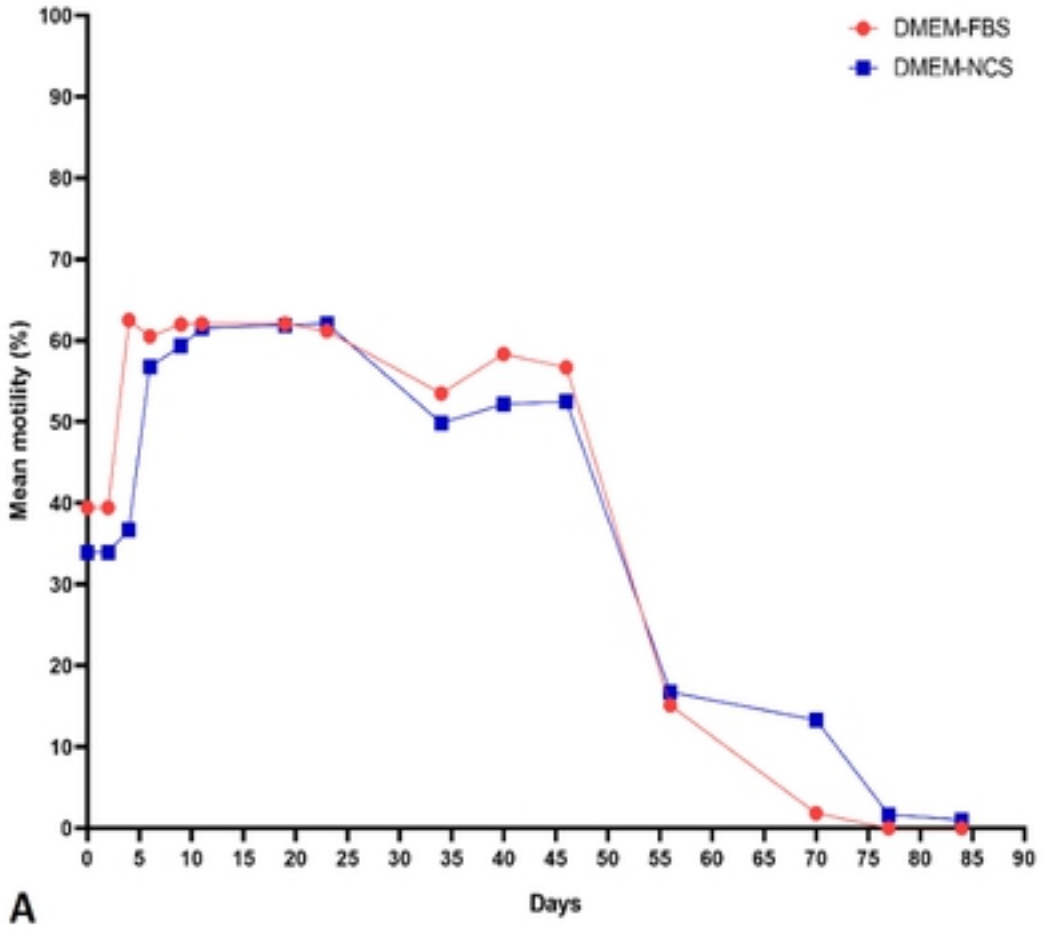
911 **S5 Table.** Experimental factors introduced in the model influencing *O. volvulus* larvae
912 viability and their standardized coefficients.

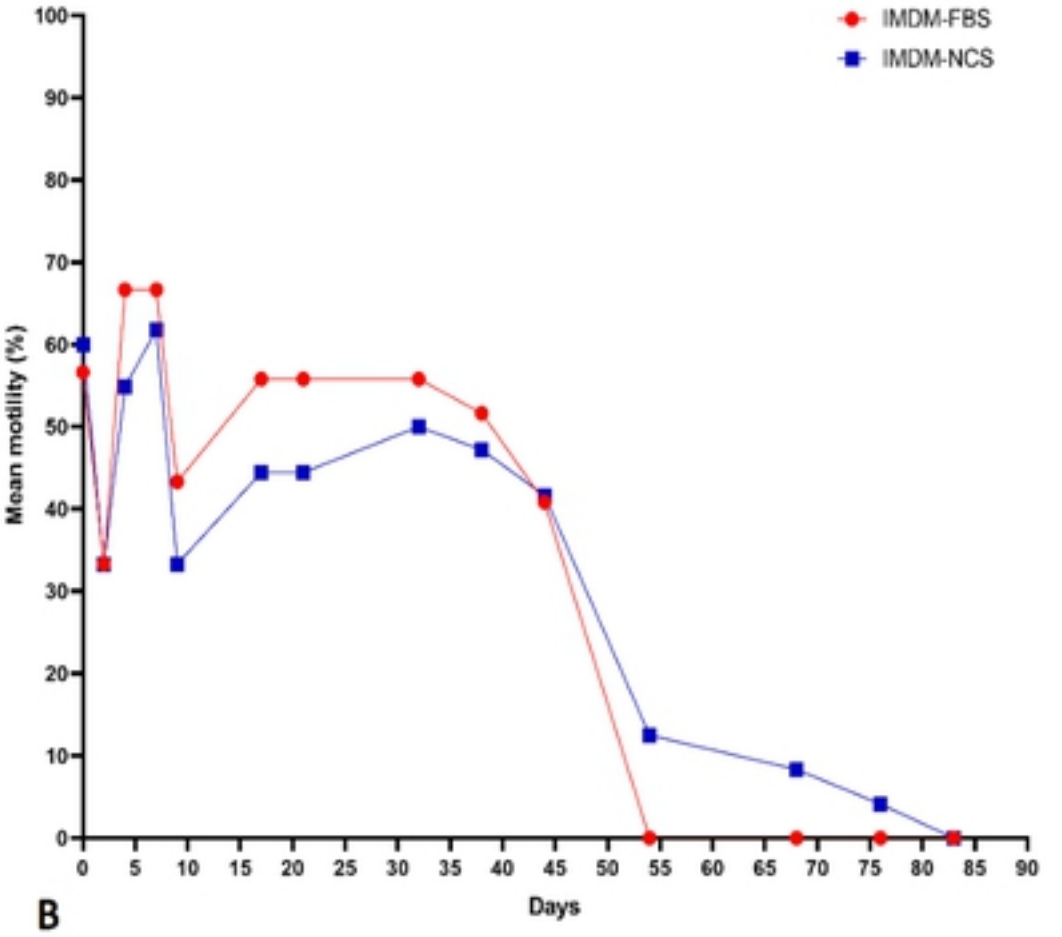
913 **S6 Figure.** Histogram of the residuals (errors) in the model for normal distribution
914 and homoscedasticity.

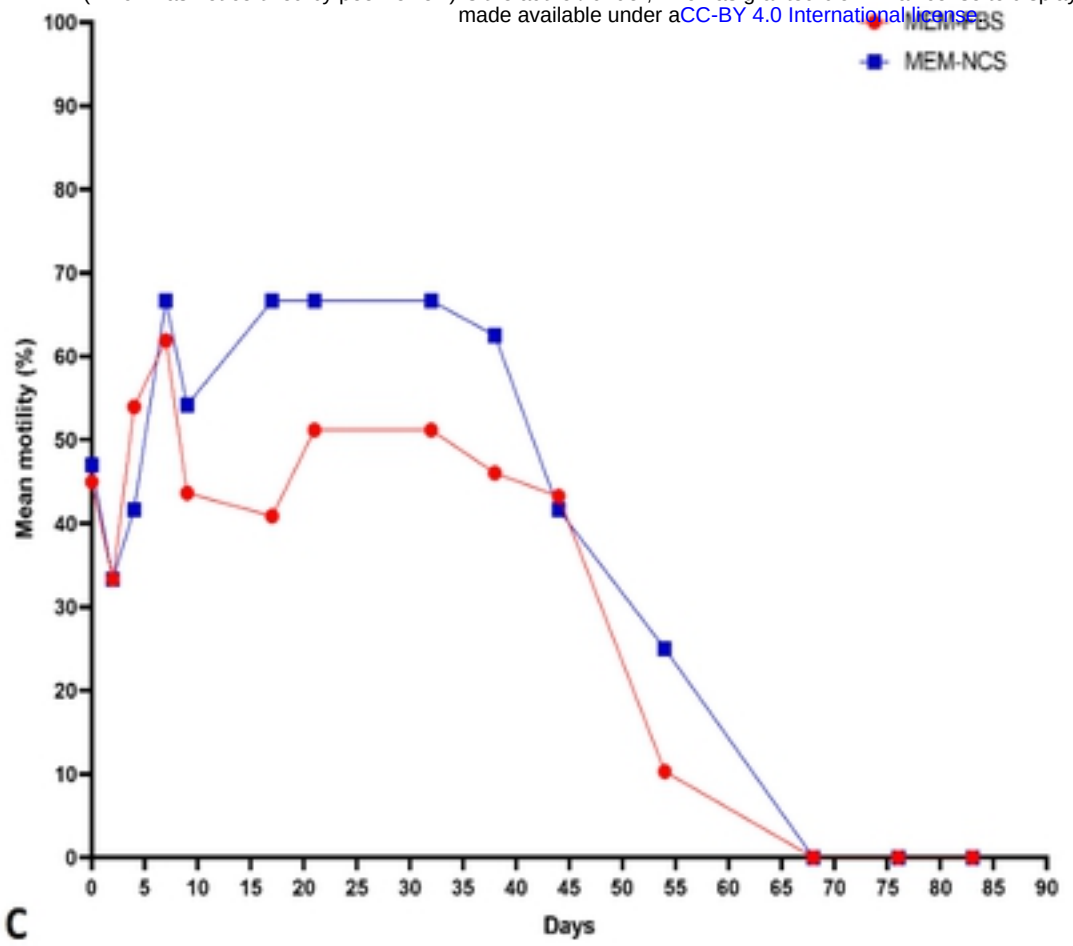
915 **S7 Figure.** Q-Q plot suggesting normal distribution of the residual.

