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## A Ferret Model of Inflammation-Sensitized Late Preterm Hypoxic-Ischemic Brain Injury --Manuscript Draft--

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**TITLE:**

A Ferret Model of Inflammation-Sensitized Late Preterm Hypoxic-Ischemic Brain Injury

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**KEYWORDS:**

Ferret, neonatal, hypoxia-ischemia, neuroprotection, lipopolysaccharide, carotid ligation, hyperoxia

**SUMMARY:**

The method describes inflammation-sensitized hypoxic-ischemic and hyperoxic brain injury in the P17 ferret to model the complex interaction between prolonged inflammation and oxidative brain injury experienced in a number of late preterm infants.

**ABSTRACT:**

There is an ongoing need for clinically relevant models of perinatal infection and hypoxia-ischemia (HI) in which to test therapeutic interventions for infants with the neurological sequela of prematurity. Ferrets are ideal candidates for modeling the preterm human brain, as they are born lissencephalic and develop gyrencephalic brains postnatally. At birth, ferret brain development is similar to a 13-week human fetus, with postnatal-day (P) 17 kits considered to be equivalent to an infant at 32–36 weeks' gestation. We describe an injury model in the P17 ferret, where lipopolysaccharide administration is followed by bilateral cerebral ischemia, hypoxia, and hyperoxia. This simulates the complex interaction of prolonged inflammation, ischemia, hypoxia,

and oxidative stress experienced in a number of neonates who develop brain injury. Injured animals display a range of gross injury severity, with morphological changes in the brain including narrowing of multiple cortical gyri and associated sulci. Injured animals also show slowed reflex development, slower and more variable speed of locomotion in an automated catwalk, and decreased exploration in an open field. This model provides a platform in which to test putative therapies for infants with neonatal encephalopathy associated with inflammation and HI, study mechanisms of injury that affect cortical development, and investigate pathways that provide resilience in unaffected animals.

## **INTRODUCTION:**

There is an ongoing need for large animal models that reflect the pathophysiology of prematurity and perinatal hypoxia-ischemia in which therapeutic interventions for infants can be tested. In 2017, 9.93% of the 382,726 infants born in the United States were born preterm, and 84% of these infants were born between 32 and 36 weeks of gestation<sup>1</sup>. In premature infants, perinatal exposure to infection or inflammation is common, where maternal immune activation due to viral or bacterial pathogens can initiate preterm labor. Postnatally, preterm infants are at high risk of early or late onset sepsis<sup>2</sup>. Preterm infants also frequently experience periods of hypoxia, hypotension, and hyperoxia due to their immature cardiorespiratory system, elevated oxygen tension in the atmosphere relative to those experienced in utero, and iatrogenic exposures. Additionally, in preterm infants, antioxidant defenses are immature<sup>3</sup> and pro-apoptotic factors are naturally upregulated<sup>4</sup>. Oxidative stress and cell death lead to activation of the immune system and neuroinflammation. These combined factors are thought to contribute to developmental and physiologic vulnerability of the brain, and result in or exacerbate the encephalopathy associated with poor developmental outcomes in preterm infants<sup>5-7</sup>.

Due to the physical and developmental similarities that the ferret brain shares with the human brain, the ferret is an attractive species in which to model brain injury<sup>8-12</sup>. Ferrets are also ideal candidates to model the preterm human brain, as they are born lissencephalic and develop gyrencephalic brains postnatally, which provides a window in which to expose the developing brain to insults that mimic those experienced by infants born preterm. At birth, ferret brain development is similar to a 13-week human fetus, with postnatal-day (P) 17 kits considered to be equivalent to an infant at 32-36 weeks of gestation<sup>13</sup>.

Our group has recently published a model of extremely preterm (< 28 weeks' gestation) brain injury in the P10 ferret by combining inflammatory sensitization with *Escherichia coli* lipopolysaccharide (LPS) with subsequent exposure to hypoxia and hyperoxia<sup>12</sup>. In the following protocol, we now describe a late preterm model in the P17 ferret, where LPS sensitization is followed by bilateral cerebral ischemia, hypoxia, and hyperoxia. This results in more severe injury in a subset of animals, and more closely models the complex interaction of prolonged inflammation, ischemia, hypoxia, and oxidative stress experienced in a number of preterm infants who develop brain injury.

## **PROTOCOL:**

Procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory

Animals and as part of an approved protocol by the University of Washington Institutional Animal Care and Use Committee.

## 1. Preparation and LPS administration

NOTE: Refer to **Figure 1** for a timeline of the procedures.

1.1. Prior to commencing the procedure, seal, sterilize and autoclave all surgical instruments and surgical drapes. Prepare pre-operative medications in sterile vials. Calculate the flow rate required to replace the air in the hypoxia/hyperoxia chamber with the experimental gas in 8–10 min.

1.2. Prepare the lipopolysaccharide (LPS from *E. coli* 055:B5) in sterile saline to produce a concentration of 1 mg/mL. Remove P17 ferret kits from their jills. Weigh and number them. Randomize animals by litter and sex to the control or injured (or treatment) groups.

1.3. Using a 300 µL insulin syringe, administer 3 mg/kg of LPS intraperitoneally to kits in the injury group, and an equivalent volume of sterile saline vehicle (3 µL/g) to control animals.

1.4. Place animals in a chamber within a water bath at 37–40 °C in order to maintain a target rectal temperature of 36–37 °C throughout the surgical procedures.

## 2. Anesthesia

2.1. During the procedure, continually monitor temperature, respiration rate, and heart rate of the animal.

2.2. Administer buprenorphine (0.05 mg/kg) subcutaneously 30 min before the surgical procedure. Induce anesthesia in a mixture of 3% isoflurane balanced with 100% oxygen. Remove the kit from the induction chamber and place it supine on a draped surgical water blanket set to 37 °C. Transfer anesthesia to the nose cone and reduce isoflurane level to 2–3%.

## 3. Surgical preparation

3.1. Using small animal clippers remove all hair on the ventral neck region. Shave in a rectangular pattern with care to avoid nicking the skin or generating razor rash. Administer local anesthesia to the shaved area using intradermal lidocaine (4 mg/kg) and bupivacaine (2.5 mg/kg).

3.2. Prepare the neck by alternating application of povidone-iodine and 70% ethanol scrub with sterile cotton swabs. Repeat the scrub such that povidone-iodine and 70% ethanol are each applied 3x in alternating fashion.

3.3. Confirm depth of anesthesia via absence of toe-pinch reflex. Maintain level of isoflurane at the minimum percentage required for a surgical plane of anesthesia. Using sterile disposable cut-out drapes that expose the neck region, drape the animal.

#### 4. Bilateral carotid artery ligation

4.1. With a single-use #11 scalpel blade, make a 1.5 cm midline incision in the center of the neck. Using fine hemostats and curved forceps, bluntly dissect down to the left carotid artery. Dissect the artery away from the associated neurovascular bundle.

4.2. Using a pair of curved fine forceps, pass a looped 10 cm length of sterile 5-0 silk suture under the artery. Cut the suture in half. Ligate the artery by securely tying both lengths of suture, leaving at least 2 mm between the knots. Transect the left carotid artery between the sutures, taking care to leave the nerve intact.

4.3. Repeat the dissection on the right side. Reversibly ligate the right carotid artery with a single sterile 1/8 inch umbilical tie. Close the wound with surgical skin clips.

4.4. Allow the animal to recover in a temperature-controlled water bath for at least 30 min before hypoxia.

NOTE: If the arteries are not fully isolated from the rest of the neurovascular bundle, increased mortality may be seen before or during later hypoxia.

