جامعة الملك سعود
King Saud University

LECTURE (7)

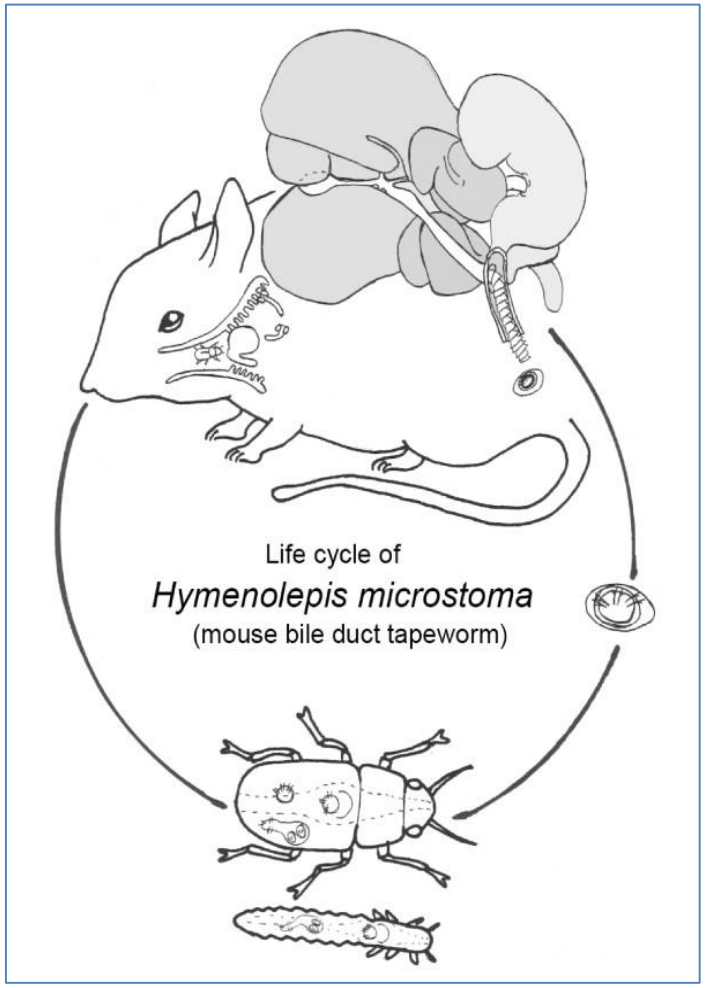
Cultivation of Cestodes & Nematodes

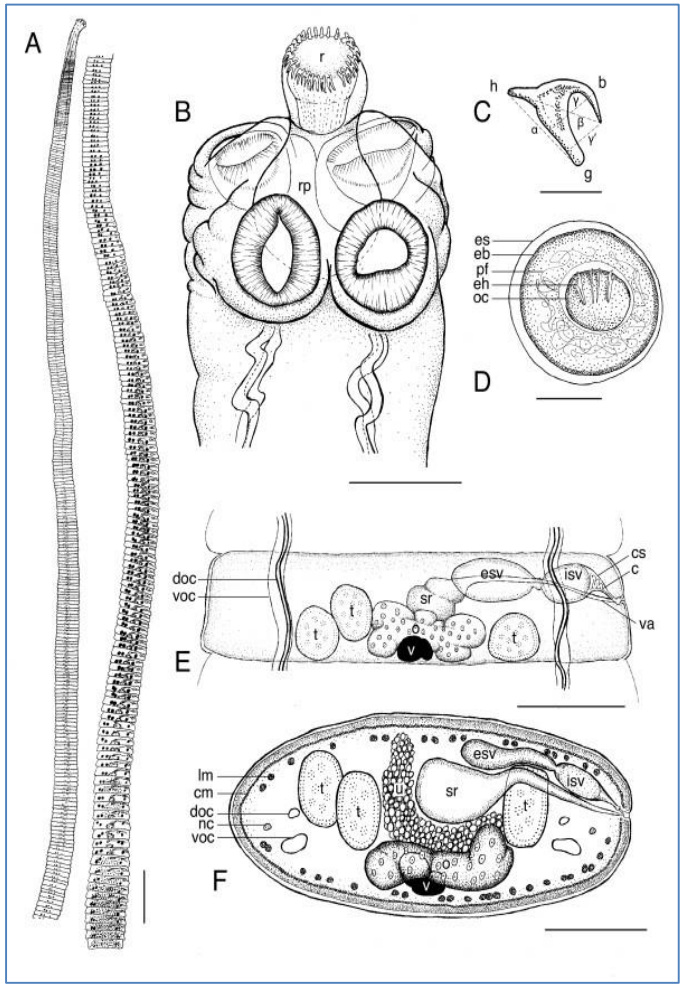
Cestodiases remain a significant threat to our health and agriculture and are gaining new ground due to climate change and globalisation.