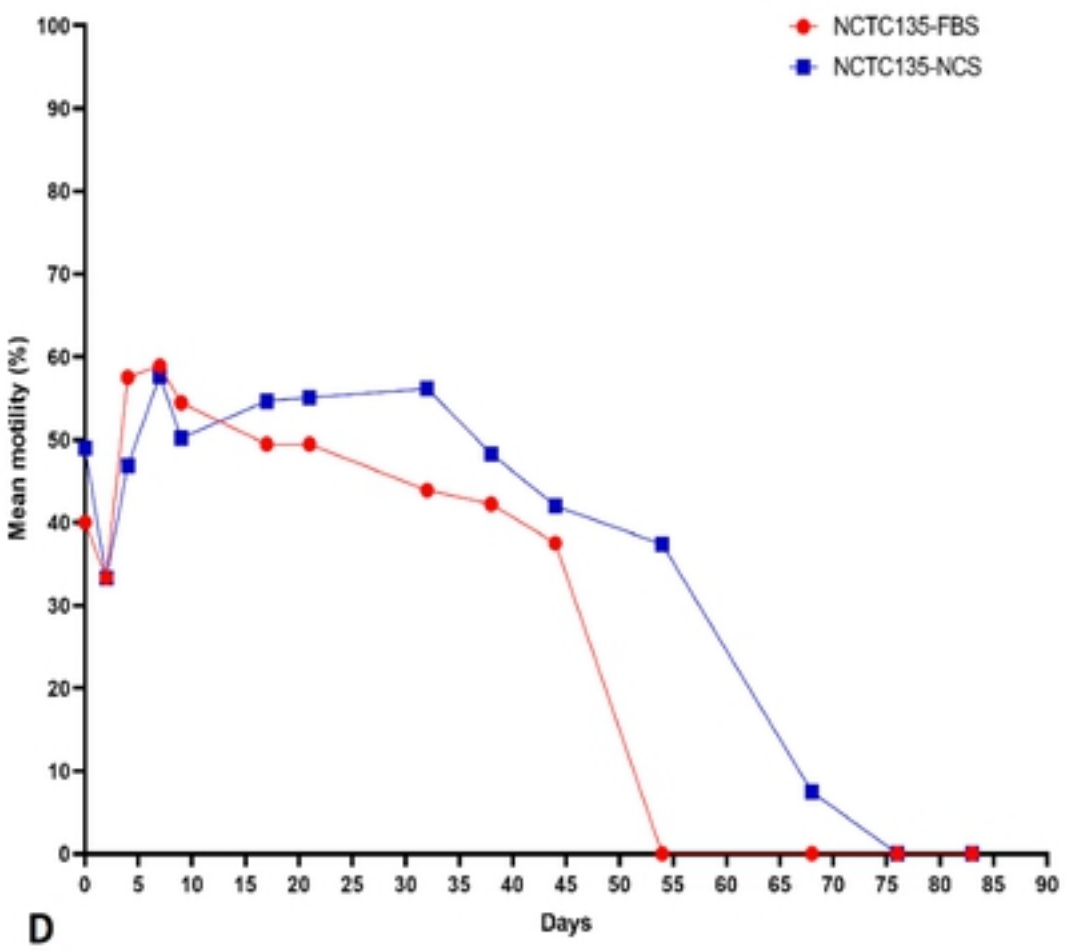
916 **S8 Figure.** Model predicted $T_{20\ and}$ T_{10} values (Days).