#### 5. Sequential hypoxia, hyperoxia, and hypoxia

5.1. During hypoxia and hyperoxia, alter water bath temperature as necessary to maintain rectal temperature during hypoxia at 37 °C in the sentinel animal(s).

5.2. Place animals in the injury group in an airtight chamber within a water bath. Continuously monitor oxygen concentration within the chamber, as well as rectal temperature in at least one sentinel animal. Flush the chamber with humidified 9% oxygen (91% nitrogen) then maintain a flow rate of 3–5 L/min, depending on chamber size. Once the concentration of oxygen in the chamber has reached 9%, continue for 30 min.

5.3. After 30 min, switch gas supply to 80% humidified oxygen (20% nitrogen), and allow the chamber to reach the target concentration based on flow rate and chamber size. Continue for 30 minutes of hyperoxia. Open the chamber to allow it to more rapidly reach normoxia by equilibrating with room air.

5.4. Seal the chamber, and flush with 9% humidified oxygen. Continuously monitor all animals visually, taking note of animals that display bradypnea. Once the concentration of oxygen in the chamber has reached 9%, continue for 30 min. If intra-hypoxic mortality (respiratory arrest) in any of the animals is seen before the end of the 30 min period, terminate hypoxia immediately.

## 6. Reversal of right carotid artery ligation

6.1. Return animals to the surgical area, and induce anesthesia in a mixture of 3% isoflurane balanced with 100% oxygen. Transfer anesthesia to nose cone and reduce isoflurane level to 2–3%. Remove the surgical wound clips and re-prepare the wound area with povidone-iodine. Confirm depth of anesthesia via absence of toe-pinch reflex. Maintain the level of isoflurane at the minimum percentage required for a surgical plane of anesthesia. Using sterile disposable cut-out drapes that expose the neck region, drape the animal.

6.2. Using curved forceps, identify and untie the umbilical tape from the right carotid artery. Close the wound with surgical skin clips.

## 7. Recovery and temperature management

7.1. Return all kits to their jills for 60 min, for nursing and recovery. After 60 min, return injured animals to the water baths at 37–40 °C for 6 h, adjusting water temperature as needed in order to maintain rectal temperature at 36–37 °C. Return kits to their jills.

7.2. Remove surgical clips 10–14 days after surgery (P27–P31).

## 8. Reflex testing

8.1. Perform all the reflex tests daily from P21–P28, and at least 3x per week from P28–P42, whilst remaining blinded to exposure (or treatment) group. Prior to reflex testing, place kits in a chamber with heat assistance (37 °C water bath, heat pad, etc.) for 1 h. For each test, complete all trials per kit before testing the next kit.

### 8.2. Negative geotaxis (25°)

8.2.1. Place a flat board (16 ½ in. x 12 in.) wrapped in an absorbent benchtop protector against an object so that the board forms a 25° angle with the table. Place a kit on the board prone and facing downhill, approximately 75% of the way up the board.

8.2.2. Ensure the kit's body is straight and that it has all four paws gripped against the board before releasing it. As soon as the kit is placed, start the time assessment.

8.2.3. Record the time when the kit manages to rotate its body 90° relative to its starting position. Record the time at which the kit rotates its body 180° and takes a full step towards the top of the board. Perform 3 trials at the 25° incline before moving onto the next test.

### 8.3. Negative geotaxis (45°)

8.3.1. Perform 3 trials of the previously described negative geotaxis test again, this time with the board set to a 45° angle.

#### 8.4. Cliff aversion

8.4.1. Place a padded platform around 1 foot below the ledge to minimize injury to kits if they fall.

8.4.2. Place a kit facing, and perpendicular to, the edge of the lab bench. Ensure the kit's body is straight, with its front paws flush with the edge. Begin time assessment from the moment the kit is placed. Take care to differentiate between conscious movement away from the cliff and other spontaneous movements that do not involve coordinated walking.

8.4.3. Record the time when the kit moves its body away from the edge (defined as the kit backing up, turning its body, or moving its front limbs away from the edge). Record the time the kit completes its first step in the opposite direction of the edge (defined as any direction or angle past a 90° rotation from its starting position facing the edge).

8.4.4. Perform 3 cliff aversion trials per kit before moving onto the next test.

#### 8.5. Righting reflex

8.5.1. Place a kit supine on the bench, holding it gently in that position before releasing it and simultaneously beginning the stopwatch. Record the time that the kit brings itself to rest with all four paws simultaneously flat against the bench in weight-bearing positions. Record the time when the kit takes a full step in any direction (defined as the placement of all four paws to achieve progress in a given direction with no spinning or dragging of the body).

8.5.2. Perform 5 trials of the righting reflex test per animal.

8.5.3. After every kit has completed the 5 righting reflex trials, return the litter to the jill.

### 9. Catwalk testing

9.1. On P42, remove kits from the jill. Place kits in plastic cages approximately 10 min before testing, so they can acclimate to the environment. Turn the lights off in the testing room to ensure ambient light does not affect catwalk function.

9.2. Create a new experiment in the relevant software. Adjust experimental settings so that the maximum run duration does not exceed 10.00 s and the minimum run duration is not less than 1.50 s. Set the maximum speed variation so it does not exceed 60%. Set a minimum requirement of three compliant runs for each animal.

9.3. Adjust the width of the walkway relative to the size of the animal so that it is able to freely locomote without touching the walls while remaining narrow enough to discourage turning. Add

a new detection setting in the **Detection settings profiles** tab using auto detection. Use the same detection settings for all litters and animals at a given age.

9.4. Thoroughly clean the walkway with a low-lint paper tissue and 70% ethanol before and after each animal. Clean the ferret's paws regularly to improve accuracy of detection and classification. Once the walkway and animal are prepared, begin trial acquisition.

9.5. Pause acquisition to clean the catwalk if footprints accumulate on the glass, or if animals pass urine or feces. Stop acquisition once the catwalk software has recognized three compliant runs based on the pre-determined experiment settings.

## **10. Open field testing (P42)**

10.1. Use a non-porous acrylic box (55 cm x 55 cm x 40 cm high) painted matte-white. Position the camera so that it is centered directly above box and all four walls are captured. Clean the testing arena with 70% ethanol before first use and between animals.

10.2. In the relevant software, select **New From Template** and **Apply a Pre-defined Template**. Continue the set-up procedure by sequentially selecting **Subject type**: Other; **Arena template**: Open field, square; **Zone template**: Center, border, corners; **Features to track**: Center-point.

10.3. Open **Arena Settings** and grab the background image from the camera input, making sure that the tops of the arena walls are visible. Calibrate the dimensions of the arena using the scale tool.

10.4. Adjust the pre-defined arena zones by resizing the outlines to fit the wall zones (NW, NE, SW, SE) and floor zones (left top, middle top, right top, left middle, center, right middle, left bottom, middle bottom, right bottom). Validate the set-up to confirm that no zones overlap.

10.5. Open the **Acquisition window** and press **Start Acquisition**. Place the ferret at the center of the testing arena oriented in such a way that is consistent for every test subject. Allow the ferret to move freely throughout the arena for a period of 5 min. At the end of the testing period, press **Stop Acquisition**. Repeat the procedure with the next ferret.

NOTE: All experimenters in the room should position themselves to be unobservable by the ferret and remain quiet during the testing period.

## **11. Fixation-perfusion**

11.1. On P42, deeply anesthetize kits with 5% isoflurane. Administer a pentobarbital overdose (120–150 mg/kg i.p.). Ensure deep anesthesia by lack of response to toe pinch and loss of respiratory movements.



11.2. Transfer the animal to a fume hood. Open the thorax and clamp the descending aorta using fine hemostats. Cut the right atrium. Using a perfusion pump, perfuse the left ventricle with 60 mL of sterile saline at a rate of 30 mL/min. Perfuse with 60 mL of formalin (10% formaldehyde) at a rate of 30 mL/min.

