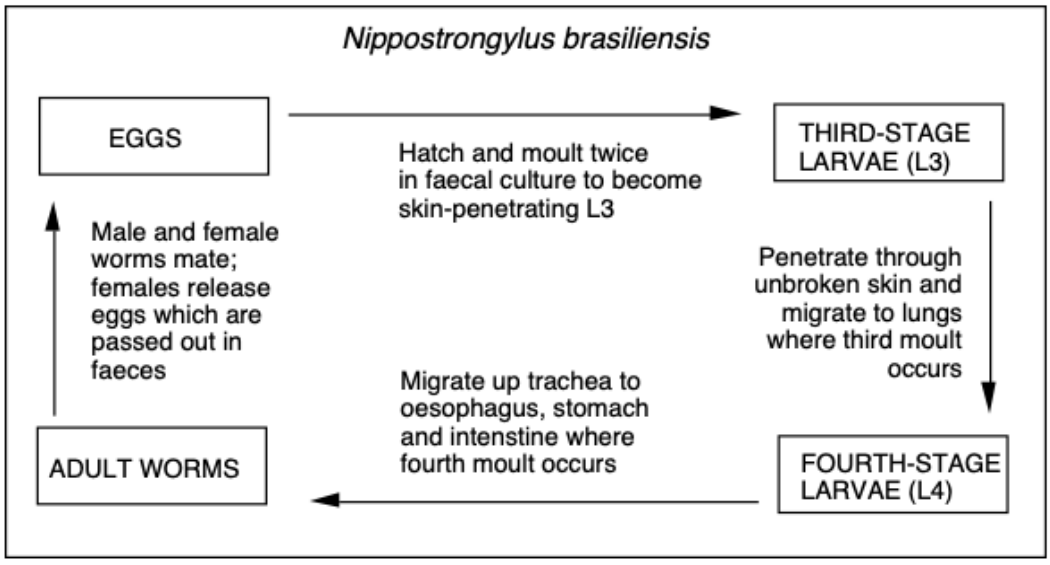
NIPPOSTRONGYLUS BRASILIENSIS LIFE CYCLE

Nippostrongylus brasiliensis is one of the most widely-studied helminth parasites, in part due to the relatively simple life cycle for parasite production. Closely related to human hookworms and the prevalent livestock parasites, *N. brasiliensis* is a natural parasite of rats which mounts a short-lived infection in mice [1]. Murine infection is marked by an overwhelming Th2 immune response, together with all the corollaries of that mode of immunity - mastocytosis, eosinophilia, goblet cell hyperplasia and mucus production. Following subcutaneous infection, 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]. The prompt explusion of worms from the mouse intestine - around 7 days post-infection [2,3] - requires neither eosinophils or mast cells, but does depend on IL-13-mediated stimulation of intestinal epithelial cells, most likely to produce mucus. See also References [4,5] for full details.



This protocol describes outlines management of the *N.brasiliensis* life cycle for consistent production of L3 larvae, recovery of adult parasites, and collection of their excretory-secretory products (NES).

Reagents and Equipment

(a) For Infection of Animals

1. L3 Larvae - see **Protocol (c)** below.
2. Dulbecco's PBS, no calcium, no magnesium (Gibco Life Tech Cat.No. 14190094).
3. P/S: Penicillin (10,000 U/ml) and Streptomycin (10,000 µg/ml), Gibco Life Tech Cat.No. 15140122.
4. For rat injection: 23 G BD Microlance Stainless Steel Needles 0.6 mm x 25 mm, BD Cat.No.300800
5. For mouse injection: 23 G Agani Single-use Sterile Hypodermic 0.6 mm x 16 mm, TerumoCat,No.8AN2316R1
6. Dissecting Microscope

7. 60 mm TC-treated Culture Dishes with 2 mm Grid (Corning Cat.No. 430196)