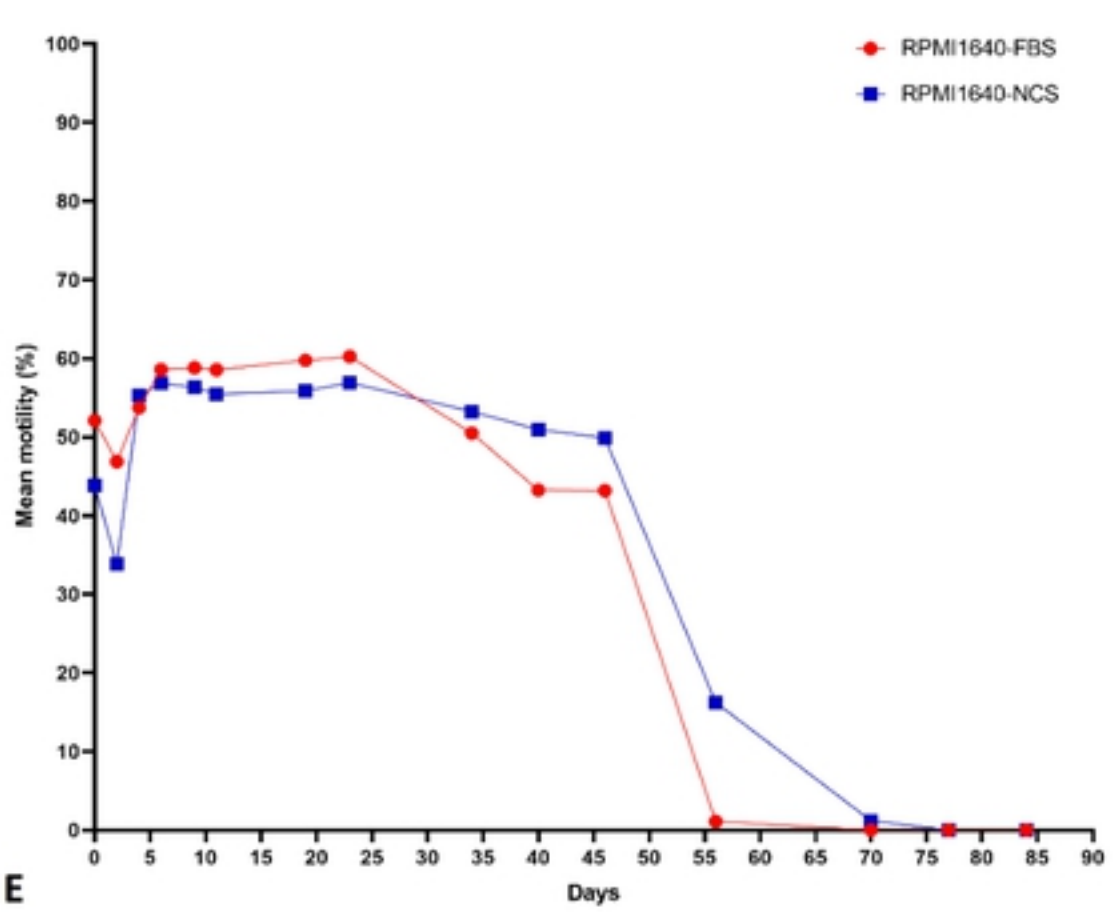
917



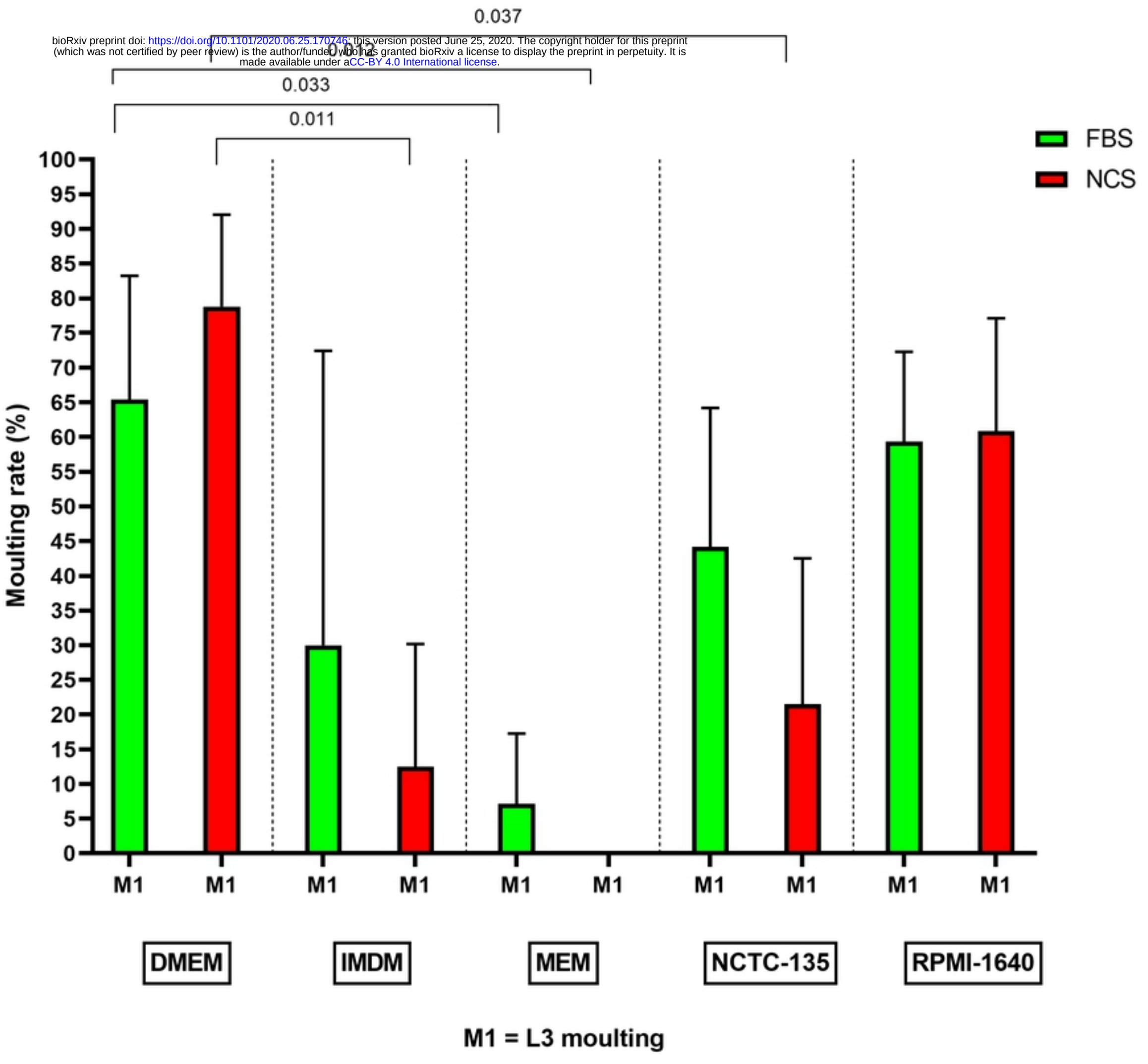




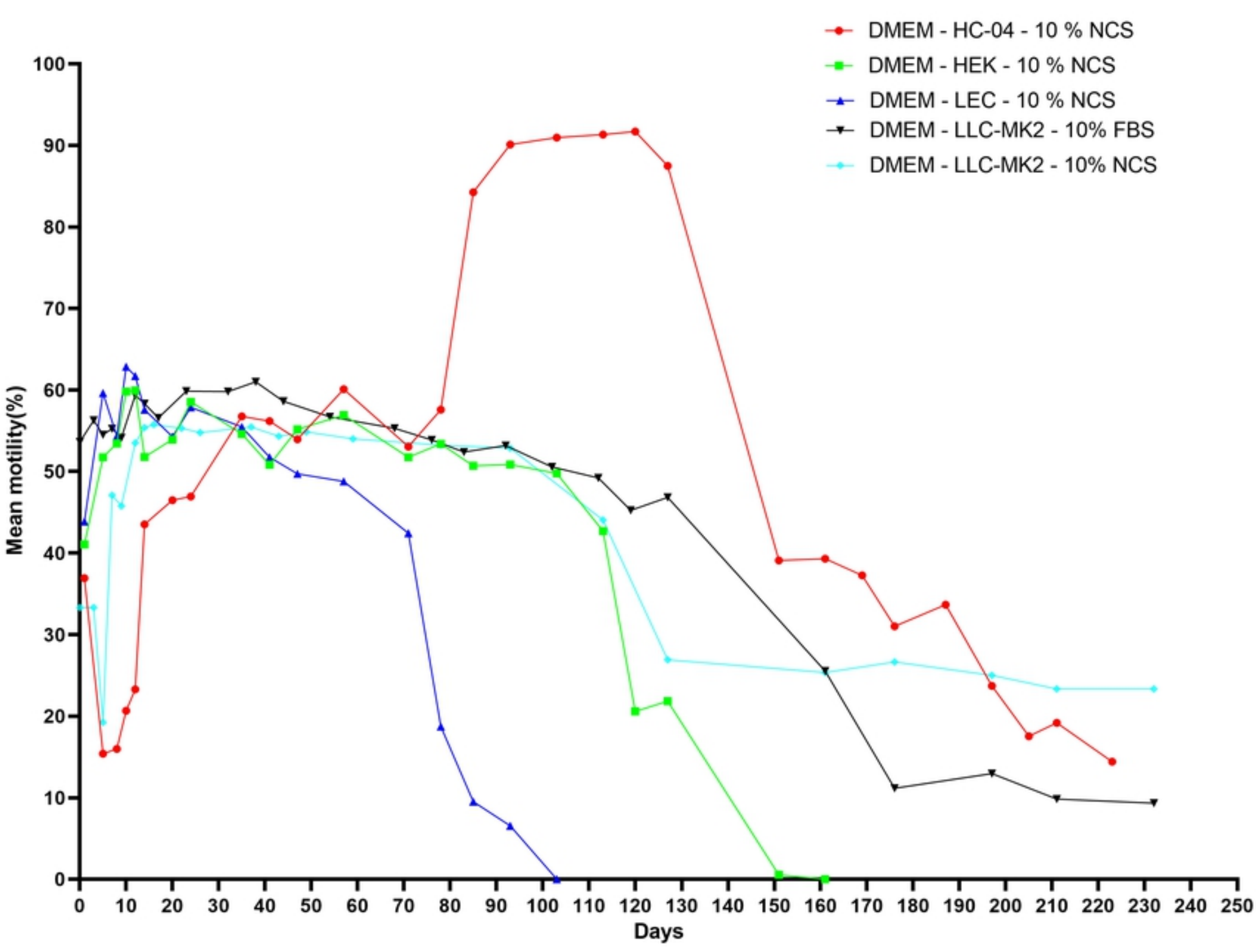




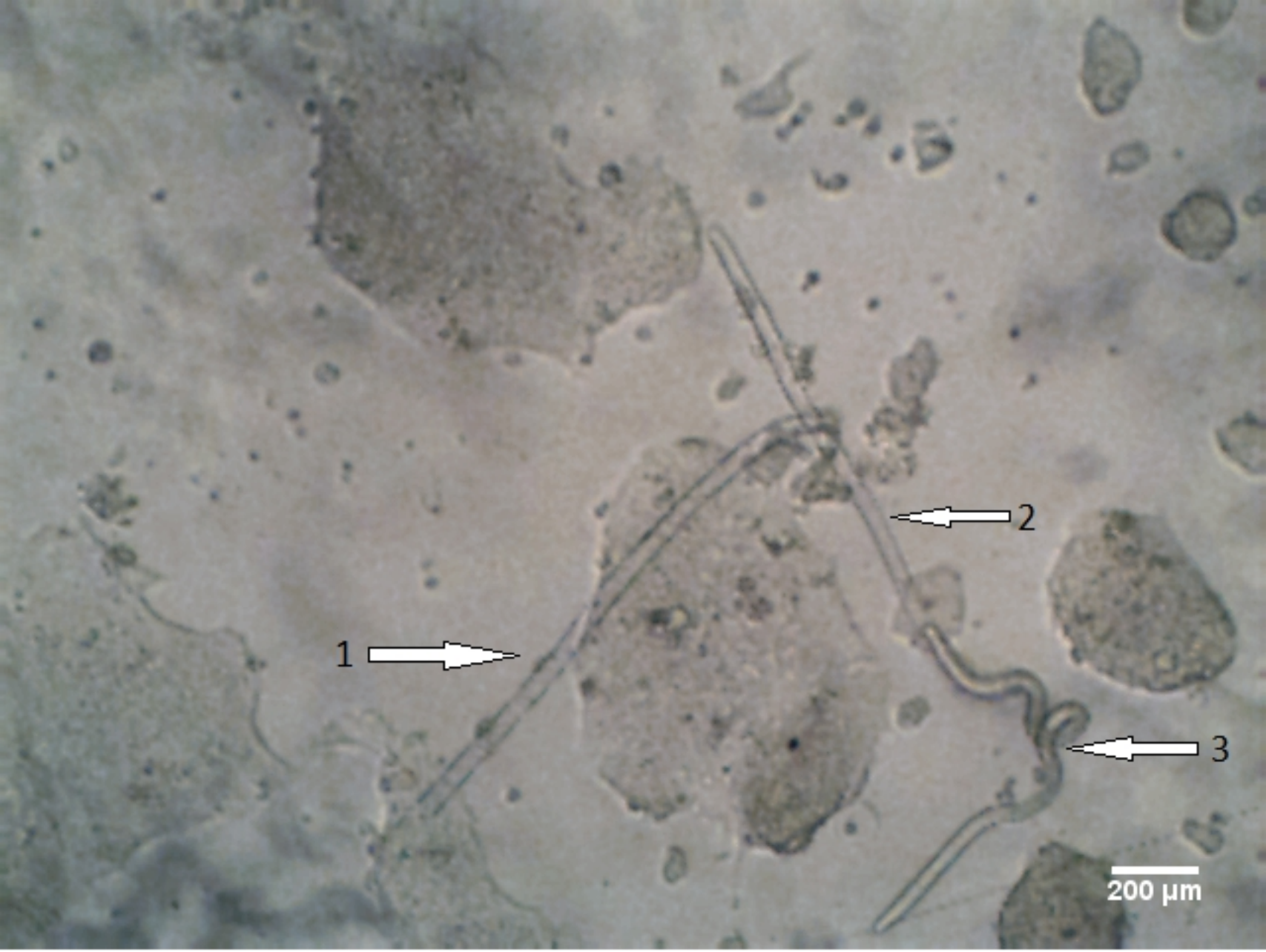
Figure



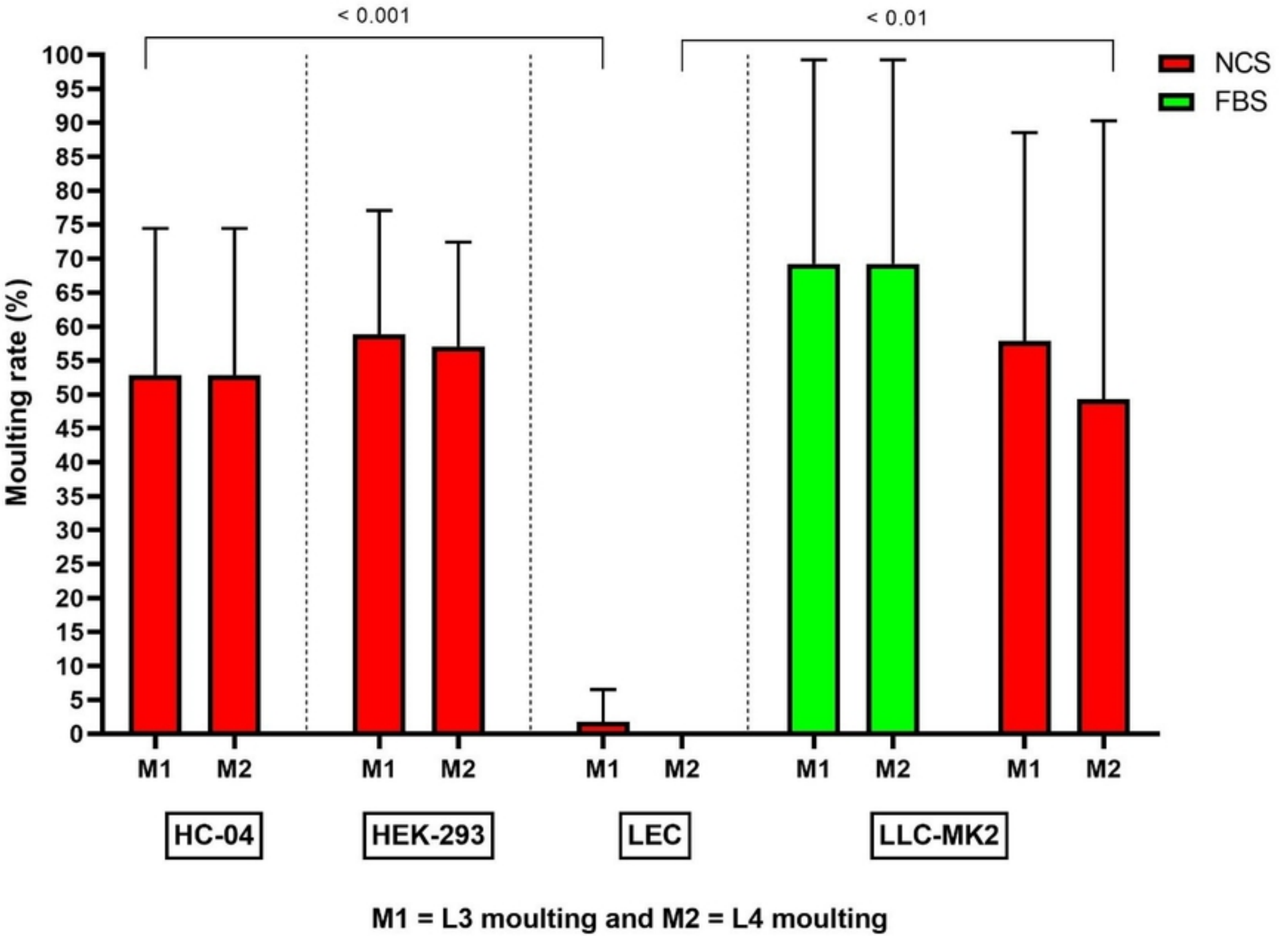
Figure


DMEM - HC-04 - 10 % NCS
DMEM - HEK - 10 % NCS
DMEM - LEC - 10 % NCS
DMEM - LLC-MK2 - 10% FBS
DMEM - LLC-MK2 - 10% NCS
Mean motility(%)
Days
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250