Species of ***Taenia*** and ***Echinococcus*** are responsible for the most significant human infections, but are limited as laboratory models due to the practical inability of maintaining adult worms *in vivo*.

Hymenolepis microstoma Blanchard, 1891, the mouse bile-duct tapeworm, is a classical rodent-hosted model system in cestodology that provides easy laboratory access to the complete life cycle utilizing natural definitive hosts that are themselves model organisms (i.e. mice and flour beetles).

Studies on the laboratory cultivation of *H. microstoma* were conducted in the 1960s and 70s, during which the entire life cycle, from egg to gravid adult, was demonstrated *in vitro*.





In vitro Cultivation of the Tapeworm *Hymenolepis nana* from Larva to Adult using Stoll's technique

Media preparation

One hundred ml. of medium was prepared by mixing the following solutions in the order stated:

Hanks saline ---------- 40 ml
Sodium penicillin *G* ---------- 10,000 units
Streptomycin sulphate ---------- 10 mg
Autoclaved 6·5 % glucose solution ---------- 5 ml
5 % 'Oxoid' yeast extract (filter sterilized)------- 10 ml
Liver extract ---------- 10 ml
Horse serum ---------- 30 ml

The *pH* of the mixture was adjusted to 7·6 by addition of 1·8 ml of 0·2 normal sodium hydroxide and 3-4 ml of 1·4 % sodium bicarbonate.

Liver extract preparation

1. Lamb liver was brought from the abattoir in an iced container.
2. Chopped into pieces of about 20 g and stored at - 15° C. When required it was thawed at 4° C for 48 h.
3. One part by weight of liver was homogenized with four parts by volume of de-ionized water at 4° C keeping the flask surrounded by crushed ice.
4. The brei was adjusted to *pH* 4·0 with 1 normal hydrochloric acid (approx. 2·5 ml./100 ml. brei), squeezed through muslin and centrifuged at 5,420g for 1 h at 4° C. The supernatant was collected, filtered through a 0·45µ 'Millipore' filter and sterilized by passing through a 0·22µ- filter.
5. Sterile extract was stored in aliquots of 5 and 10 ml at -15° C.

After mixing, the medium was dispensed in 5 ml quantities in 125 x 25 mm roller tubes and gassed for 40 sec with 95 % nitrogen and 5 % CO2. The culture tubes were immediately closed with 'Esco' *RWH* grade rubber bungs, placed in a roller drum incubator and rotated (0· 15 r.p.m.) for at least 2 h before inoculation with freshly excysted worms.

Infected beetles *(Tribolium conjusum*) were dissected in Hanks balanced salt solution (BSS). Freed cysticercoids washed twice in sterile BSS and thereafter all procedures were carried out at 37° C using sterile solutions containing 100 u of penicillin and 100 µg of streptomycin. Cysticercoids placed in 1% pepsin in BSS at *pH* 1·7 for 12-15 min.