(b) For Culture of Larvae from Eggs

8. Forceps, scissors
9. Activated Charcoal NORIT GAC 1240 12-40 Mesh, Acros Organics Cat.No. 395712500 (washed and autoclaved, see *Note 1*)
10. Activated Charcoal, slug 6-8 mesh, Alfa Aesar Cat.No. 88764-A1 (washed and autoclaved)
11. 60 mm with 2 mm Grid TC-treated Culture Dish (Corning, Cat.No. 430196)
12. Standard 90mm Petri Dishes, (Thermo Scientific, Cat.No. 101VR20)
13. Dissecting Microscope
14. 26°C incubator

(c) For Recovery of Larvae from Cultures

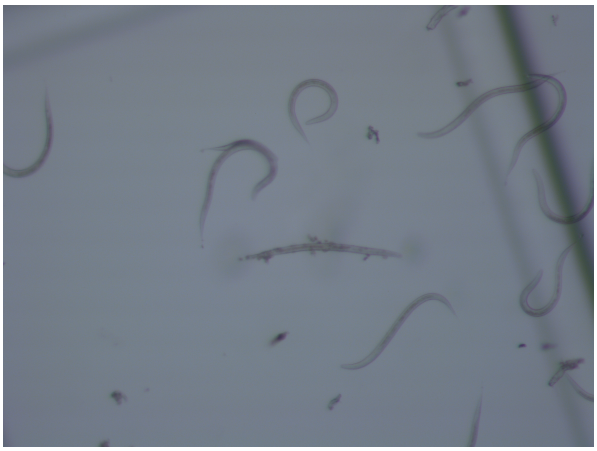
15. Baermann apparatus (see below).
16. P/S: Penicillin, Streptomycin (10,000 U/mL), Gibco Life Tech Cat.No. 15140122
17. Muslin fabric, paperclips and stapler
18.

(d) For Recovery of Adult Worms

19. Round-ended scissors
20. Baermann apparatus (see below).
21. Muslin fabric, paperclips and stapler
22. Microscope Slides Clarity 76x26 1.0mm Silane Treated (DixonScience Cat.No. N/C360).
23. HBSS, no calcium, no magnesium (Gibco Life Tech Cat.No. 14170138)
24. Gentamicin (10 mg/ml), Gibco Life Tech, Cat.No. 15710049
25. RPMI 1640 Medium, no glutamine (Gibco Life Tech Cat.No. 31870025)
26. P/S: Penicillin, Streptomycin (10,000 U/mL), Gibco Life Tech Cat.No. 15140122
27. L-Glut: L-Glutamine (200mM), Gibco Life Tech Cat.No. 25030024.
28. D-glucose (Fisher Cat.No. G/0500/53).
29. 15 ml and 50 ml Falcon tubes (Greiner Cat.Nos. 188271 and 227261).
30. 37°C incubator

***Caution*: Wear gloves when handling larvae as they may penetrate human skin although any infection would be short-lived.**

