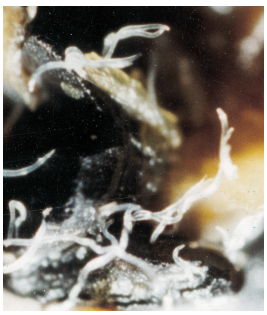
Maintenance of *Nippostrongylus brasilensis*

1. Infection of Rats

Inject 3000-6000 infective larvae (L3) subcutaneously in 0.5 ml saline into an anaesthetised Sprague-Dawley (SD) male rat. The 'take' is higher in male animals. For routine maintenance 3,000 L3 are adequate for a 100-200 g rat. For maximum production of adult worms, 6000 larvae can be given to a 175-200 g animal. Use a 21 G needle, insert under the flank skin for 2-3 cms and inject smoothly causing a bleb to form under the skin. Routine maintenance is usually carried out in outbred SD (Sprague-Dawley) rats. The larvae migrate to the lungs, where they moult to L4 after 24 hours. From 48 hours L4 begin to arrive in the gut, where they moult once more to reach the adult stage by day 6 [1,2]. *N. brasiliensis* will also infect mice [3] but are expelled a little faster. See also References [4, 5] for full details.

2. Culture of Larvae

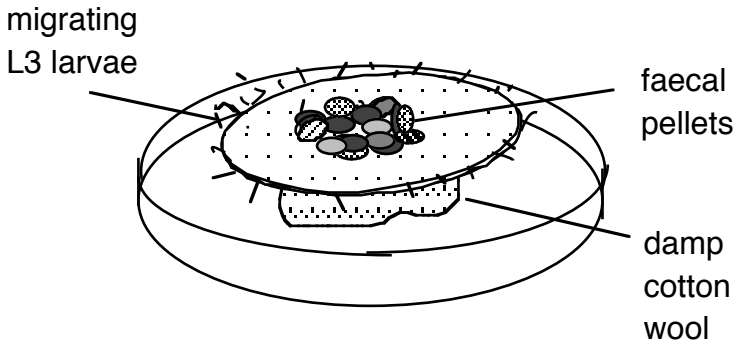
From day 6 post-infection, *Nippostrongylus* eggs appear in faeces of infected rats. Rats should be kept in wire-bottomed cages and faeces collected on a tray beneath covered with well-dampened paper. The paper should be absorbent because urine can adversely affect the eggs. Collect faeces on days 6, 7, 8 and 9, and each day soak them for 30 mins in water to soften. Day 6 collection can be omitted for routine purposes as numbers will be low. Then make a paste with an equal volume of granulated charcoal (BDH 33034), adding water if necessary to keep moist but not too wet. Distribute the paste onto 90 mm sterile plasti petri dishes, and incubate in the dark at approximately 26°C [6]. It is preferable to use petri dishes which are deeper than standard (eg Corning 430167, 100 mm x 20 mm).



Eggs will hatch, and larvae moult twice over the following six days. Cultures can be inspected with a dissecting microscope and top illumination. Larvae remain infective for at least four weeks, and a proportion are still infective at six weeks. Up to 50,000 larvae can be recovered from a single petri dish. Fungal infection is common, and can be combatted by adding Nystatin to the starting paste at 240 U/ml.

An alternative "cleaner" method of larval culture is to place the faecal-charcoal paste in a small mound on filter paper. A damp filter paper circle is placed on a pad of damp cotton wool or folded tissue in the centre of the petri dish. If

the filter paper remains moist, larvae will migrate to the periphery of the dish after about 5 days.



Culture of L3 larvae

3. Faecal Egg Counts

Routinely, the *Nippostrongylus* life cycle is reliable and robust, and faecal cultures can be set up with confidence. If problems arise, or if experimental protocols require, eggs in the faeces can be enumerated. For rats, each animal must be housed in an individual wire-bottomed cage from the day before faecal samples are required. This method uses salt flotation to concentrate eggs at the top layer of a suspension, and a specialised counting chamber (McMaster) designed for this purpose.