They next washed three times with BSS and transferred to trypsin bile salt solution (0·5% trypsin and 0·3% sodium tauroglycocholate in BSS at *pH* 7·2). About 90% of worms excysted within 8-10 min; then washed three times in BSS and collected in Petri dish of 5cm diameter. From this dish groups of 16-20 excysted worms transferred to small glass containers, examined under x 12 magnification and pipetted into a culture tube.

The tube was gassed a second time with 95% nitrogen and 5% CO2 and returned to incubator. The *pH* of medium 2-3 h after setting up was 7·2 ± 0·2. Medium changed on days 3, 6, 9, 11 and 13.

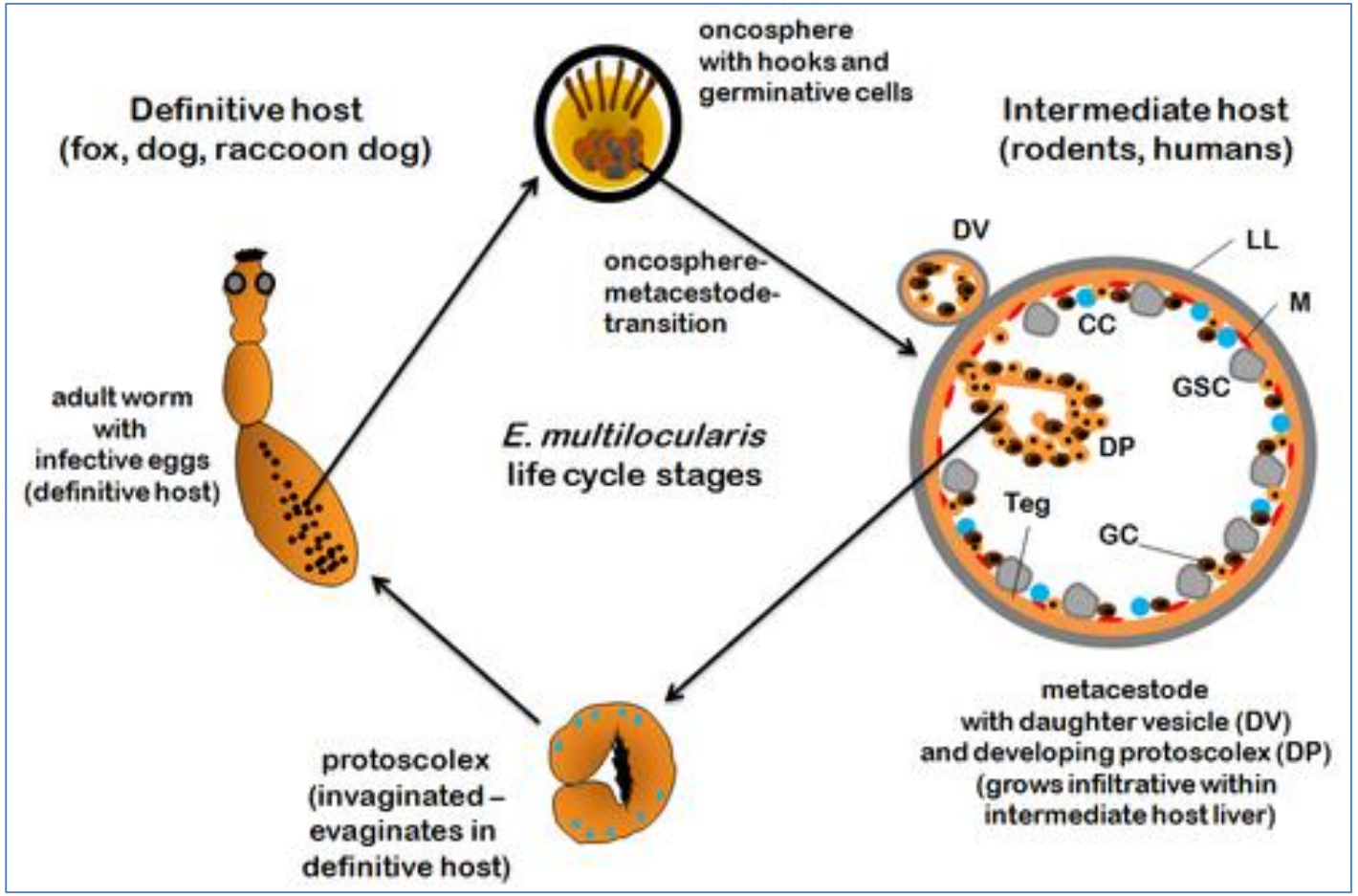
Principal results as follows. Cysticercoids, during 14 days of cultivation, grew to strobilated worms 7-30 mm long, 60-70% had mature genitalia with active sperm and 20-30% produced eggs in some proglottids. Discharged eggs were found in medium from 10 day onwards and these eggs and those from proglottids have been fed to intermediate host where they developed into normal infective cysticercoids.