(a) Protocol For Infection of Animals

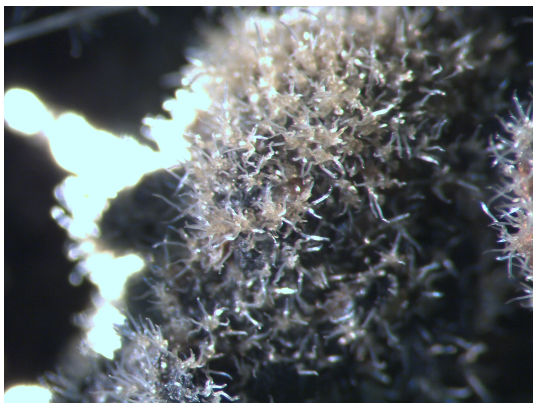


1. Before injection, wash L3 larvae at least 6 times in sterile warmed PBS, containing Pen/Strep.
2. Dilute L3 larvae in 10-20 ml of PBS, aspirate 10 samples of 25 µl and place them on the surface of 60 mm culture dishes.
3. Count the L3 larvae (should be mobile, and best viewed under 50X magnification with a dissecting microscope) and do an average. If counts vary by >20%, increase the number of counts. Centrifuge 5 minutes 800-1000 rpm and resuspend in PBS to the concentration of 6,000 L3 larvae per ml.
4. For life cycle production, infect 275-300g male Wistar or Sprague-Dawley (SD) rat with 3,000 *N.brasiliensis* L3 larvae in 500 µl of PBS by subcutaneous injection (these rats are docile strains); either anaesthetise the rat, only 1 person needed; or restrain conscious rat, 2 people needed). Agitate thoroughly prior to each injection (larvae settle quickly in PBS) and aspirate 500 µl in a 1 ml syringe. Use a single use 23 G needle (25 mm), insert under the flank or neck skin for 2-3 cms and inject smoothly causing a bled to form under the skin.
   Note : For maximum production of adult worms, 6,000 larvae can be given to a 275-300 g animal,
5. *N. brasiliensis* will also infect mice [3] but are expelled a little faster. Infect mice (6-8 weeks old male from inbred strains) with 250 L3 larvae in 200 µl of PBS (use 16 mm 23G needle and inject in neck skin).
   Note: To maintain mouse-adapted N. brasiliensis in mice, alternate cycles between wild-type and immune-deficient (IL-4R$^{-/-}$ or RAG$^{-/-}$) mice. Male mice are more susceptible than female.

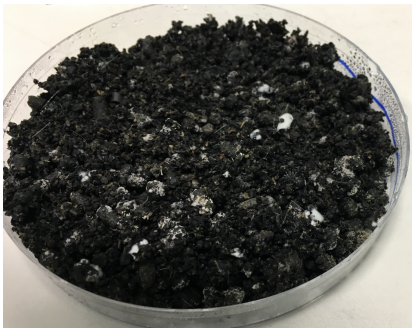
(b) For Culture of Larvae

N. brasiliensis eggs can be collected in faeces of infected rats (steps **1-3** below), and from the caecum and colon of infected rats (steps **4-7** below).

1. From day 6 post-infection, *N. brasiliensis* 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 (ask the animal house staff to put the rats on grids on the evening of day 5). The paper should be absorbent because urine can adversely affect the eggs. Collect faeces on days 7 and 8, and each day soak them for 1-2 hours at 37°C in sterile water to soften.
2. In a fume hood, make a paste by mixing with fine (12-40 mesh) and chunky (6-8 mesh) charcoals, adding water to keep moist but not too wet. Use a clean spatula and an autoclaved plastic bucket to avoid external contamination with fungi. Distribute the paste onto 90 mm sterile petri dishes, label them with Life cycle number and date, then place them in a plastic box with well-dampened paper and incubate in the dark at approximately 26°C [6].
3. At day 8 after infection, rats may be culled to collect adult worms (see below), and intestinal contents for eggs. Cull by a Schedule 1 method routinely used, e.g. exposure to rising concentration of CO_2 and confirmation of cessation of blood circulation.
4. Wash the abdomen with 70% ethanol. Cut the skin over the abdomen and pull back to reveal the anterior abdominal wall. Make midline incision to enter the peritoneal cavity.
5. Remove complete gut, from bottom of stomach to end of colon. Place in dry petri dish.
6. Draw out caecum and colon part and remove gut to full length into a 50 ml Falcon tube for later adult worm recovery (see below).
7. In a fume hood (in the main lab) scrape out contents of the lower gut with forceps/scissors. Then continue with steps 1 and 2.
8. 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 (as also seen by their motility) at six weeks. Up to 50,000 larvae can be recovered from a single petri dish.