11.3. Decapitate the carcass, and remove the brain from the skull using scissors, forceps, rongeurs, and a spatula. Take high-resolution photographs of the dorsal, ventral, and lateral aspects of each brain. Post-fix the brain in formalin for at least 48 h.

## **12. Ex vivo brain measurement**

12.1. Remove the brain from formalin (step 11.3) and place onto a paper towel to absorb excess liquid.

12.2. Using an electronic caliper, measure the height of the brain by placing the tips of the caliper at the dorsal and ventral aspects of the brain. Measure the length of the brain by placing the tips of the caliper at the olfactory bulb and the most posterior border of the occipital lobe. Measure the width of the brain by placing the tips of the caliper at the most lateral portions of the temporal lobes. Weigh the brain.

12.3. Measure the longitudinal fissure (anterior and posterior to the cruciate sulcus), lateral sulci, suprasylvian sulci, coronal sulci, pseudosylvian sulci, ansinate sulci, cruciate sulci, presylvian sulci, lateral gyri, suprasylvian gyri, sigmoid gyri (anterior and posterior), coronal gyri, ectosylvian gyri (anterior and posterior), and orbital gyri. Measure all sulci from the beginning and end of the most distinct portion of the corresponding sulcus. Measure all gyri from the widest aspect of each corresponding gyrus.

12.4. Measure the amount of cerebellum exposed by placing one tip of the caliper at the most posterior point of the longitudinal fissure and placing the other tip of the caliper at the most posterior part of the cerebellum.

NOTE: The ferret brain atlas, as found in Biology and Diseases of the Ferret<sup>14</sup>, was used to develop the ex vivo ferret brain measurements.

## **13. Gross injury scoring**

13.1. Using the photographs taken in step 11.3., apply the scoring criteria in steps 13.2–13.4 to assess gross brain injury (0–9 scale) whilst remaining blinded to exposure (or treatment) groups.

13.2. Assess the longitudinal fissure. If it appears normal, assign a score of 0. If it is mildly widened (approximately 2x normal width), but the increase in width is incomplete along length of fissure, assign a score of 1. If it is moderately widened (approximately 2–3x normal), assign a score of 2. If it is markedly widened, with a visible gap >3x normal width along most of the length of fissure, apply a score of 3.

13.3. Assess the lateral sulci. If they show normal definition, with separation of the lateral gyri and suprasylvian gyri, assign a score of 0. If mild unilateral or bilateral reduced definition of sulcus is seen, particularly in the caudal portion, with minimal narrowing of frontal and temporal lobes relative to the occipital lobes, assign a score of 1.

13.3.1. If moderately reduced definition of the sulci is seen, with depression of the suprasylvian gyri, narrowing of the coronal and ectosylvian gyri, and mild narrowing of frontal and temporal lobes relative to occipital lobes, assign a score of 2. If unilateral cystic degeneration is seen, with minimal change of the contralateral hemisphere, assign a score of 3. If poor definition of the lateral sulci is present, with bilateral cystic or severe degeneration of the occipital and temporal lobes, assign a score of 4.

13.4. Assess the visible portion of the cerebellum. If it appears normal, with (<75% of the vermis and <66% of the hemispheres visible, assign a score of 0. If 75–90% of the vermis and ≥66% of the hemispheres are visible, assign a score of 1. If most of the cerebellum is visible, showing all of the vermis and ≥66% of hemispheres, assign a score of 2.

#### 14. Data analysis

14.1. For reflex testing data, assign failures a score of 61 s to allow them to be compared to successes at the end of time (60 s), but with a worse ranking in the statistical analysis. Calculate an area under the curve for each animal over time in each of the reflex tests.

14.2. Adjust catwalk data that involves paw size of pressure by the weight of the animal.

14.3. Analyze data using non-parametric statistical methods, describing data using the median and interquartile range (IQR).

#### REPRESENTATIVE RESULTS:

Of 34 (n = 18 males, n = 16 females) animals from six litters exposed to the insult, eight animals (24%; n = 4 males, n = 4 females) in the injured group died during the second hypoxia period (n = 5), during temperature management (n = 2), or overnight after the insult (n = 1). In the injured group, nine of 26 survivors (35%) had visible gross injury. Five animals (n = 5 males) had moderate injury, and four animals (n = 2 males, n = 2 females) had severe injury, defined as gross pathology scores of 2–5 and 6–9, respectively (**Figure 2A**). Animals exposed to the insult therefore have a 50% risk of death or significant gross injury. With increasing injury, narrowing of gyri in the temporal and/or occipital lobes is seen, with associated sulcal shortening, widening of the longitudinal fissure, and large areas of areas of cystic tissue loss in the most severely-injured animals (**Figure 2B**). In surviving injured animals (n = 26; n = 14 males, n = 12 females), significantly greater exposure of the cerebellum is seen (**Figure 3A**), as well as shortening of the longitudinal fissure (**Figure 3D**). There is also significant narrowing of the coronal and anterior ectosylvian gyri (**Figure 3B,E**), as well as shortening of the lateral and suprasylvian sulci (**Figure 3C,F**). Median (IQR) brain weight was 8.1 g (7.9–9.7g, n = 6) in control animals, and 7.0 g (6.5–7.7

g) in injured animals ( $n = 26$ ,  $p = 0.005$ ). In control animals, median (IQR) brain length was 28.9 mm (27.8–29.6 mm,  $n = 6$ ) compared to 27.5 mm (25.5–38.0 mm,  $n = 26$ ) in injured animals ( $p = 0.007$ ). Similar patterns are seen across the brain, with median width and height 5–7% smaller in injured animals. Anatomical structures on both the left and right side are affected in a similar manner, with no difference between hemispheres. See **Figure 1B** for depictions of the anatomical locations. Over the reflex testing period (P21–P39), injured animals display slower time to rotate in the negative geotaxis task (**Figure 4A**), slower time to rotate away from the edge in the cliff aversion task (**Figure 4B**), and slower time to right (**Figure 4C**). In the catwalk, injured animals have a similar average speed to controls (**Figure 5A**), but display a significantly greater degree of speed variation during each run (**Figure 5B**). The weight-adjusted distance between fore paws and hind paws (print position) is significantly greater in injured animals (**Figure 5C**), with less pressure exerted per unit paw area through the fore paws (**Figure 5D**). In the open field, injured animals cover less total distance (**Figure 6A**), and stop more frequently (**Figure 6B**). They spend significantly more time in the center of the field, and less time in the corners (**Figure 6C,D**). Representative heat maps of control and injured animals are shown in **Figure 7A,B**.

#### FIGURE LEGENDS:

**Figure 1: Timeline.** On P17, animals are administered 3 mg/kg LPS before undergoing bilateral carotid artery ligation and 30 min each (not including time for the chamber to equilibrate) of hypoxia (9% oxygen), hyperoxia (80% oxygen) and hypoxia (9%). The right carotid artery ligation is then reversed. Animals are exposed to 6 h of normothermia to ensure they do not become spontaneously hypothermic in the nest in the period after injury. Reflex testing is then performed daily from P21–P28, and three times per week from P28–P42. On P42, animals are tested in the catwalk and open field before sacrifice.