Cystic echinococcosis is a zoonotic infection caused by the larval stage (metacestode) of the parasite *Echinococcus granulosus*.

The metacestode stage is a fluid-filled bladder which consists of an inner germinal or nucleated layer supported externally by a tough, elastic, acellular laminated layer surrounded by a host produced fibrous adventitial layer.

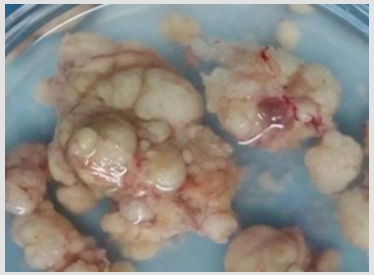
The germinal layer contains several cell types, including undifferentiated cells with large nuclei and nucleoli, as well as muscle and tegumentary cells.

Metacestodes develop in various anatomic sites of the human body from oncospheres released from ingested eggs, but the liver and the lung are the most frequently affected organs.



Collection of protoscoleces and *in vitro* cultivation

Preparation of protoscolex (PSC)



Metacestode masses collected from peritoneal cavity of infected jirds then washed with sterile phosphate-buffered saline (PBS). The masses minced into small tissue pieces.

Parasite tissues then passed through a sterilized sieve (100 mesh sieve) by grounding with a glass pestle. The passed through parasite tissues containing PSC washed five times with PBS containing 100 U/ml penicillin + 100 µg/ml streptomycin (PBS-PS) by placing tube on bench for 5 min to allow PSC to sediment at room temperature.

The sedimented PSC digested with 1 % (w/v) pepsin at pH 2.0 in Hank's Buffer for 20 min at 37 °C. After digestion procedure, PSC washed five more times with PBS-PS to remove debris by natural sedimentation for 5 min at room temperature. Viability of PSC determined by staining with 0.1 % methylene blue, with dead PSC staining blue.

Primary culture

Only samples with ≥ 95 % viability subsequently cultured in RPMI 1640 medium containing 25 % (v/v) fetal bovine serum (FBS), 0.45 % (w/v) yeast extract, 0.4 % (w/v) of glucose, 100 U/ml penicillin and 100 µg/ml streptomycin in a culture flask at 37 °C in presence of 5% CO2. Medium changed every 3 days. Each culture was monitored weekly under an optical microscope to check the growth status of the larval vesicles.

Primary Culture

After 24 h
Washed plate and just remain attached cells

Subculture every 7 days up to 1 month

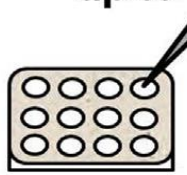
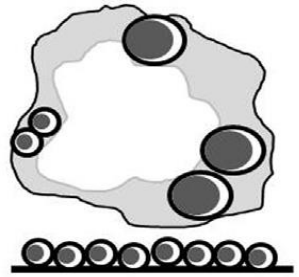