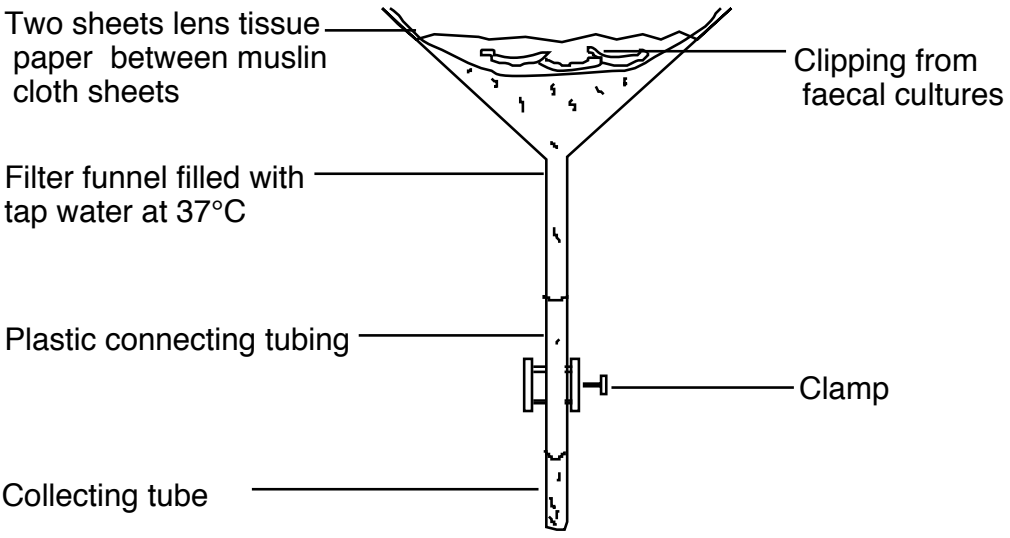
Protocol

1. Take ~4 pellets from each rat in a preweighed 50 ml screwcap tube and weigh (estimate ~1 g).
2. Add 22.5 ml of distilled water and stand for 1 hr, RT°
3. Vortex well, add 22.5 ml of saturated NaCl (add 100 g to 280 ml water), and vortex again; do not allow to settle.
4. Fill a McMaster chamber with suspension, holding pipettor at shallow angle (approximately 800 µl volume).
5. Slide gridded cover slip, grid side down, trying not to trap any air bubbles
6. Eggs will rise to the top of the chamber, by the grid, while debris sinks to the bottom. Count the area covered by the grid by focussing on the top plane: 10x10x1.5 mm = 150 µl. Multiply the count by 300 (150 µl/45 ml total).
7. Count 3 separate aliquots of each sample, making sure each aliquot is taken from well shaken suspension.

4. Collection of Larvae

An adapted Baermann apparatus is set up with tap water at 30-37°C. At the top of the funnel, use paper clips to fix a double muslin sheet containing 1-3 folded sheets of lens tissue under 1 cm of water. Do not let the muslin sheet

overhang the funnel or water will leak out; the water should be about 1 cm below the rim of the funnel. Add the contents of 1-3 petri dish cultures and stand for 60 minutes to allow larvae to collect in the bottom tube. If separation from all faecal debris is essential, repeat the process (this is unnecessary for routine maintenance). Disconnect the collection tube, and wash 3 times in PBS, allowing the larvae to settle by gravity.



Collection of L3 Larvae

Count the larvae by mixing well in 10 ml water, and taking two 10 µl aliquots. Cut the end from the Gilson tip to provide a wide aperture, and transfer onto 60 mm gridded petri dishes for counting. The count should tally with an "eyeball" estimate of 1 million larvae per packed ml volume.

Larvae can be stored in water or saline for 1-2 days at 4° C. Wear gloves when handling larvae as they may penetrate human skin although any infection would be short-lived.