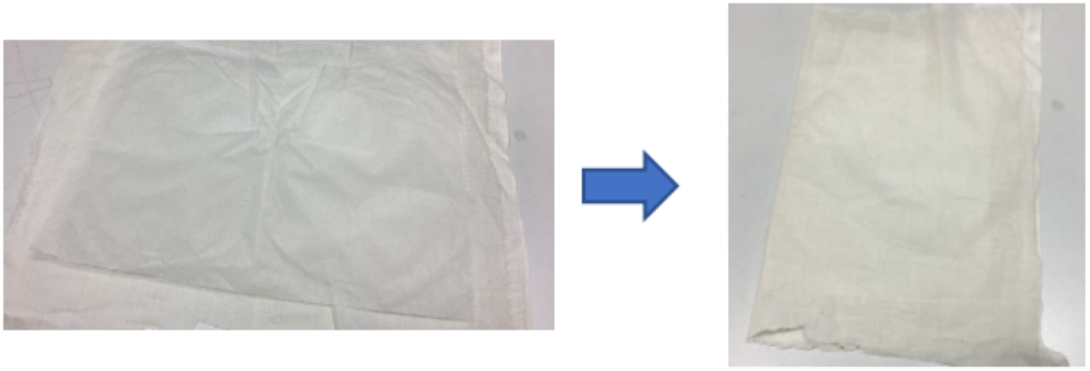


9. Fungal infection is common, and can be combatted by adding Nystatin to the starting paste at 240 U/ml.

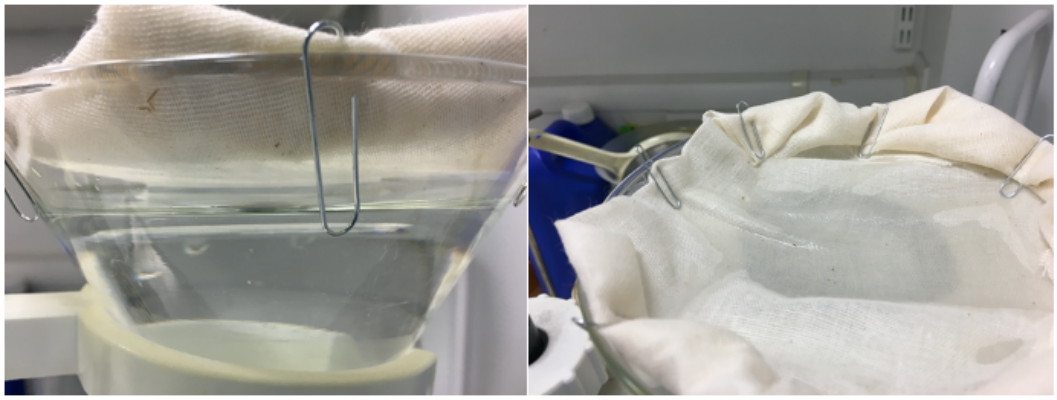


(c) For Recovery of Larvae from Cultures

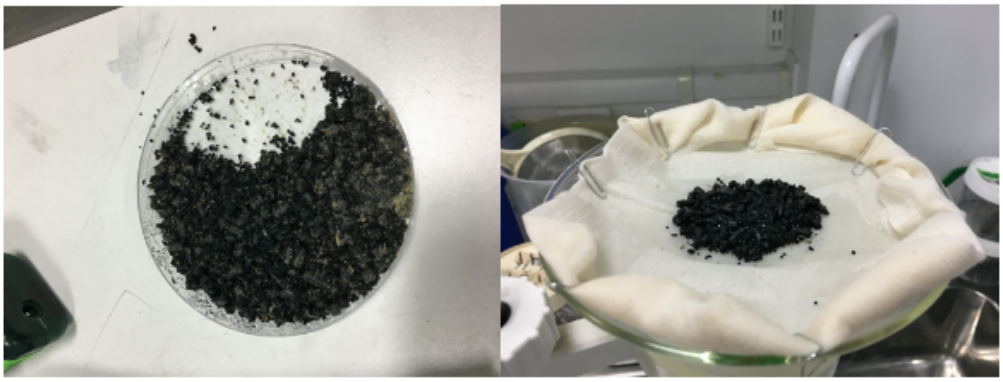
1. An adaptated Baermann apparatus is set up: funnel should be attached to rubber tubing connected to a glass tube to collect the larvae in and be sitting on a stand. Fill funnel with tap water at 30-37°C (do not exceced 37°C) and check for any leaks.
2. At the top of the funnel, use paper clips to fix a double muslin sheet containing 1 folded sheets of lens tissue.



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.