Plate 2 x 10^6 cells/cm^2

A

Subcultured every 7 days

B



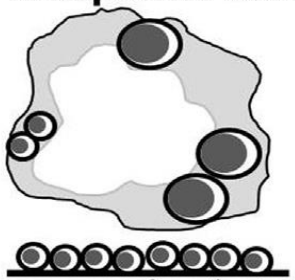
Mantained without splitting for 2-3 month

Agreggated cells

Two phases: aggregate formation + cells attached

Monolayer cells

C **Criopreservation**

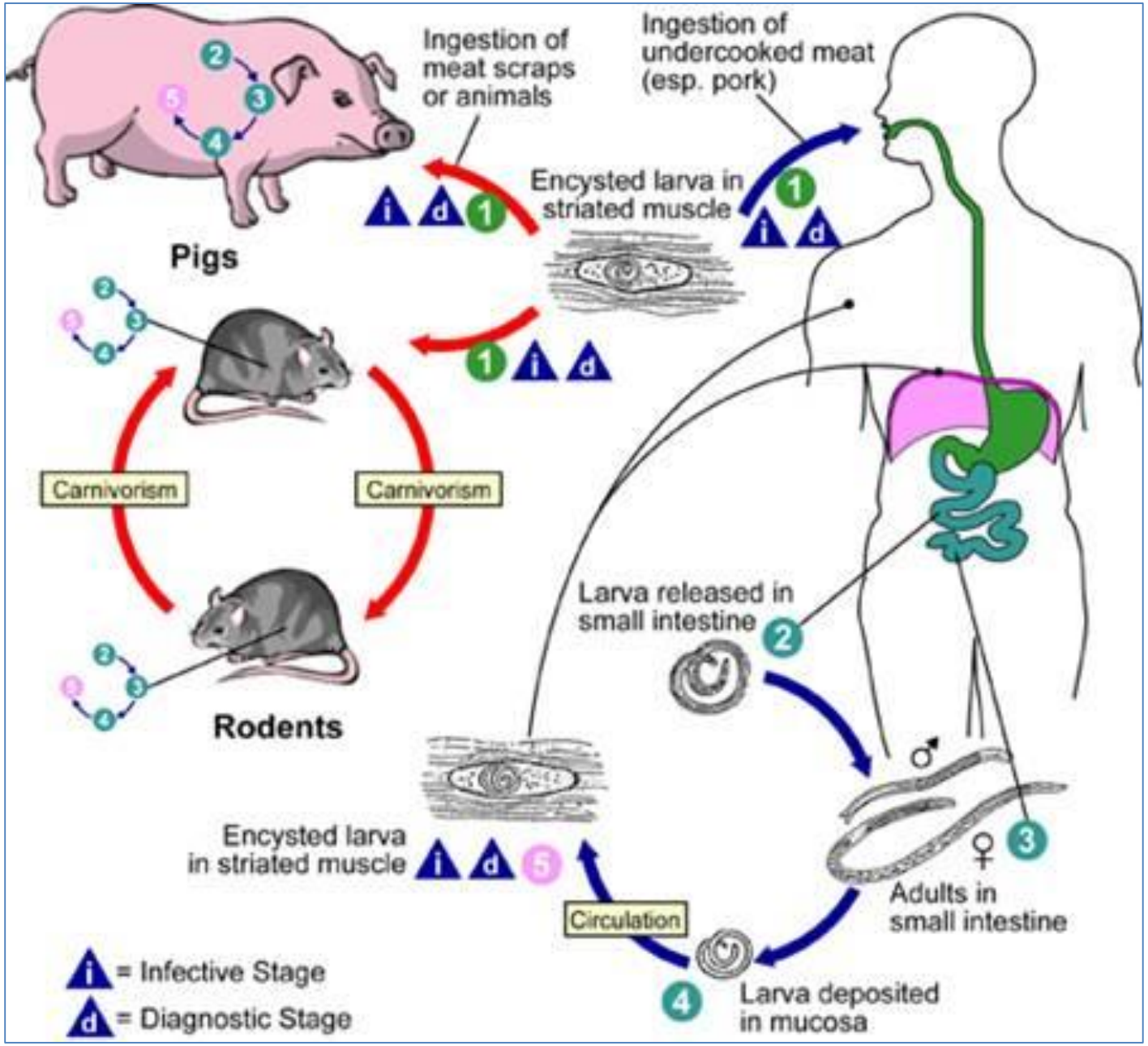


Two phases: aggregate formation + cells attached

Trichinella spp.