5. Recovery of Adult Worms

Adult worms are found loosely attached to the anterior half of the small intestine. Remove the top half of the intestine and cut along the whole length with scissors. Ideally, use scissors which are rounded on one blade, inserting the rounded end into the lumen. Place the open intestine into a muslin bag, tied with cotton, and immerse in warm (37°C) saline (0.9% NaCl) in a Baermann apparatus, similar to that used for L3 collection. (Note that no lens tissue is used to collect the larger adult worms.) Connect a large funnel to flexible tubing and a 10-15 ml glass or plastic collection tube. Parasites, dull red in colour, will collect in the bottom tube within 1 hour. The collection apparatus should be incubated at 37°C for optimal recovery. It is important to withhold food from rats for the 24 hours before harvest, although recovery is still possible if this has not been observed.

6. Fourth-Stage Larvae

For experimental purposes, L4 larvae can also be recovered. Around 26 hours, lung L4 can be harvested by coarsely mincing the lungs with scissors and placing the tissue in a muslin bag as described for adult worms. From 48 hours, L4 arrive in the gut, from where they can be recovered by a similar process. Beyond 96 hours the parasites will be adult worms.

7. Trickle Infection

Low-dose infection repeated over a number of weeks represents more closely the natural pattern of exposure. When an infection regimen of 5 L3 per rat per weekday for 4 to 12 weeks is used, explusion does not occur and a longer-lived population of adult worms becomes established [7, 8].