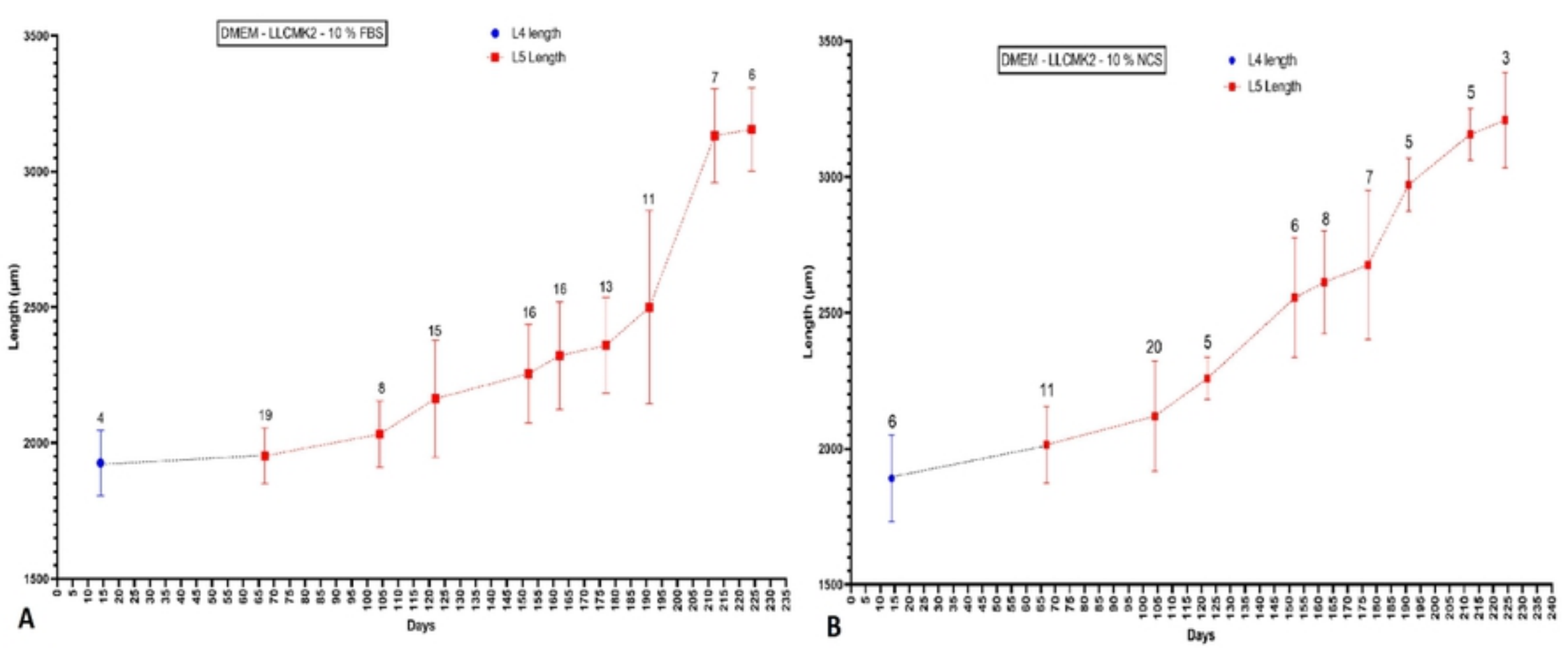
Figure



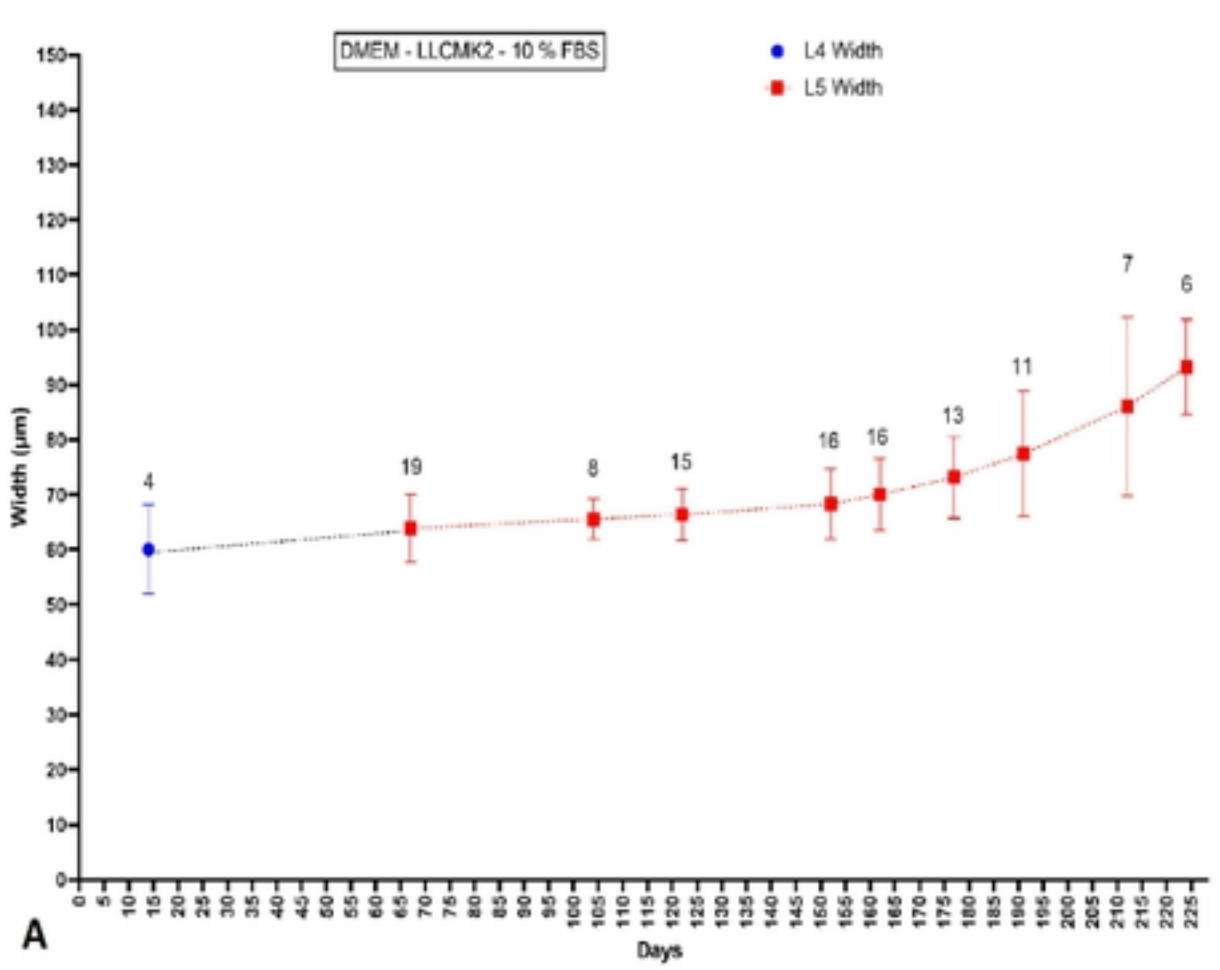
Figure

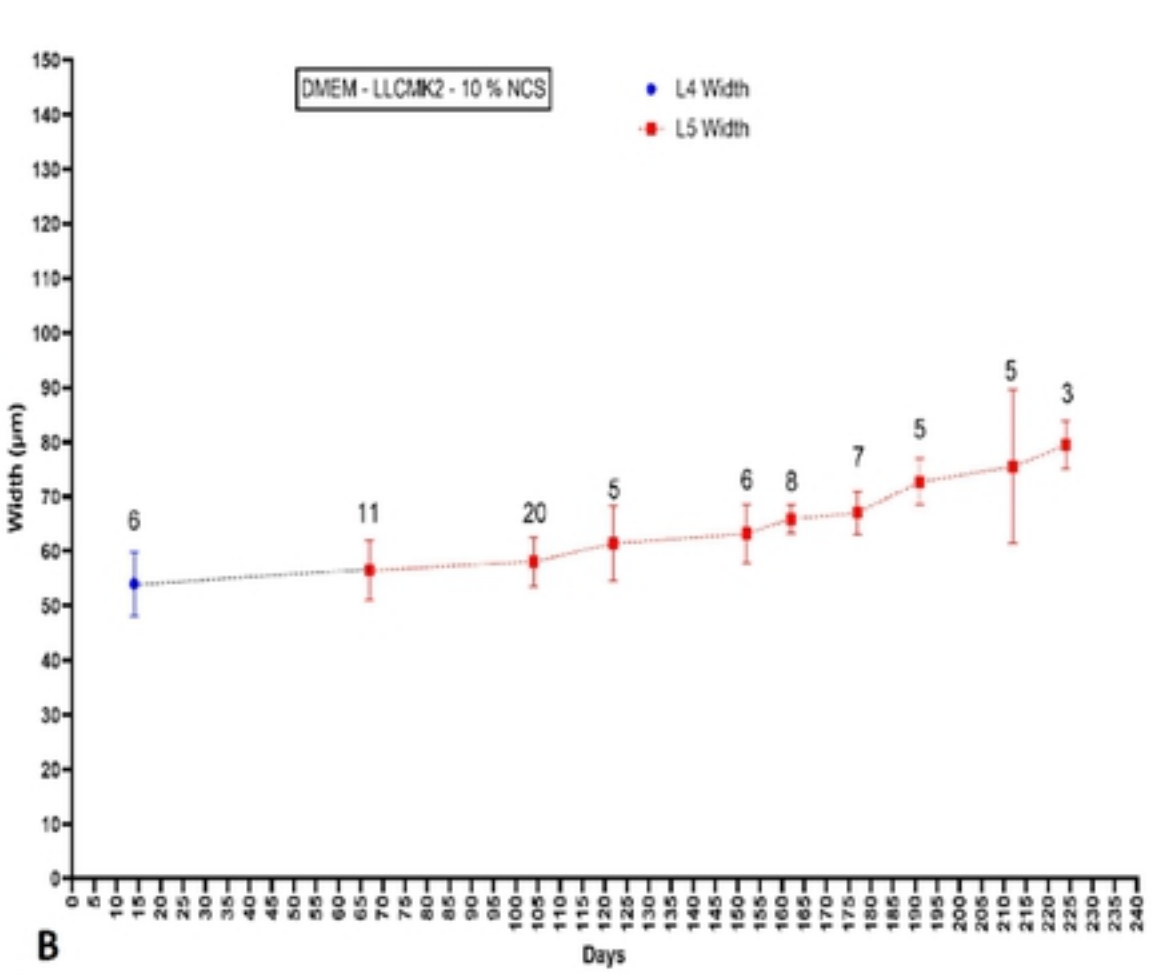

< 0.001
< 0.01
NCS
FBS
Moulting rate (%)
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
M1
M2
M1
M2
M1
M2
M1
M2
M1
M2
HC-04
HEK-293
LEC
LLC-MK2
M1 = L3 moulting and M2 = L4 moulting