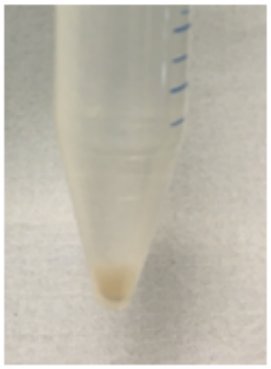


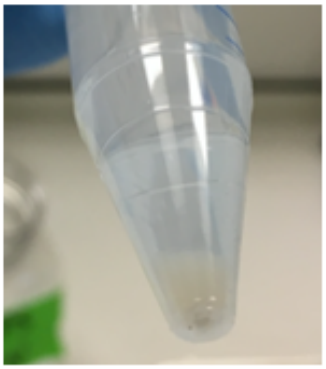
3. Add the contents of 1 petri dish culture (or less) on top gently (mix carefully to moisten the charcoal).



4. Stand for 1-3 hours at RT° to allow larvae to collect in the bottom tube. Carefully detach the test tube from the connecting rubber hose over the sink (taking care to avoid losing worms at this point or splash yourself with media).

5. Use a plastic pipette to put larvae in a 15 ml tube and wash 5-6 times with PBS (prewarmed to 37°C; use sterile TC grade PBS as these will be injected into animals). Spin larvae for 5 min/800-1000rpm between each wash.
6. Count larvae at each wash: resuspend the pellet well in a large volume of PBS, then aspirate 2 samples of 25 µl and place them on the surface of 60 mm culture dishes. Count the L3 larvae (usually mobile, and best viewed under 50X magnification with a dissecting microscope) of each sample and do an average.
7. If charcoal is found in the bottom of the tube after first spin, take the larvae carefully from the top of the pellet and put them in a new 15 ml Falcon. Wash only the larvae from the new tube (without charcoal).

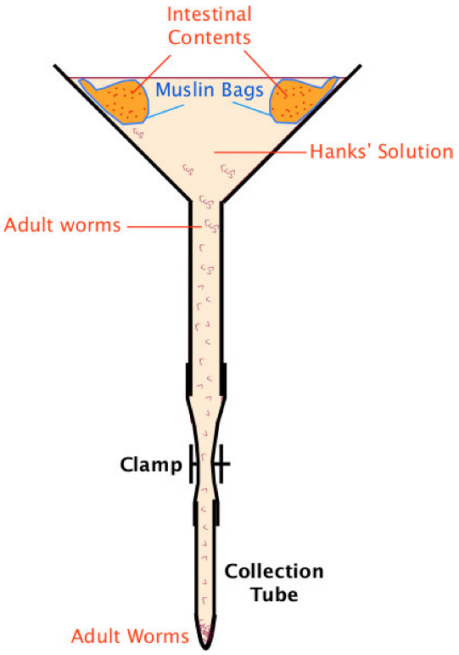




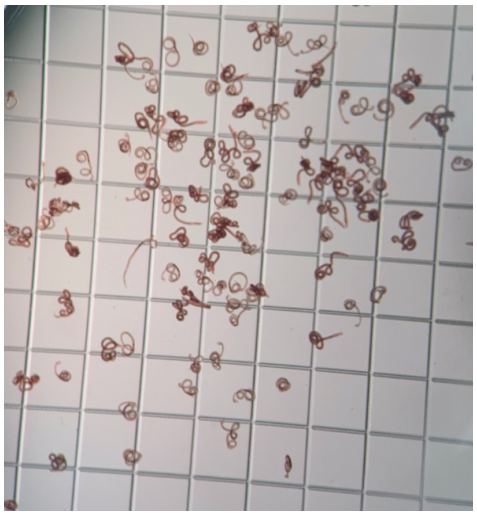
8. Use larvae on the day of collection; if unavoidable, they can be stored in water or saline for 1-2 days at 4° C and should still be infective.

(d) For Recovery of Adult Worms

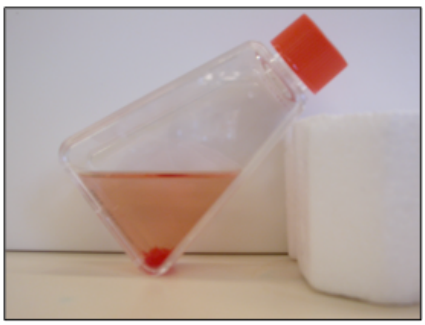
1. Cut off top 2/3 of small intestine that contains the worms – identified by the relatively thick wall of the duodenum and often a red appearance due to the intra-luminal worms.
2. Into a 90 mm Petri dish, open the worm-filled proximal gut portion longitudinally with scissors (round-ended scissors are best for this).
3. Transfer into a new petri dish with HBSS (prewarmed to 37°C), scrape down inside of gut lining with a glass slide and a forceps to remove the worms. Then discard the clean gut wall.