Trichinella is the genus of parasitic roundworms of the phylum Nematoda that cause **Trichinosis** (also known as trichinellosis).

Members of this genus are often called **trichinella** or **trichina worms**. *Trichinella* is known as the smallest human nematode parasite, yet it is also the largest of all intracellular parasites.



Cultivation of the newborn larva

- Adult worms were obtained from the intestine of rats, which were experimentally infected with infective muscle larva three days before. These adult worms were then incubated in a culture medium and allowed to shed newborn larvae in the medium, which were then used for culture.

- Muscle cells, obtained after digestion with trypsin of finely cut muscle of 15 days old fetus of CF-1 mouse, were seeded (106 cells/ml) in a culture medium with 20% chicken embryo extract and allowed to form muscle cell sheet on the inner surface wall of the culture flask.

- When newborn larvae were introduced into the flask with the muscle sheet, many larvae could be seen penetrating into the cultured muscle cells.

- Five days later, the larvae had started to coil up in the muscle cell and they also showed the presence of stichocytes on them. However, between day 3-14, the infected muscle cells began to degenerate and became separated from the interior surface wall of the culture flask. The larvae within the muscle cells were already dead.

Cultivation of excysted muscle larva

- Excysted muscle larvae were obtained by digestion with artificial gastric juice and then artificial intestinal juice of the Waring blender-treated muscle of guinea pig that had been orally infected with infective muscle larvae 3 months previously.

- These excysted larvae were then cultured in NCTC135 supplemented with 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 20% inactivated bovine serum, under 90%N2-5% C02~5%02. Between 12-120hr of culture, sheath formed on the larva was observed.

- Only a portion of the larvae was able to escape from the sheath but most could not. When the Berntzen exsheathment apparatus was applied the larvae, the exsheathed male worm began to show copulatory papillae, vesicula seminalis and non-sperm containing testis, and the female worm vaginal opening, uterus and non-ova containing ovary.

- Two to three days after exsheathment, a portion of the male worm was found to possess sperm and the female worm ova. Sometimes copulation of the worms was also observed. They existed under this condition for 3 weeks and by the 4th week, their internal organs had degenerated and they died.

Cultivation of the fifth stage larva

- *Trichinella* worms were obtained by the modified Baermann apparatus after digesting the intestine of male Wistar rat that had been orally inoculated with 10,000 infective muscle larvae and then starved 24hr before, with 2% trypsin and 1% pancreatin.

- The worms were washed with Hank‘s solution containing antibiotics and then cultured. Most of the worms had already been released from their capsule as well as from their sheath but were still sexually immature. The composition of the culture medium was the same as that for the excysted muscle larva described above.

- After culturing the worms for 36hr, the male worm could be seen to possess vesicula semi nailswithout sperms, testis, copulatory papillae and cloaca, while the female worms showed well-developed ovary and uterus. Two days after the beginning of the culture, most of the male worms showed an abundant sperms in their vesicula seminalis and the female had attained sexual maturity as evidenced by the presence of eggs in the posterior of their uterus.

- By day 4 of the culture, most of the female worms had completely developed newborn larvae in their uterus and these newborn larvae were then shed into the culture medium, resulting in the medium teeming with these newborn larvae. The adult worms died between day 14-25 post-culture and during their survival period, there was no difference between the male and the female worms. When the newborn larvae, which were separated from the adult worms using 200 mesh filter, were put into fresh culture medium they became very active again but after 4 days their internal structures disintegrated and by day 9, all the larvae had died.

thank you