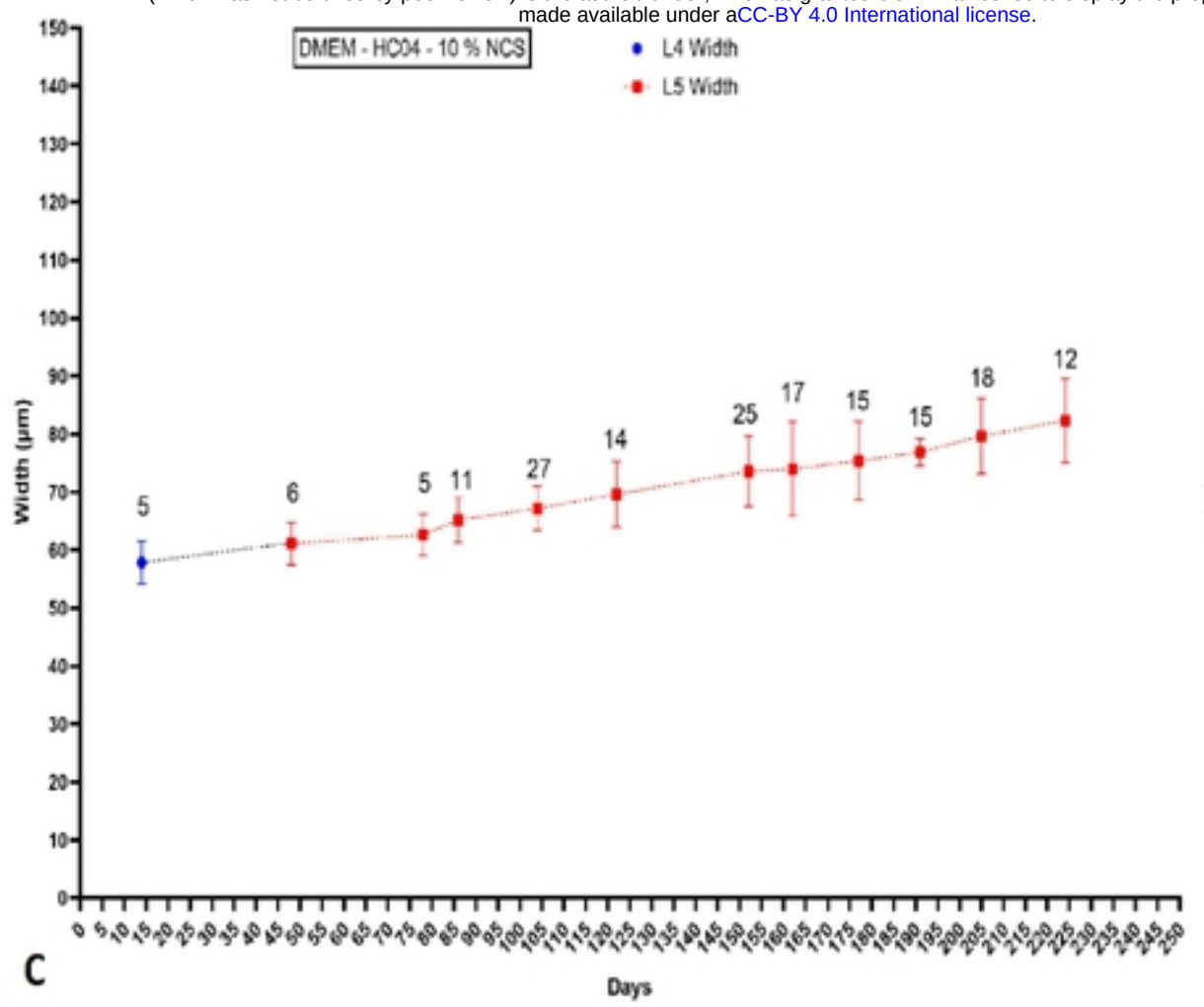
Figure

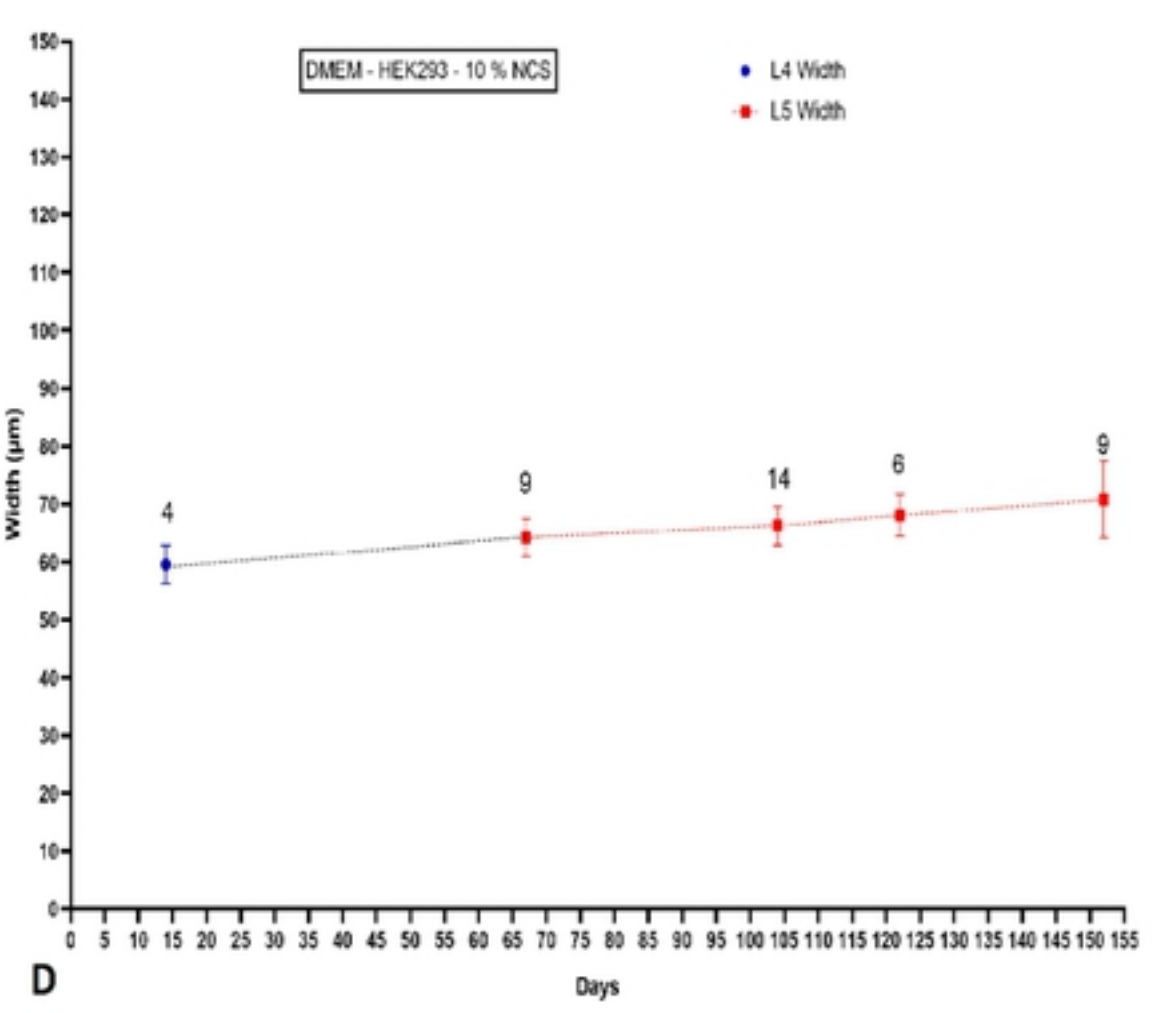


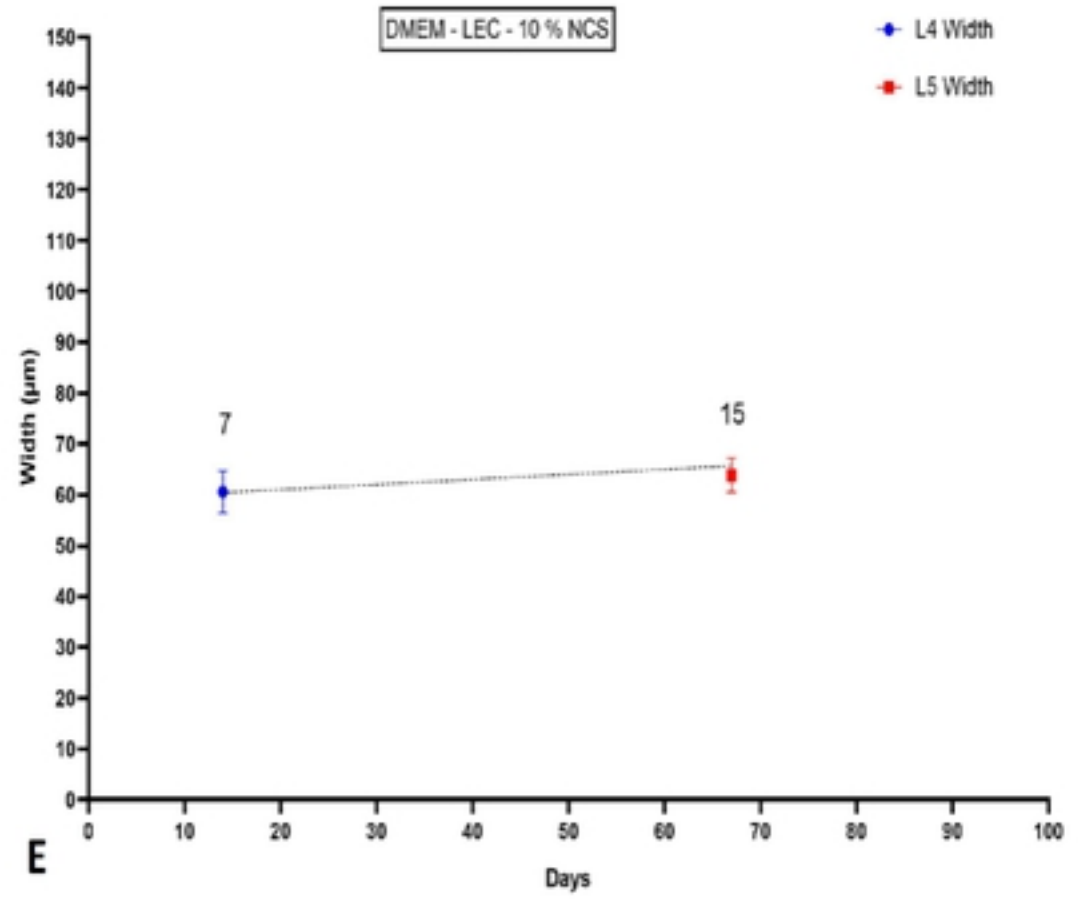
Figure



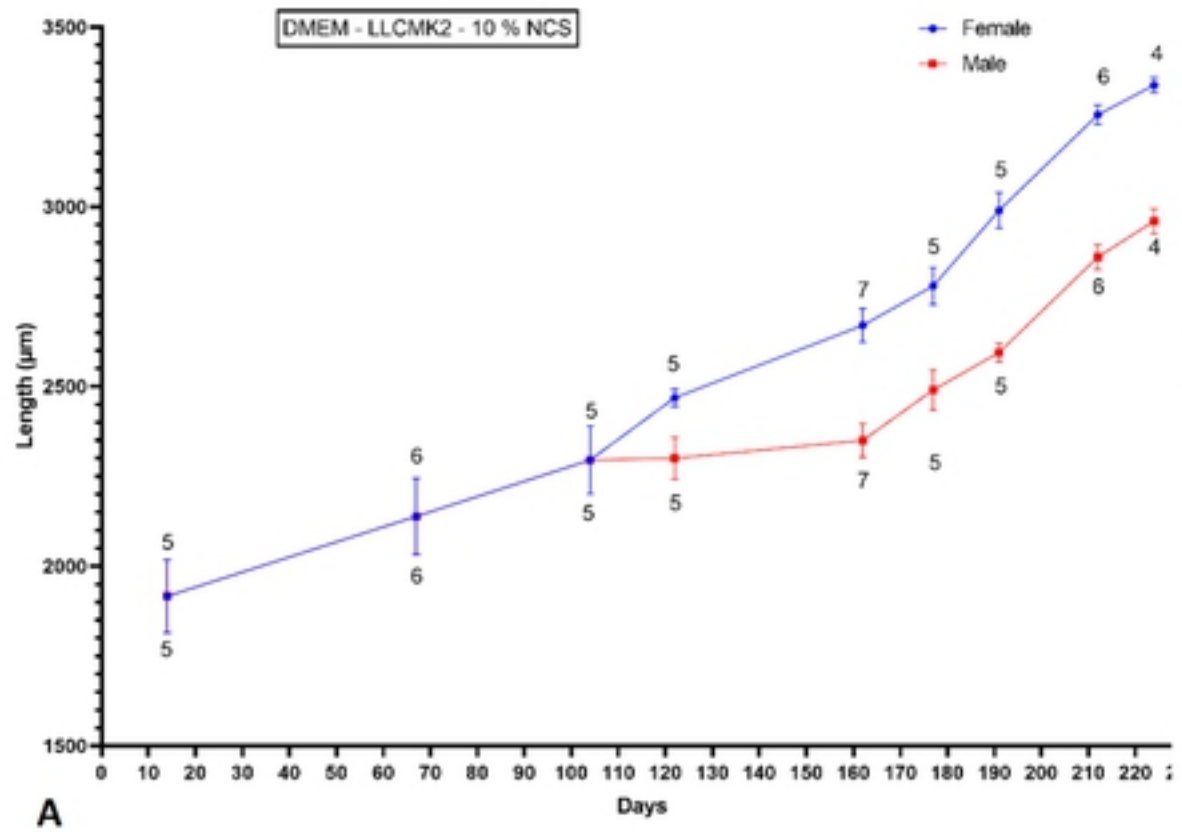