4. An adapted Baermann apparatus is set up: funnel should be attached to rubber tubing connected to a glass tube to collect the worms in and be sitting on a stand. First fill funnel with water to detect eventual leaks, remove the water, then fill it with HBSS (prewarmed to 37°C)
5. Tip worms into little muslin bags, staple closed and secure with paperclips around the edge of glass funnel (4 bags maximum).
6. Place apparatus in 37°C incubator for 1-2 hr, gently agitating half way through to dislodge debris from the gut preparation that may occlude the muslin filter. Take care to avoid spillage of debris outside the muslin bag – this will cause contamination of thea dult worm preparation.
7. Adult worms should have slowly migrated through the muslin cloth and settled at the bottom of the glass test tube. Carefully detach the test tube from the connecting rubber hose over the sink (taking care to avoid losing worms at this point or splash yourself with media).
8. Use a plastic pipette to transfer worms to a 50 ml tube and wash 6 times with HBSS (prewarmed to 37°C), leaving to settle under gravity before removing media.
9. If adult worms are to be used in tissue culture, *they must be kept sterile from this point onwards.* Move to a laminar flow hood (room B642) and wash another 6 times in RPMI (prewarmed to 37°C) containing 5 ml P/S per 500 ml of RPMI.



NES Prep



The ES of adult worms is a major source of immunologically active parasite products [7]. Collect NES-containing culture media from worm cultures (flasks) at days 1, 3, 5 and 7, setting aside the first collection after 24 hr of culture, and terminating the culture at day 7. Replace with equal volume of media each time, using the same protocol as for *Heligmosomoides polygyrus* ES (HES).

NES media

Most important NEVER add FCS!!!!!!!)
To 500 ml RPMI1640
+ 20 ml of 25% glucose solution, final concentration 1%
+ 5 ml P/S final concentration 100 U/ml penicillin, 100 µg/ml streptomycin;
+ 5 ml L-Glutamine, final concentration 2 ml
+ 5 ml Gentamicin, final concentration 100 µg/ml (optional)

The spent media at 24 hr may have contaminating levels of LPS and host proteins [8] , and so should be processed separately or discarded).

Faecal Egg counts

Routinely, the *N. brasiliensis* life cycle is reliable and robust, and faecal cultures can be set up with confidence. If problems arise, 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.

→ *See details in Faecal Egg counts protocol*

Typical Schedule

Day 0	Weds or Thurs	Infect 2 male rats with 3,000 L3
Day 5	Mon or Tue	Put the rats on wire-bottomed cages with absorbent paper beneath
Days 6-8	Tue-Thurs or Weds-Fri	Collect faeces and prepare cultures as above
Day 8	Thurs or Fri	Cull rat, collect faeces from caecum and prepare cultures as above
Day 13 onwards	Tue or Wed	Larvae can be harvested for a new cycle of infection

Notes

1. Charcoal must be washed well and dried before use with worms. Buy charcoal in active form. Deactivate in batches by filling large (5L) plastic beaker ½ way with charcoal, slowly add water. Air bubbles will come off. Run the water continuously for an hour. Pour off the water. Scrape out the wet charcoal onto paper towel inside a large rectangular tray. Spread into a thin layer and leave to dry at room temp. When the paper towel is dry the charcoal is fine to use and can be transferred to storage tub.
2. Withholding food overnight from rats before adult harvest reduces contamination of adult worms with intestinal contents.

3. 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 [6, 7].
4. 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.

References

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[6] Jennings FJ, Mulligan W & Urquhart GM (1963) Variables in X-ray 'inactivation' of *Nippostronglyus brasiliensis* larvae. Exp Parasitol 13: 367-373

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Updated by Claire Ciancia and Rick Maizels 26 April 2020