**Figure 2: Representative injury distribution and depiction.** (A) Gross injury scoring from 26 survivors ( $n = 14$  males,  $n = 12$  females) in the injured group, compared to six litter mate controls. Five animals ( $n = 5$  males) had moderate injury, and four animals ( $n = 2$  males,  $n = 2$  females) had severe injury, defined as gross pathology scores of 2–5 and 6–9, respectively. Graph shows median with interquartile range. (B) Control brain (left panel, score 0), with brains depicting increasing gross injury scores of 2, 5, and 8 out of a total possible score of 9, from left to right. The control brain shows anatomical structures particularly susceptible to injury; 1 = longitudinal fissure, 2 = lateral sulcus, 3 = suprasylvian sulcus, a = coronal gyrus, b = anterior ectosylvian gyrus.

**Figure 3: Representative brain measurements.** Compared to controls ( $n = 6$ ), injured animals ( $n = 26$ ) display significantly increased exposure of the cerebellum (A), shortening of the longitudinal fissure (D), narrowing of the coronal (B) and anterior ectosylvian (E) gyri, and shortening of the lateral (C) and suprasylvian (F) sulci. Graphs show median with interquartile range. \*denotes  $p < 0.05$  (Wilcoxon-Mann-Whitney U-test).

**Figure 4: Representative reflex development.** Compared to controls ( $n = 6$ ), injured animals ( $n = 26$ ) display slower development (area under the curve, AUC) of negative geotaxis (A), cliff aversion (B), and righting reflex (C). Graphs show median with interquartile range. \*denotes  $p <$

0.05 (Wilcoxon-Mann-Whitney U-test).

**Figure 5: Representative catwalk results.** Compared to controls (n = 6), injured animals (n = 26) walk at a similar average pace **(A)**, but with a greater variability in speed during walking **(B)**. Injured animals also display a longer average print position **(C)**, with less pressure applied per unit area **(D)**. Graphs show median with interquartile range. \* denotes  $p < 0.05$  (Wilcoxon-Mann-Whitney U-test).

**Figure 6: Representative open field behavior.** Compared to controls (n = 6), injured animals (n = 26) cover a smaller total distance **(A)**, as well as stopping more frequently **(B)**. Injured animals also spend more time at the center **(C)** than in the corners **(D)**. Graphs show median with interquartile range. \*denotes  $p < 0.05$  (Wilcoxon-Mann-Whitney U-test).

**Figure 7: Representative open field heat maps.** **(A)** control female, **(B)** injured female. Injured animals cover a significantly smaller distance within the open field.

## DISCUSSION:

Due to the physical and developmental similarities shared between the ferret brain and human brain, the ferret is increasingly being used to model both adult and developmental brain injury.<sup>8-12</sup> However, research to date suggests that the ferret brain is both resistant to initial injury as well as highly-plastic, with behavioral deficits diminishing over time even in the setting of visible pathological injury<sup>10,12</sup>. Here, we describe the first model of inflammation-sensitized hypoxic-ischemic (HI) brain injury in the late preterm-equivalent ferret, which results in significant bilateral injury and sustained behavioral deficits in survivors. As with any preclinical model, the goal was not to accurately reproduce the exposures encountered by preterm infants clinically, but to provide a confluence of the mechanistic factors thought to be involved in premature brain injury. These include inflammation, hypoxia, and oxidative stress<sup>7</sup>.

One critical aspect of LPS administration in our ferret models is a single high dose given around 4 h before hypoxia. LPS exposure in near-term equivalent rodents results in a circulating inflammatory cytokine peak around 4 h after exposure, which corresponds with sensitization of the brain to hypoxia-ischemia, and a significant increase in brain injury<sup>15-17</sup>. A similar time course of inflammatory cytokine release (peak TNF- $\alpha$  and IL-6 release 2–4 h after LPS exposure) is seen in isolated ferret peripheral blood mononuclear cells<sup>18</sup>. Assuming a single surgical set-up, administering LPS 30–60 min before the start of surgery allows for adequate time to perform 12–15 bilateral carotid artery ligations and initiate the first hypoxia exposure 4 h after LPS administration. During model development, an LPS dose of 5 mg/kg was initially used, as described in our P10 injury model<sup>12</sup>. However, this LPS dose was associated with significant intra-hypoxic mortality and pulmonary edema on necropsy. Both mortality and pulmonary edema were reduced by decreasing the LPS dose to 3 mg/kg.

During hypoxia exposure, a number of factors appear to be critical to ensuring significant gross injury whilst also preventing high levels of mortality. Due to laboratory ferrets being outbred, there is an inherent variability in hypoxia tolerance across litters. In our experience, cross-

fostering animals or combining animals from different litters in the same hypoxia chamber predominantly results in the earlier death of larger animals or animals from the most susceptible litter. If more susceptible animals die before the target 30 min hypoxia exposure and hypoxia is stopped early, smaller animals from less susceptible litters will receive suboptimal hypoxia exposure, and are unlikely to sustain significant injury. As a result, each litter of animals should be exposed to hypoxia within their own separate chamber. The second hypoxia period was added as part of an iterative model development process that we have previously described<sup>12</sup>. A single hypoxia period resulted either in death or survival with no significant injury, regardless of length. As ferrets are able to tolerate long periods of acute hypoxia or bilateral carotid artery ligation without showing significant brain injury, our current hypothesis is that the period of hyperoxia results in elevated metabolism and vasodilatation that facilitates brain ischemia during the second hypoxia period. To minimize variability in the model, we used pre-ordered sex-balanced litters of 8 ferrets that arrived in our facility on P15. In each litter, 6–7 animals underwent surgery followed by hypoxia within a single chamber.

After hypoxia and reversal of the right carotid artery ligation, animals should be returned to their jills for a period of time to feed due to a risk of dehydration and hypoglycemia from the prolonged injury protocol. If significant mortality is experienced during the temperature management period, animals may need additional fluid resuscitation (subcutaneous saline and/or hand feeding with formula and water) before being placed in the water baths for 6 h. The temperature management period is, however, a critical determinant of long-term injury, as animals may otherwise experience neuroprotection from relative hypothermia in the nest. This risk of hypothermia is at least partly due to the low temperature of housing conditions required for the ferret (60–70 °F).

The behavioral tests described were largely developed within the laboratory, with some basis in reflex tests previously described in the developing ferret<sup>19</sup>, with catwalk and open field tests adapted from adult rodents to be used in juvenile ferrets. Other groups have also described open field, maze, and gait testing in adult ferrets after traumatic brain injury<sup>10</sup>, as well as the effect of in utero inflammation on social interaction in adult ferrets<sup>20</sup>. Though a long fasting period is not recommended in ferrets due to their short gut transit time, placing them in an animal carrier for 30–60 min before any of the tests is beneficial in order to allow them to pass urine and feces before the tests. As the ferret is by nature an inquisitive animal, it often behaves in an opposite manner to rodents in these behavioral tests. This is particularly evident in the catwalk, where lights and sounds, particularly recordings of another ferret vocalizing (“dooking”), can be used to motivate the ferret to walk forwards.

The current protocol does have some limitations. As it was developed iteratively using previously developed methods in the P10 ferret<sup>12</sup>, we do not currently know the relative contributions of LPS, hypoxia, hyperoxia, and reperfusion to the final degree of injury seen. However, it is worth noting that development of the method described here included using the original Vannucci model (unilateral carotid artery ligation followed by a single period of hypoxia) in the ferret<sup>21</sup>, which did not result in any significant injury. Therefore, interactions between the multiple parts of the injury protocol is likely to be necessary for sustained injury. Despite this, there remains a

distinct variability in gross injury in surviving animals, which is another potential limitation. Though animals without significant gross injury may have injury that is detectable using MRI or histopathology<sup>12</sup>, future work on the model will include iterations to try and increase the number of animals that sustain significant injury, for instance by using permanent bilateral carotid artery ligation. Finally, in order for this model to be maximally useful to test putative neuroprotective therapies for developmental brain injury, it should be validated by assessing the efficacy of neuroprotective agents that are either established for the treatment of HI brain injury in human neonates, or have been successful in a range of other animal models of neonatal brain injury. Future studies will therefore assess the efficacy of therapeutic hypothermia and erythropoietin in this model, including sex-based therapeutic responses and ex vivo MRI<sup>12</sup>.