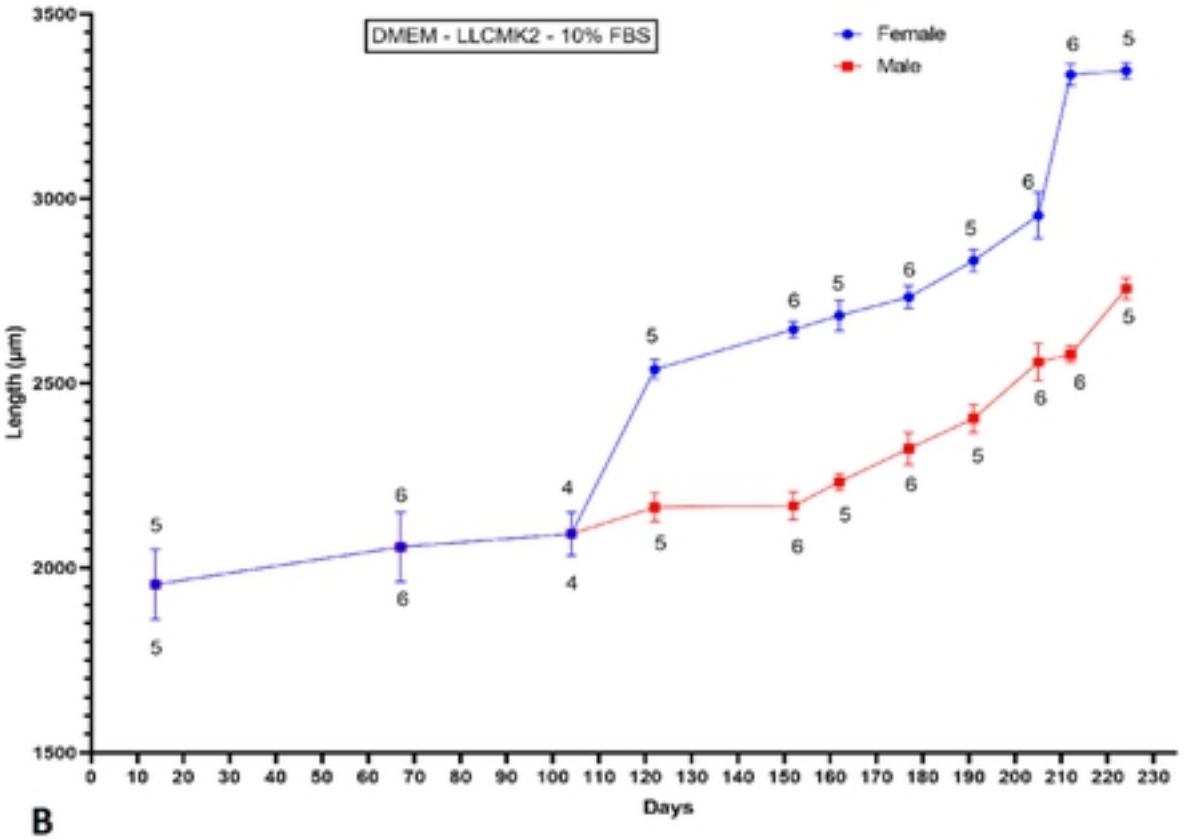


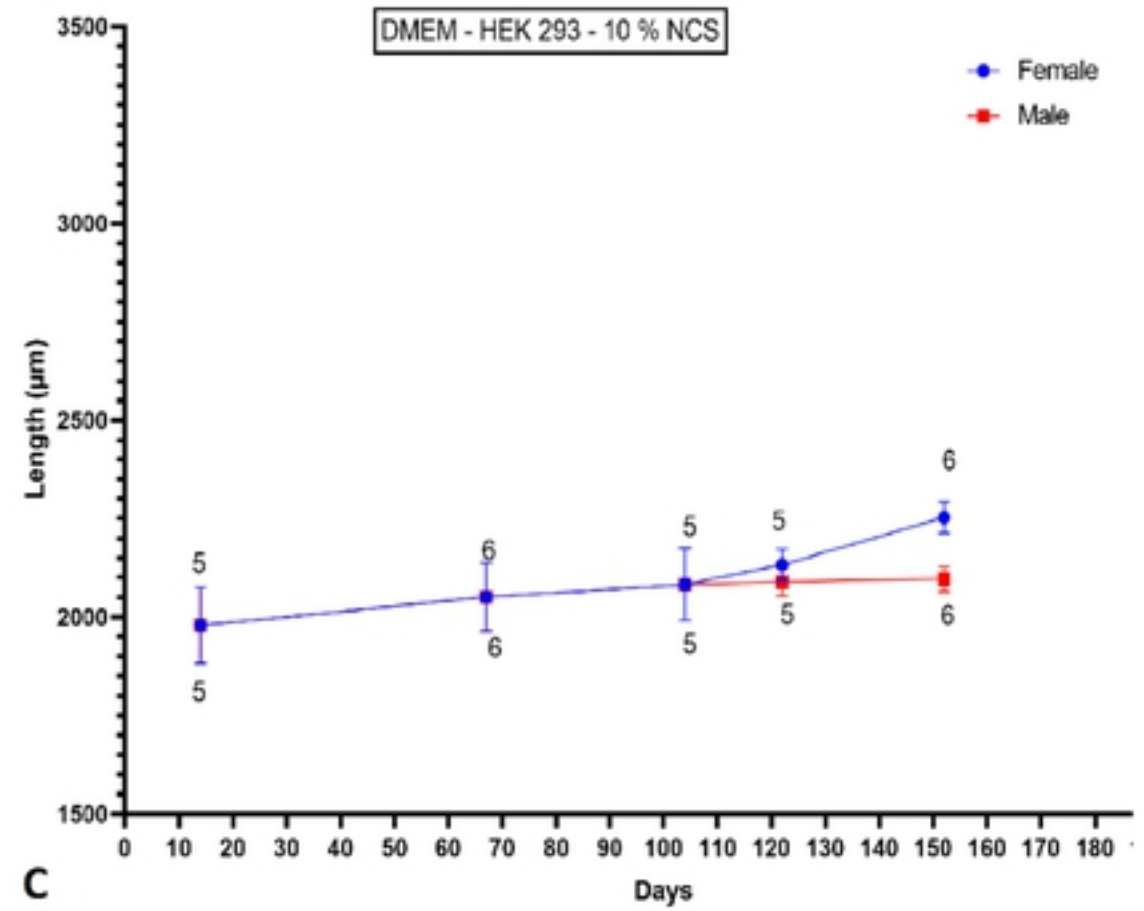


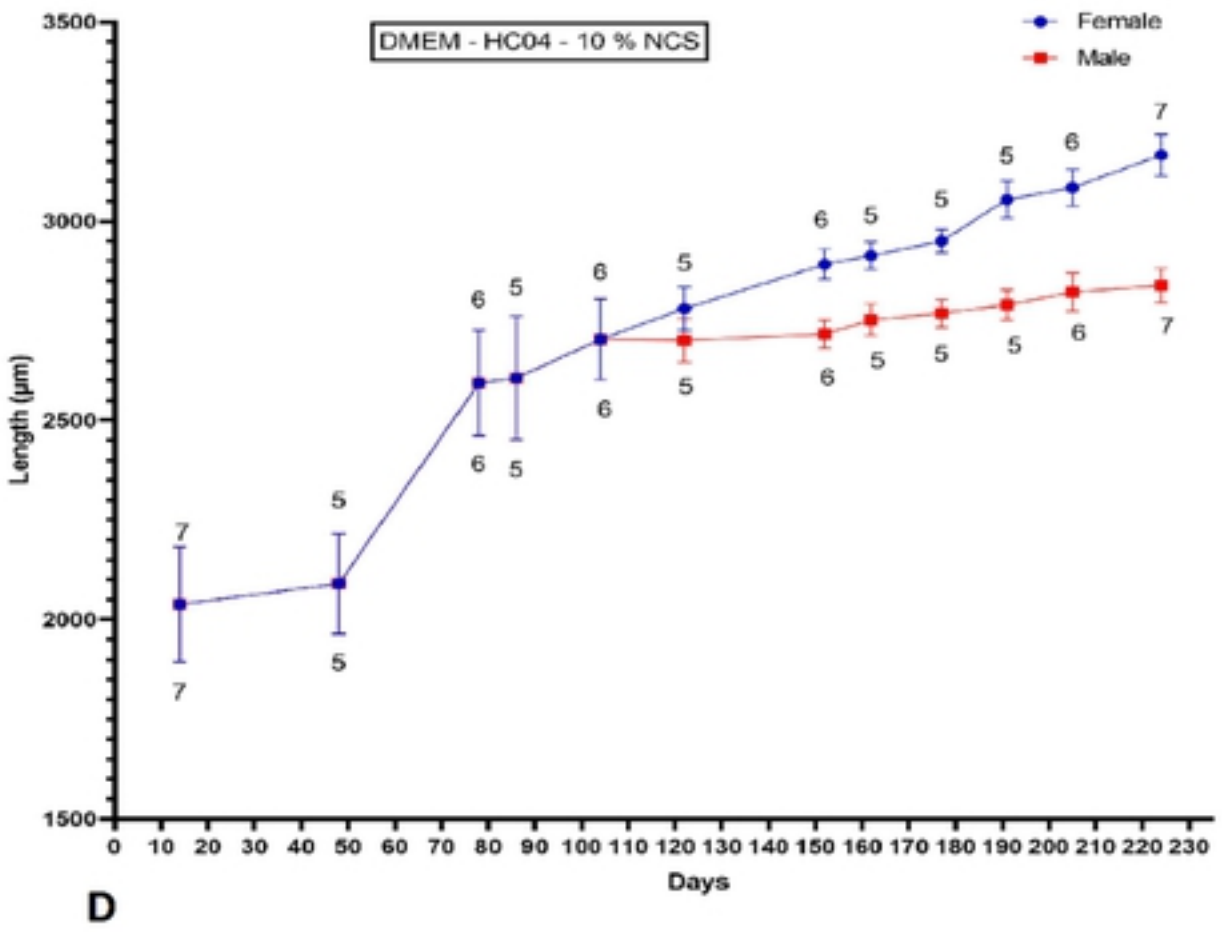


Figure


DMEM - LLCMK2 - 10 % NCS
Female
Male
Length (µm)
Days
A

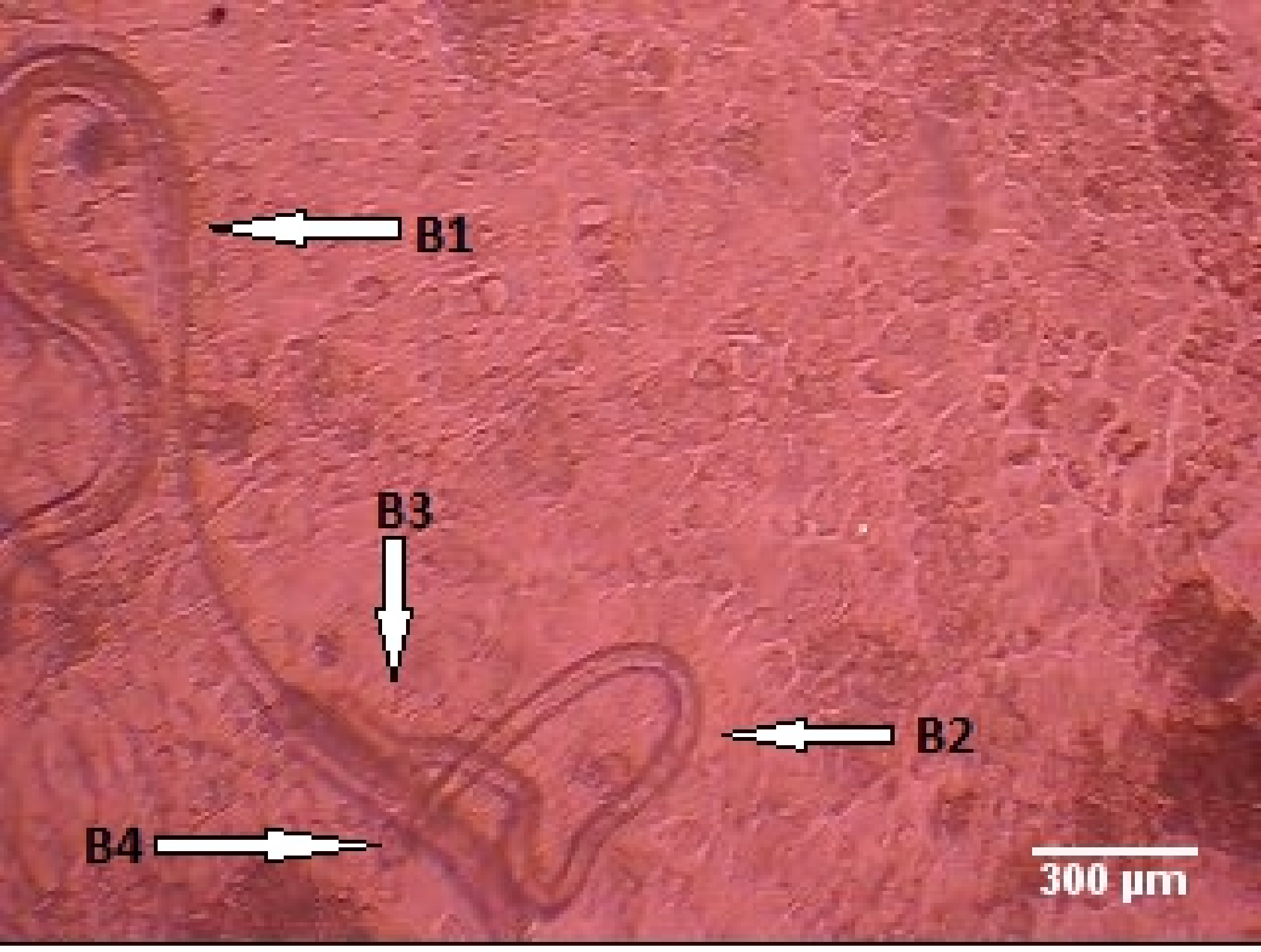