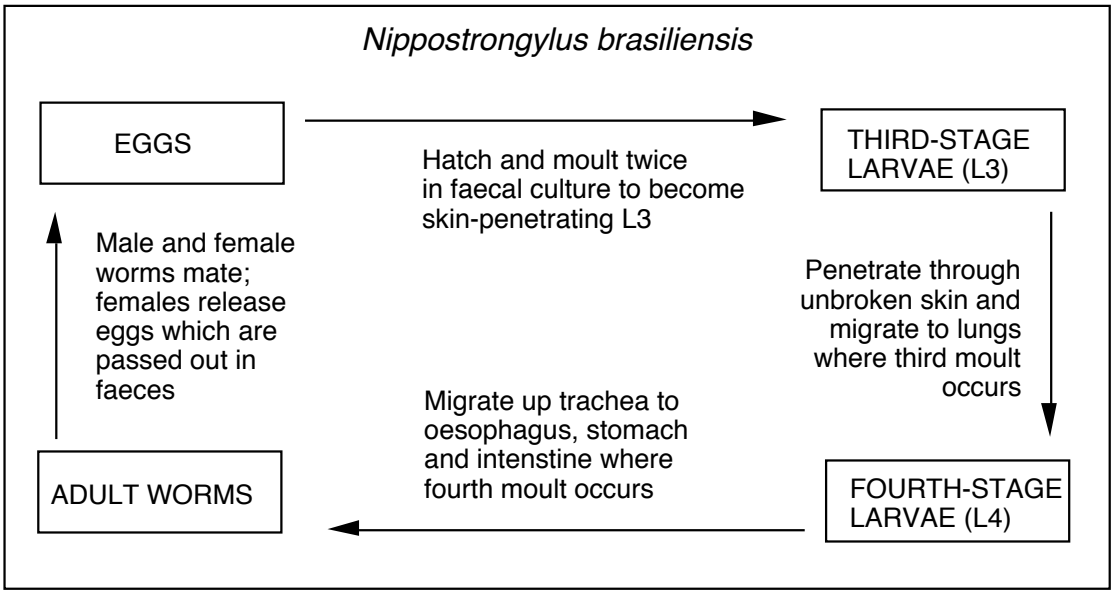
8. Culture of Adults to Collect ES

The ES of adult worms is a major source of immunologically active parasite products [9]. Adult worms are washed 5 x in sterile saline, 5 x in RPMI 1640 containing 1x penicillin and streptomycin, and then incubated for 20 mins at room temperature in RPMI 1640 containing 1 mg/ml Gentamicin (Gibco 15710-049). Worms are then washed 5 x in RPMI 1640 containing 1x penicillin and streptomycin. All procudures are performed in a sterile hood, and worms are settled by unit gravity within 1-2 minutes in each case. Worms are counted (taking 2 x 10 µl aliquots from a 10 ml suspension, using a Gilson tip cut with a blade to provide a broad ~1 mm opening). Parasites are then cultured in RPMI 1640 supplemented with glutamine, 1% glucose and 1x penicillin/streptomycin at a density of approximately 100/ml. Typically, 2000 are cultured in 20 mls of medium in a T25 tissue culture flask. Cultures are incubated at 37°C, in 5% CO_2, and the supernatant collected and replaced after 24 hours, and subsequently every 2 days. Viability remains excellent for 7 days, although secretion of ES appears to decline beyond this period.

Because host (rat) proteases are present in adult ES released in the first 24 hours [10], this fraction is set aside. Material collected between days 1-7 is pooled and stored at –70°C. Unconcentrated ES contains ~10 µg/ml protein, which can be concentrated by Amicon ultrafiltration using a PM-10 (10,000 Da cut-off) membrane. Routinely, batches of NES are prepared from ~1 litre of culture supernatant, which is expected to yield ~10 mgs of concentrated NES. Each batch of NES is analysed by SDS-PAGE, numbered and aliquotted at –70°C until required. To produce heat-inactivated NES (hiNES), NES is treated for 15 minutes at 95°C.

(2D of Adult NES by Yvonne Harcus)

9. Summary of Life Cycle



10. Typical Schedule

Day 0 : Infect 4 male SD rats with 5,000 L3 (*Wednesday*)
Day 5 : Put the rats on wire-bottomed cages with absorbent paper beneath (*Monday*)
Days 6-9 : Collect faeces and prepare cultures as above (*Tuesday-Friday*) and/or
Day 8 : Harvest adult worms from intestine (*Thursday*)
Day 13 onwards : larvae can be harvested for a new cycle of infection

11. References

[1] Yokogawa S (1922) The development of *Heligmosomum muris* Yokogawa, a nematode from the intestine of the wild rat. *Parasitology* 14: 127-166

[2] Ogilvie BM & Jones VE (1971) *Nippostrongylus brasiliensis*: A review of immunity and the host/parasite relationship in the rat. *Exp Parasitol* 29: 138-177

[3] Westcott RB & Todd AC (1966) Adaptation of *Nippostrongylus brasiliensis* to the mouse. J Parasitol 52: 233-236

[4] Kassai T (1982). Handbook of *Nippostrongylus brasiliensis*. Budapest: Akadémiai Kiadó.
[5] Camberis, M., Le Gros, G. and Urban, J., Jr. (2003). Animal model of *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. *Current Protocols in Immunology*. R. Coico, John Wiley and Sons, Inc.**:** 19.12.1-19.12.27.
[6] Jennings FJ, Mulligan W & Urquhart GM (1963) Variables in X-ray 'inactivation' of *Nippostronglyus brasiliensis* larvae. *Exp Parasitol* 13: 367-373
[7] Jenkins DC & Phillipson RF (1970) The kinetics of repeated low-level infections of *Nippostrongylus brasiliensis* in the laboratory rat. *Parasitology* 62: 457-465
[8] Jenkins DC & Phillipson RF (1972) Increased establishment and longevity of *Nippostrongylus brasiliensis* in immune rats given repeated small infections. *Int J Parasitol* 2: 105-111
[9] Holland, M. J., Harcus, Y. M., Riches, P. L. and Maizels, R. M. (2000). Proteins secreted by the parasitic nematode *Nippostrongylus brasiliensis* act as adjuvants for Th2 responses. *European Journal of Immunology* **30**: 1977-1987.
[10] Healer, J., Ashall, F. and Maizels, R. M. (1991). Characterization of proteolytic enzymes from larval and adult *Nippostrongylus brasiliensis. Parasitology* **103**: 305-314.

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