#### ACKNOWLEDGMENTS:

Development of the model was funded Bill and Melinda Gates Foundation, as well as by NIH grant 5R21NS093154-02 (NICHD).

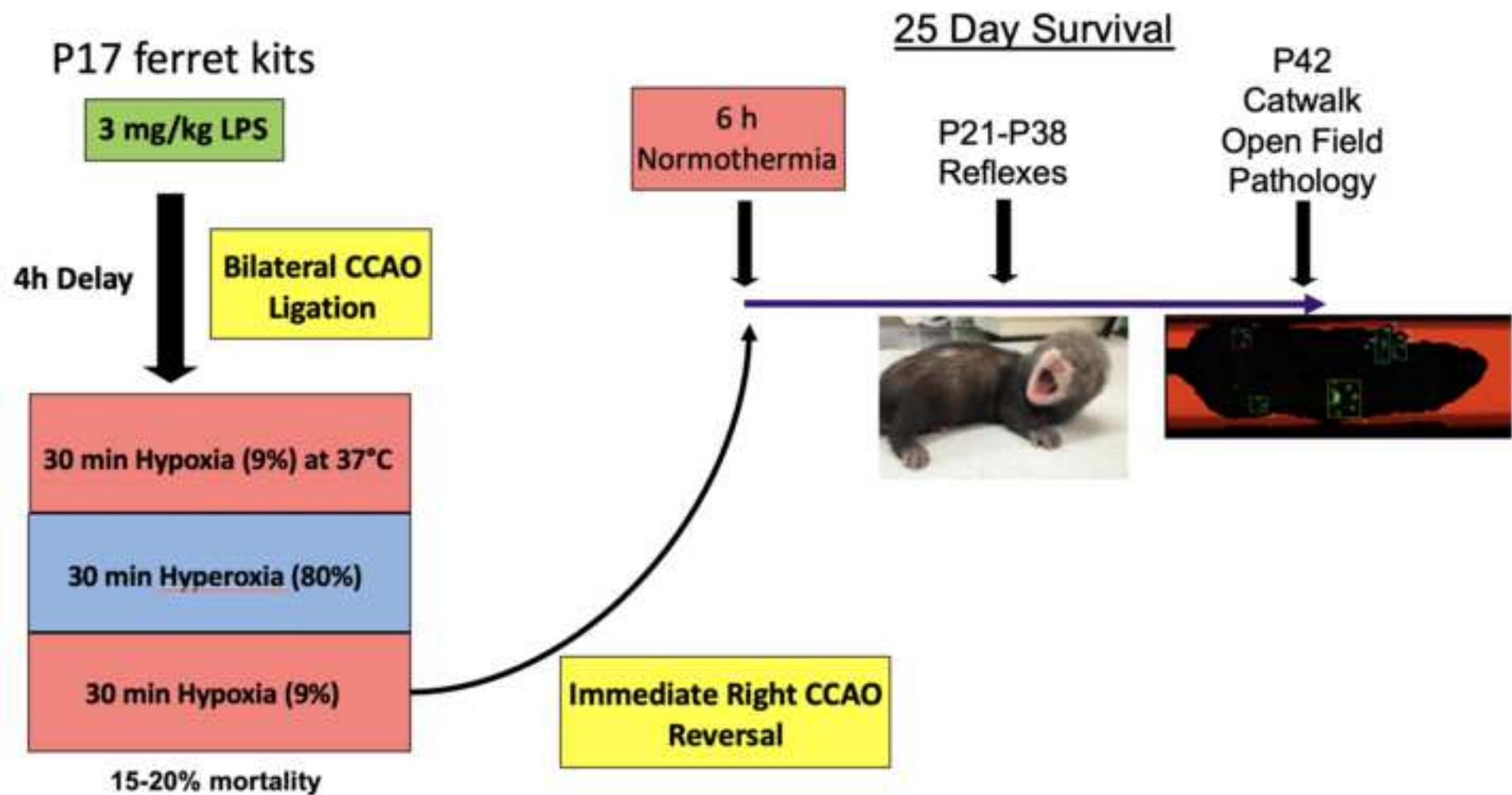
#### DISCLOSURES:

The authors have nothing to disclose.

#### REFERENCES:

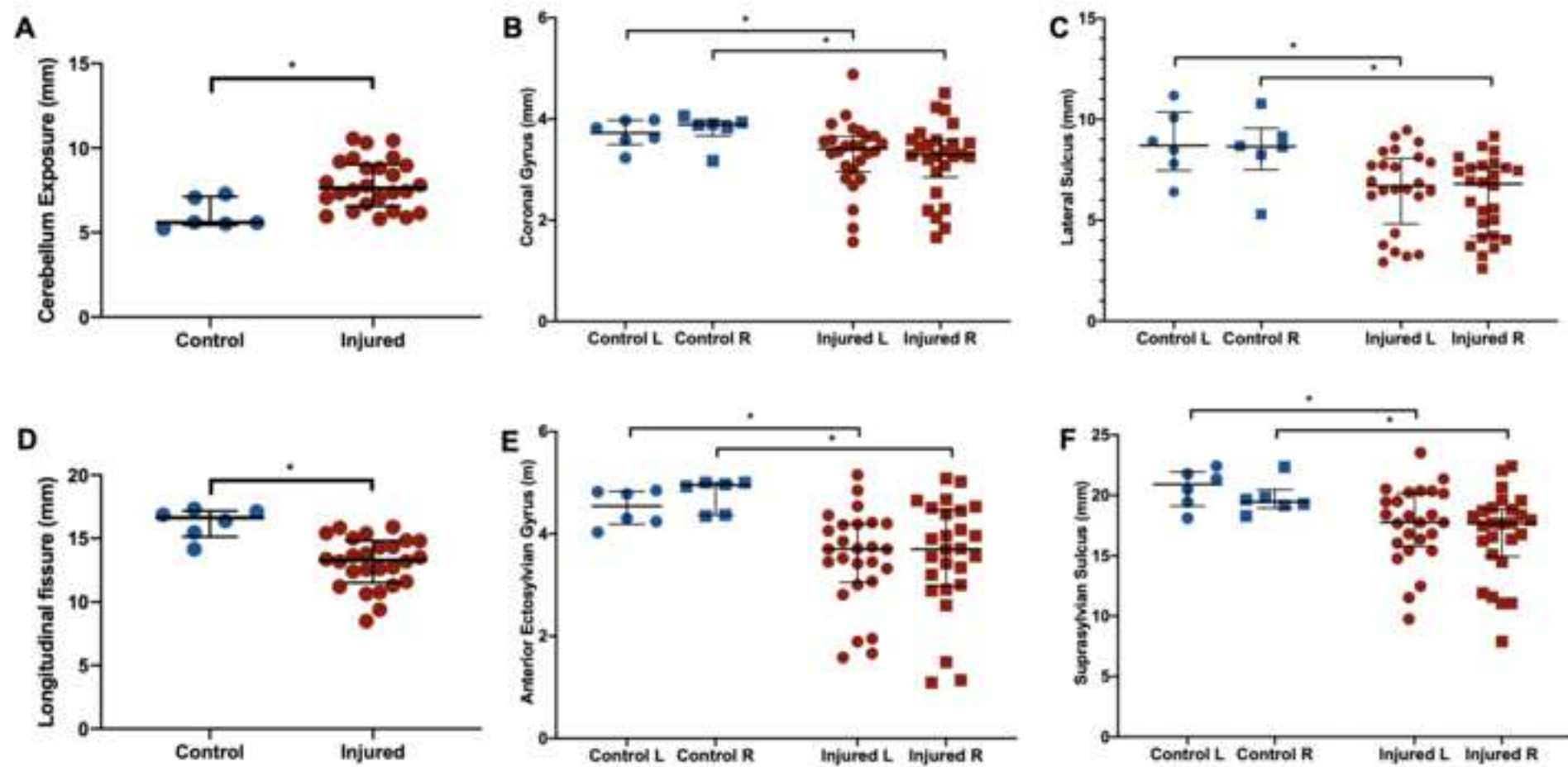
- 1 Martin, J. A., Hamilton, B. E., Osterman, M. J. K., Driscoll, A. K., Drake, P. Births: Final Data for 2017. *National Vital Statistics Report*. **67** (8), 1-49, (2018).
- 2 Vanhaesebrouck, P. *et al.* The EPIBEL study: outcomes to discharge from hospital for extremely preterm infants in Belgium. *Pediatrics*. **114** (3), 663-675, (2004).
- 3 Raju, T. N. *et al.* Long-Term Healthcare Outcomes of Preterm Birth: An Executive Summary of a Conference Sponsored by the National Institutes of Health. *Journal of Pediatrics*. 10.1016/j.jpeds.2016.10.015, (2016).
- 4 Raju, T. N. K., Buist, A. S., Blaisdell, C. J., Moxey-Mims, M., Saigal, S. Adults born preterm: a review of general health and system-specific outcomes. *Acta Paediatrica*. **106** (9), 1409-1437, (2017).
- 5 Bennet, L. *et al.* Chronic inflammation and impaired development of the preterm brain. *Journal of Reproductive Immunology*. **125** 45-55, (2018).
- 6 Reich, B., Hoeber, D., Bendix, I., Felderhoff-Mueser, U. Hyperoxia and the Immature Brain. *Developmental Neuroscience*. **38** (5), 311-330, (2016).
- 7 Galinsky, R. *et al.* Complex interactions between hypoxia-ischemia and inflammation in preterm brain injury. *Developmental Medicine & Child Neurology*. **60** (2), 126-133, (2018).
- 8 Empie, K., Rangarajan, V., Juul, S. E. Is the ferret a suitable species for studying perinatal brain injury? *International Journal of Developmental Neuroscience*. **45** 2-10, (2015).
- 9 Snyder, J. M. *et al.* Ontogeny of white matter, toll-like receptor expression, and motor skills in the neonatal ferret. *International Journal of Developmental Neuroscience*. 10.1016/j.ijdevneu.2018.05.006, (2018).
- 10 Schwerin, S. C. *et al.* Progression of histopathological and behavioral abnormalities following mild traumatic brain injury in the male ferret. *Journal of Neuroscience Research*. **96** (4), 556-572, (2018).
- 11 Rafaels, K. A. *et al.* Brain injury risk from primary blast. *Journal of Trauma and Acute Care*