DMEM - LLCMK2 - 10% FBS
Female
Male
Length (µm)
Days
B


DMEM - HEK 293 - 10 % NCS
Female
Male
Length (µm)
Days
C

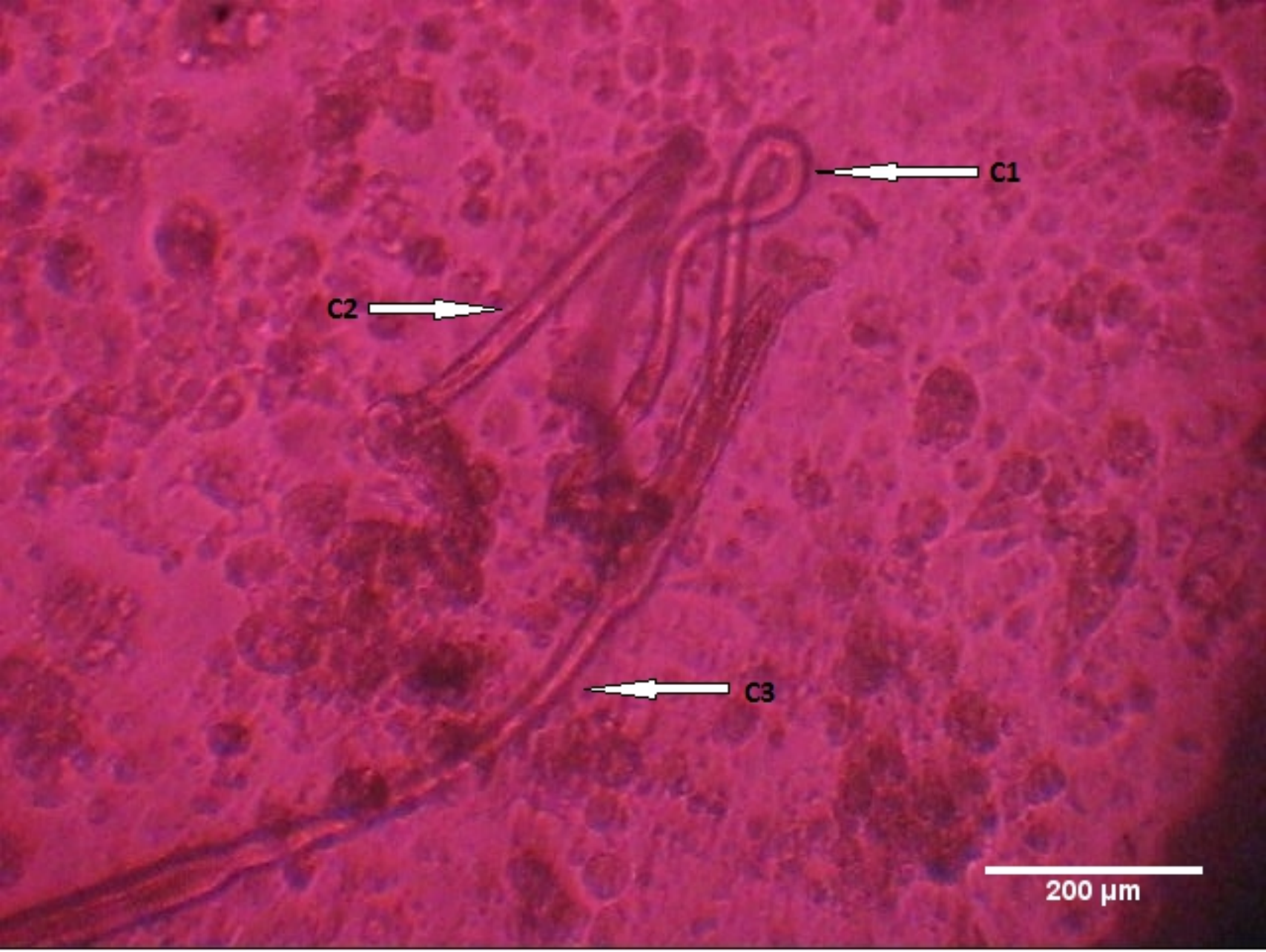

DMEM - HC04 - 10 % NCS
Female
Male
Length (µm)