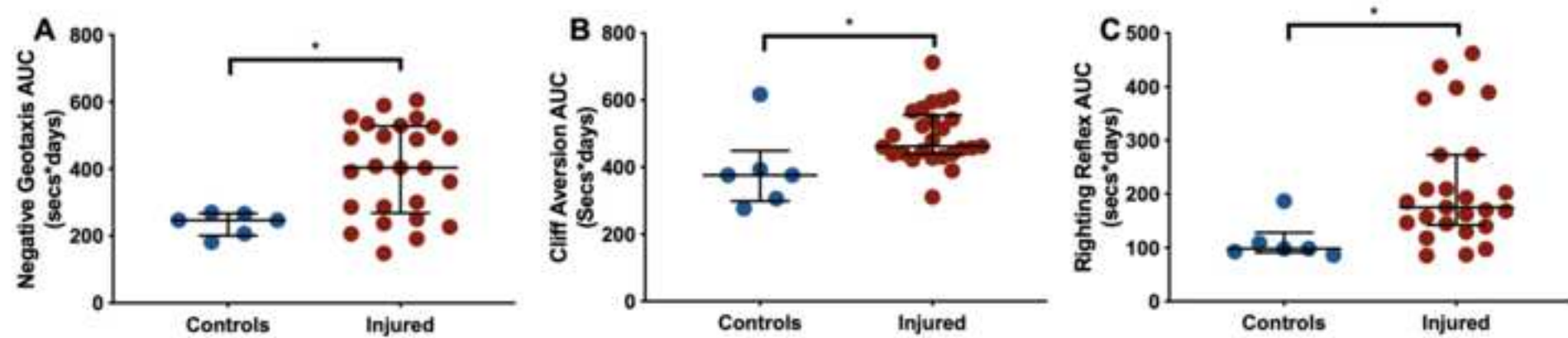
- Surgery*. **73** (4), 895-901, (2012).
- 12 Wood, T. *et al.* A Ferret Model of Encephalopathy of Prematurity. *Developmental Neuroscience [In Press]*. (2019).
- 13 Barnette, A. R. *et al.* Characterization of Brain Development in the Ferret via Magnetic Resonance Imaging. *Pediatric Research*. **66** (1), 80-84, (2009).
- 14 Kroenke, C. D., Mills, B. D., Olavarria, J. F., Neil, J. J. in *Biology and Diseases of the Ferret* doi:10.1002/9781118782699.ch3 (Wiley, 2014).
- 15 Eklind, S. *et al.* Bacterial endotoxin sensitizes the immature brain to hypoxic--ischaemic injury. *European Journal of Neuroscience*. **13** (6), 1101-1106, (2001).
- 16 Falck, M. *et al.* Neonatal Systemic Inflammation Induces Inflammatory Reactions and Brain Apoptosis in a Pathogen-Specific Manner. *Neonatology*. **113** (3), 212-220, (2018).
- 17 Osredkar, D. *et al.* Hypothermia Does Not Reverse Cellular Responses Caused by Lipopolysaccharide in Neonatal Hypoxic-Ischaemic Brain Injury. *Developmental Neuroscience*. **37** (4-5), 390-397, (2015).
- 18 Nakata, M., Itou, T., Sakai, T. Quantitative analysis of inflammatory cytokines expression in peripheral blood mononuclear cells of the ferret (*Mustela putorius furo*) using real-time PCR. *Veterinary Immunology and Immunopathology*. **130** (1-2), 88-91, (2009).
- 19 Christensson, M., Garwicz, M. Time course of postnatal motor development in ferrets: ontogenetic and comparative perspectives. *Behavioral Brain Research*. **158** (2), 231-242, (2005).
- 20 Li, Y., Dugyala, S. R., Ptacek, T. S., Gilmore, J. H., Frohlich, F. Maternal Immune Activation Alters Adult Behavior, Gut Microbiome and Juvenile Brain Oscillations in Ferrets. *eNeuro*. **5** (5), (2018).
- 21 Rice, J. E., 3rd, Vannucci, R. C., Brierley, J. B. The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Annals of Neurology*. **9** (2), 131-141, (1981).

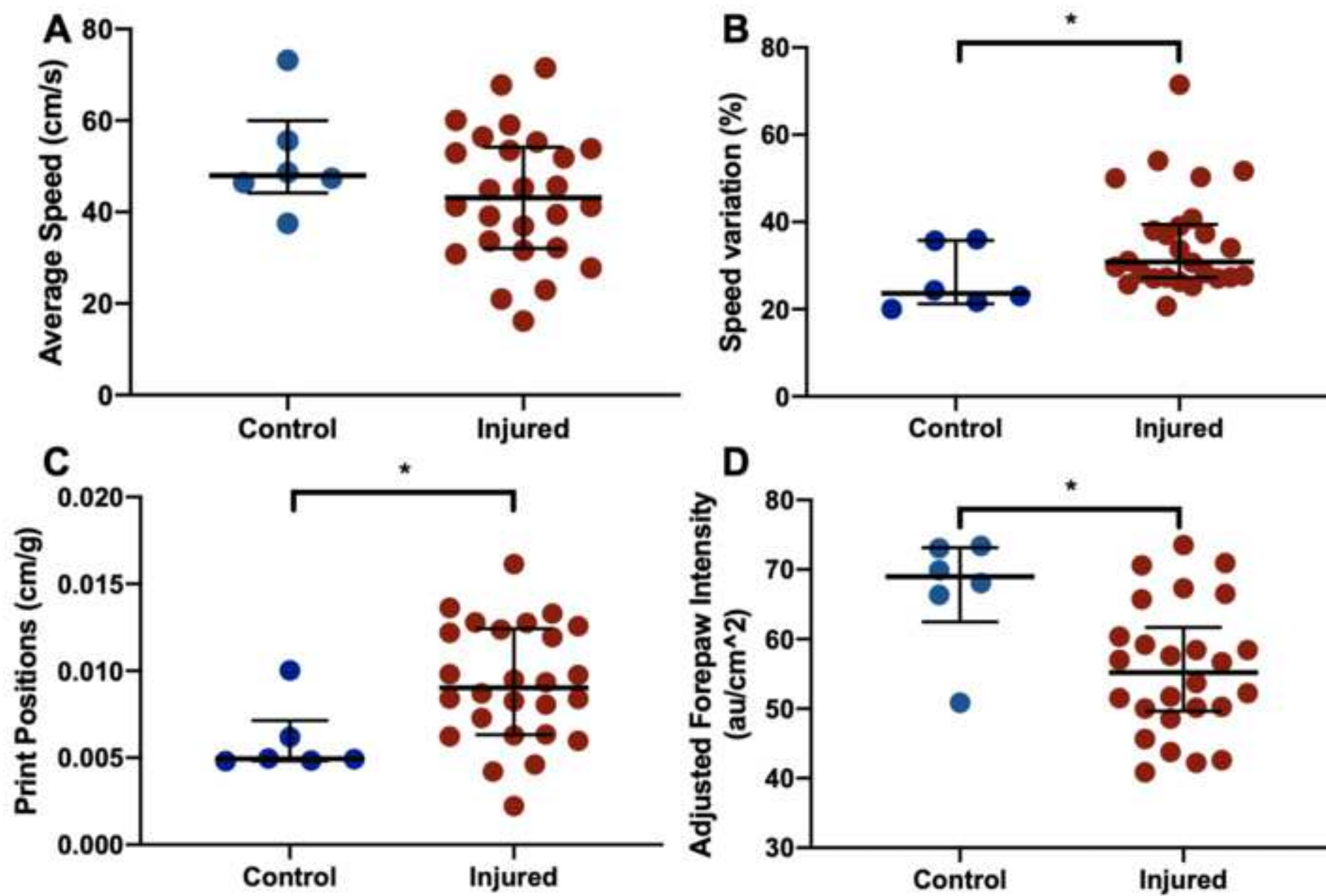


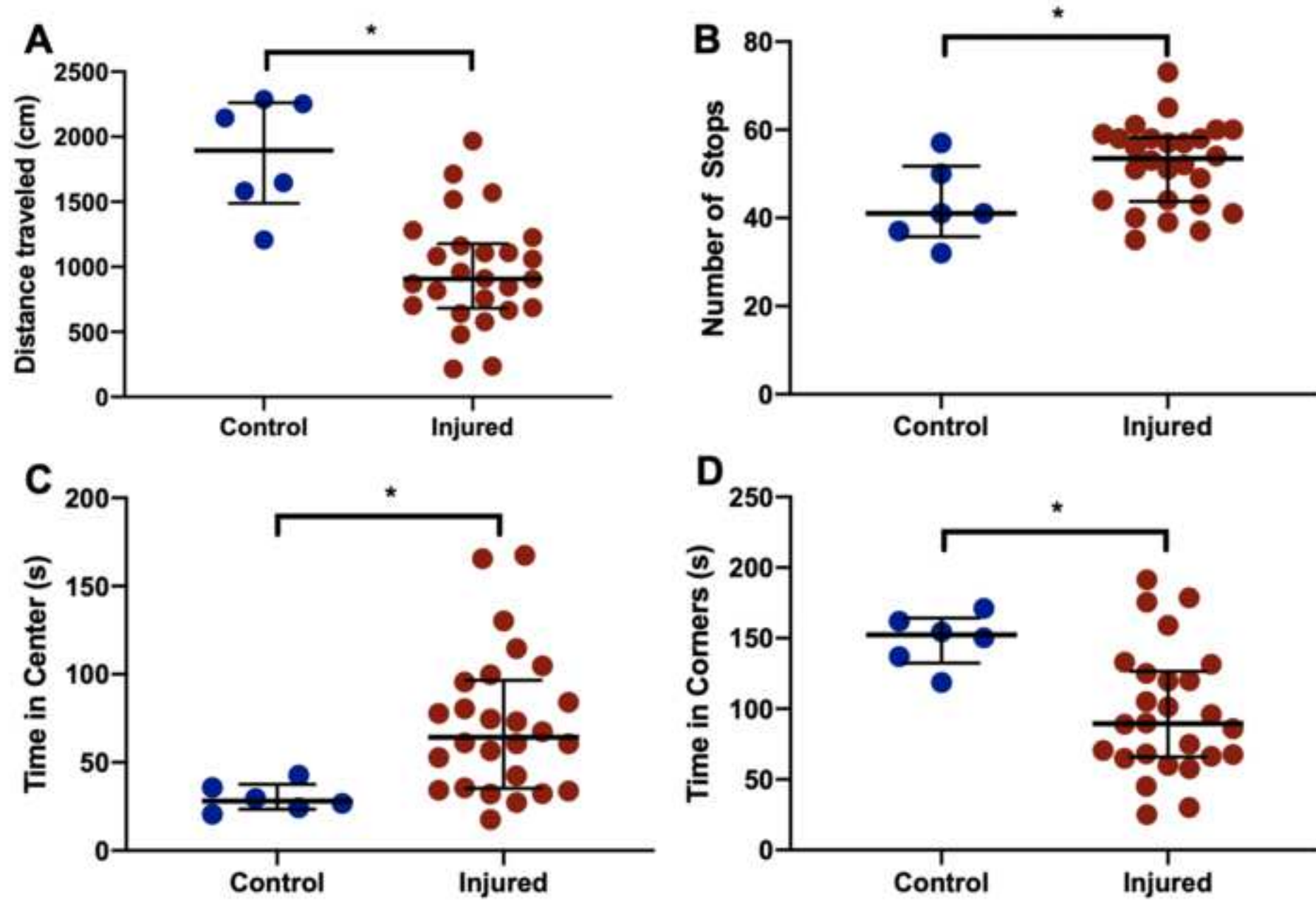








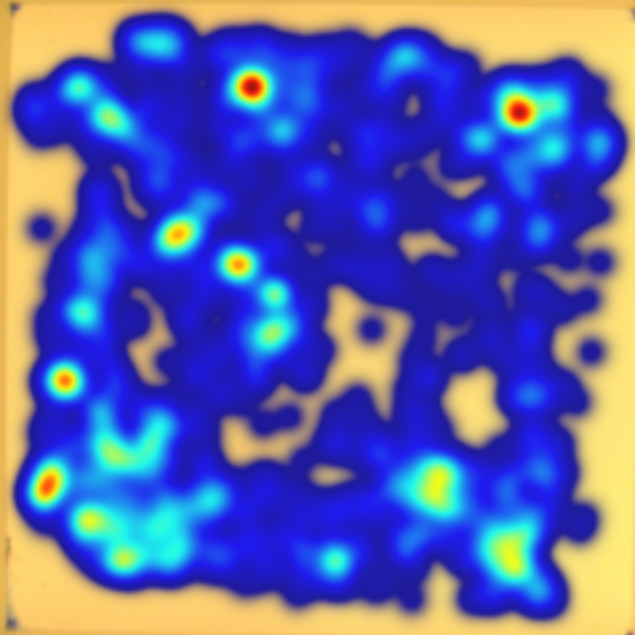




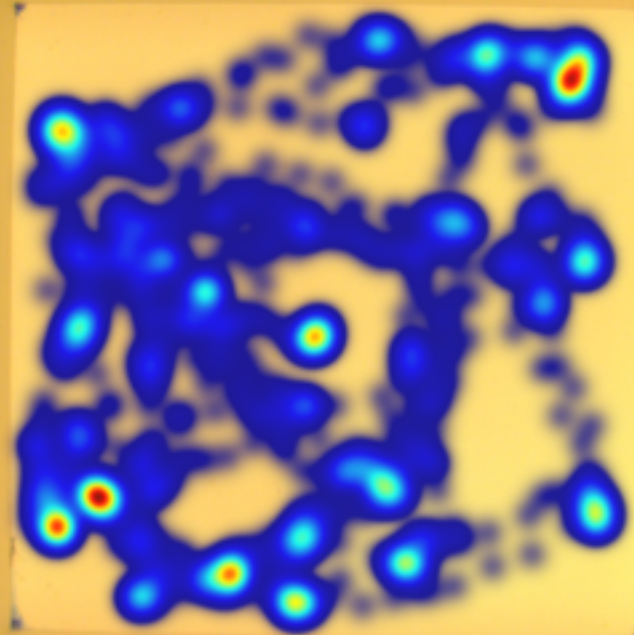


Figure

**A**



**B**



| Name of Material/ Equipment              | Company                     | Catalog Number          | Comments/Description             |
|--|-----------------------------|-------------------------|----------------------------------|
| 80% Oxygen                               | Praxair                     |                         |                                  |
| 9% Oxygen                                | Praxair                     |                         |                                  |
| Absorbent benchtop protector             | Kimtech                     | 7546                    |                                  |
| Automated catwalk                        | Noldus                      |                         |                                  |
| Betadine surgical scrub                  |                             |                         |                                  |
| Bupivacaine                              | Patterson Veterinary        | 07-888-9382             |                                  |
| Buprenorphine                            |                             |                         |                                  |
| Calipers                                 | SRA Measurement Products    | ME-CAL-FP-200           | 200mm range, .01 mm resolution   |
| Cotton Gauze Sponge                      | Fisher Scientific           | 22028556                |                                  |
| Curved fine hemostat                     | Roboz                       | RS-7101                 |                                  |
| Curved forceps                           | World Precision Instruments | 501215                  |                                  |
| Curved suture-tying hemostat             | Roboz                       | RS-7111                 |                                  |
| Ethovision tracking software             | Noldus                      |                         |                                  |
| Eye Lubricant                            | Rugby                       | NDC 0536-1970-72        |                                  |
| Ferrets ( <i>Mustela putorius furo</i> ) | Marshall Biosciences        |                         | Outbred (no specific strain)     |
| Formalin                                 | Fisher Scientific           | SF100-4                 | 10% (Phosphate Buffer/Certified) |
| Hair Clippers                            | Conair                      | GMT175N                 |                                  |
| Insulin Syringes                         | BD                          | 329461                  | 0.3 cc 3 mm 31G                  |
| Isoflurane                               | Piramal                     | 66794-017-25            |                                  |
| Lidocaine                                | Patterson Veterinary        | 07-808-8202             |                                  |
| LPS                                      | List Biological             | LPS Ultrapure #423      |                                  |
| Oxygen sensor                            | BW Gas Alert                | GAXT-X-DL-2             |                                  |
| Pentobarbital                            |                             |                         |                                  |
| Plastic chamber                          | Tellfresh                   | 1960 10L; 373x270x135mm |                                  |
| Saline Solution, 0.9%                    | Hospira                     | RL-4492                 |                                  |
| Scalpel blade                            | Integra Miltex              | 297                     |                                  |