Days
D

Figure

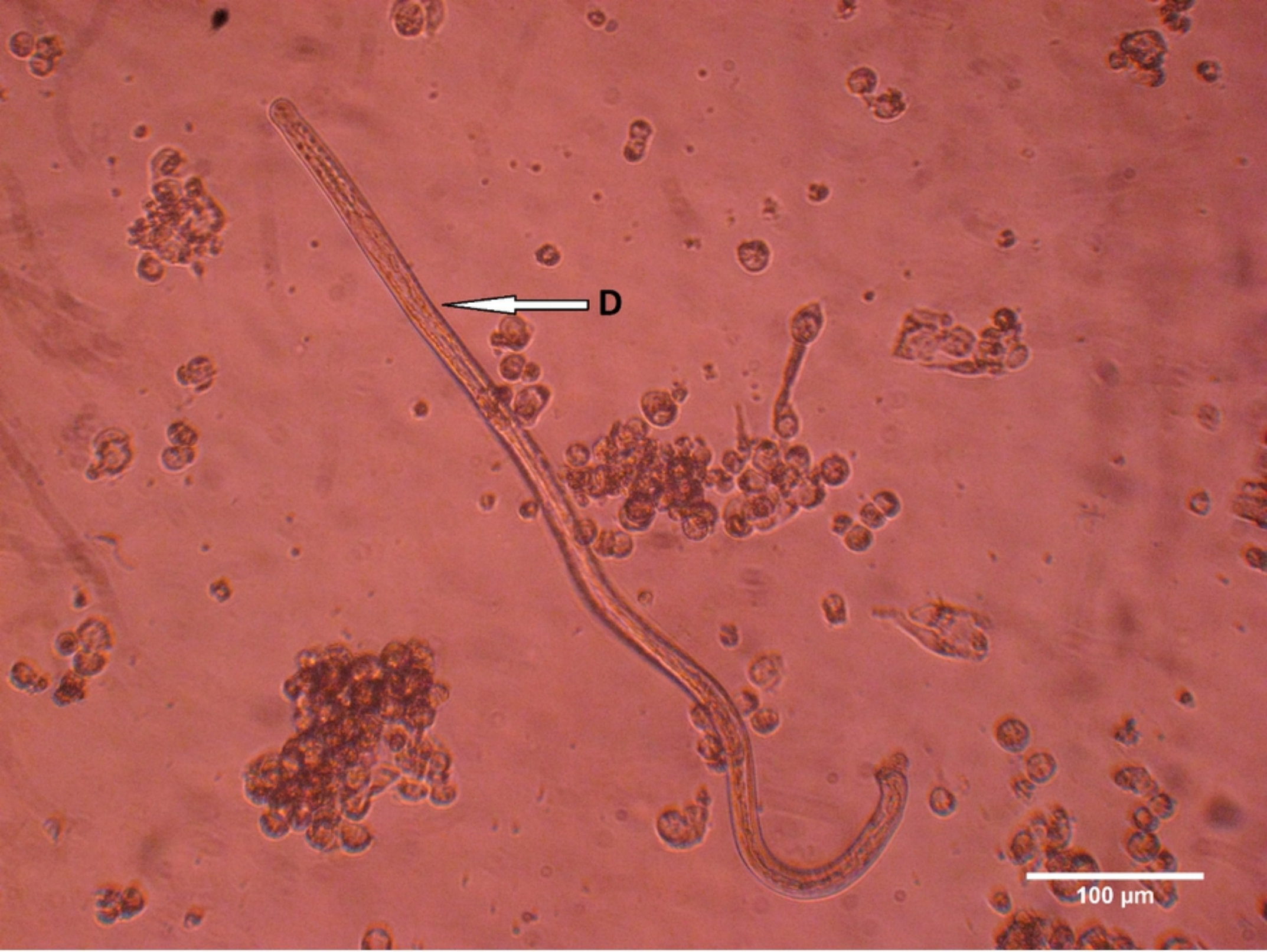
Figure



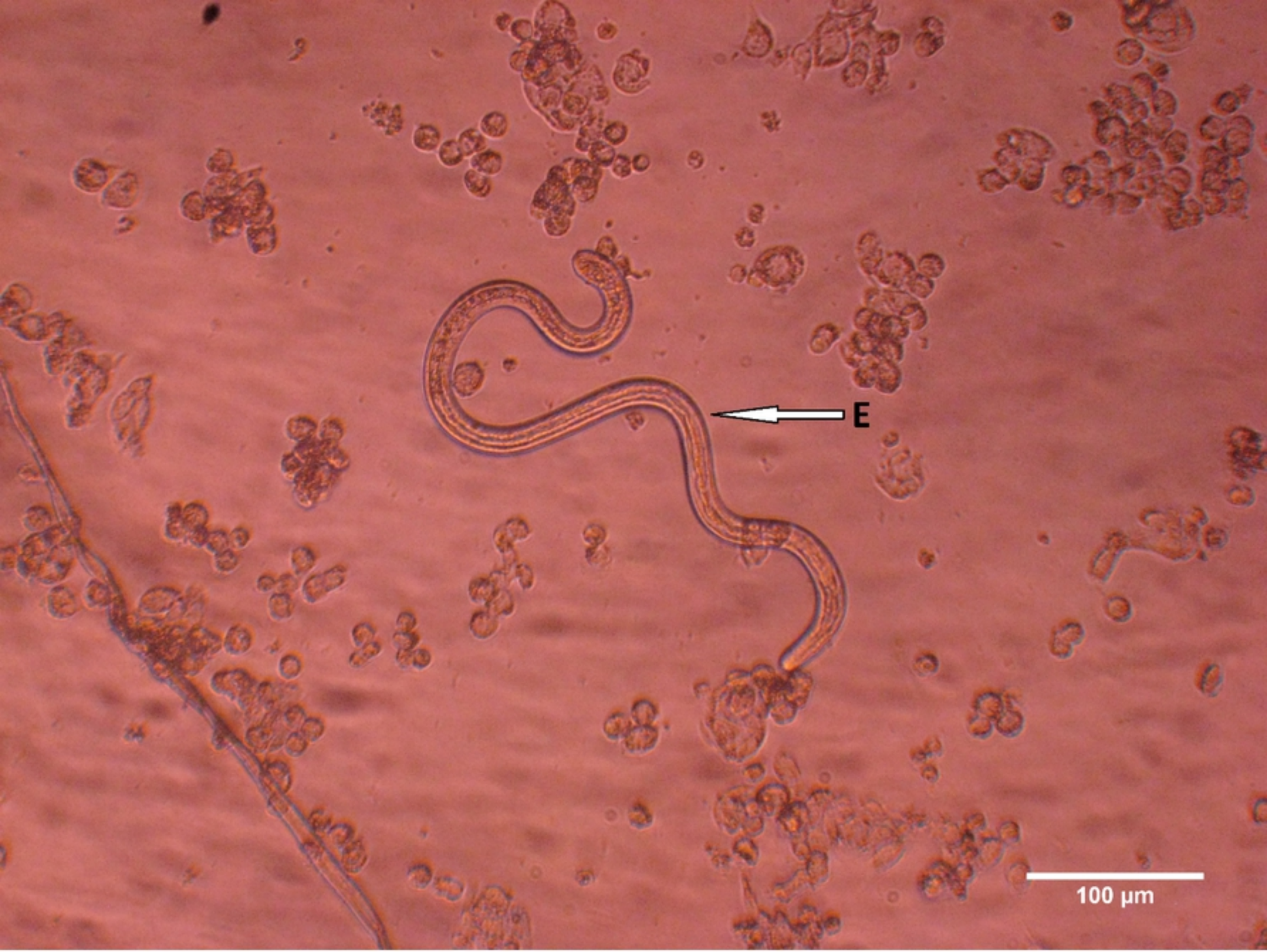
Figure



Figure

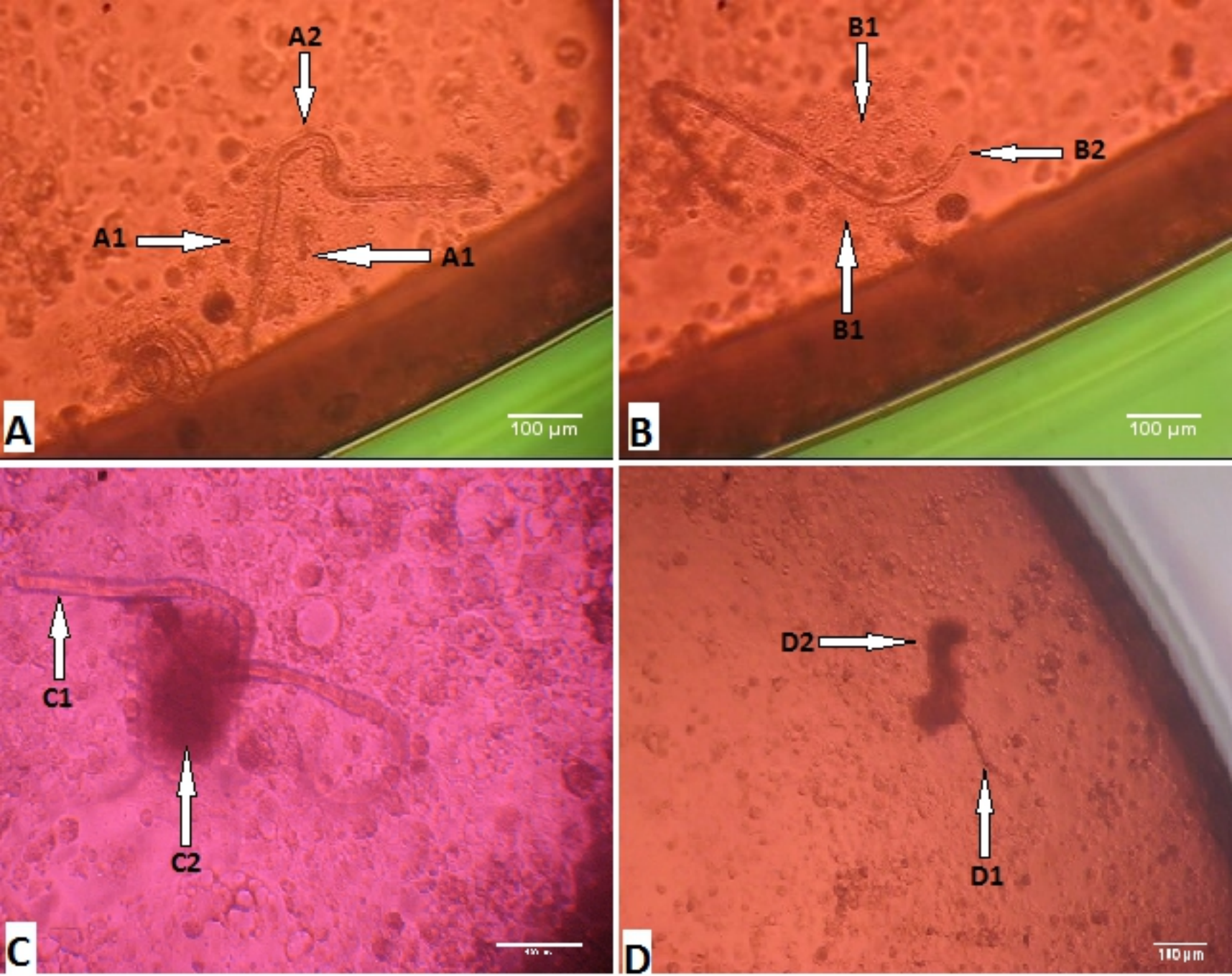


Figure



Figure

Figure



Figure