| Scalpel handle                           | World Precision Instruments | 500236                  | #3, 13cm                         |
| Sterile suture                           | Fine Science Tools          | 18020-50                | Braided Silk, 5/0                |
| Surgical clip applicator                 | Fine Science Tools          | 12020-09                |                                  |
| Surgical clip remover                    | Fine Science Tools          | 12023-00                |                                  |
| Surgical drapes                          | Medline Unidrape            | VET3000                 |                                  |
| Surgical gloves                          | Ansell Perry Inc            | 5785004                 |                                  |

|                      |                    |                 |         |
|----------------------|--------------------|-----------------|---------|
| Surigical clips      | Fine Science Tools | 12022-09        |         |
| Thermometer (rectal) | YSI                | Precision 4000A |         |
| Thermometer (water)  | Fisher Scientific  | 14-648-26       |         |
| Umbilical tape       | Grafco             | 3031            | Sterile |
| Water bath           | Thermo Scientific  | TSCOL19         | 19L     |



This piece of the submission is being sent via mail.

**UW Medicine**  

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**DEPARTMENT OF PEDIATRICS**  

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**DIVISION OF NEONATOLOGY**

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Dr. Aaron Berard  
Science Editor, *JoVE*

April 14<sup>th</sup>, 2019

Dear Dr. Berard,

Please find attached our revised manuscript entitled “**A Ferret Model of Inflammation-Sensitized Late Preterm Hypoxic-Ischemic Brain Injury**”.

We thank the editorial staff and reviewers for their time and input, and have revised the manuscript accordingly. A number of critical components to reproducing the model were rightly requested by the reviewers, and these have been added to the protocol and discussion. Comments are repeated in the following pages, along with responses and details of associated edits made to the text of the manuscript. All authors have approved the version submitted.

Yours sincerely,

A handwritten signature in blue ink, appearing to read 'Thomas Wood', with a long, sweeping underline.

Thomas Wood BM BCh, PhD  
Research Assistant Professor, Division of Neonatology, University of Washington

## **Editorial Comments:**

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
- Please print and sign the appropriate Author License Agreement, then scan and upload it with your manuscript files.
- **Protocol Language:** *Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please edit sections 13 and 14 as much as possible to use complete sentences in the imperative voice. Section 14 should be re-written to have a few steps under it.*

**Authors’ response:** The protocol has been edited to minimize the use of notes, combine short steps, and ensure all steps are written in the imperative tense. Sections 13 and 14 have also been re-written in the imperative.

- **Protocol Detail:**

- 1) 12.1: unclear how and when the brain was excised.

**Authors’ response:** More details have been added to steps 11.3 and 12.1.

- **Protocol Highlight:** *Please edit the highlighting to make it <2.75 pages.*
  - 1) Will animals be available for filming?
  - 2) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
  - 3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
  - 4) Notes cannot be filmed and should be excluded from highlighting.
  - 5) Please do not highlight anesthetization as it will not be filmed.

**Authors’ response:**

- 1) Animals will be made available for filming.
- 2) This has been done.
- 3) The highlighted region is <2.75 pages, though these are non-continuous in the protocol. It includes the main experimental methods involved in the injury protocol (minus anesthesia), followed by gross brain measurements after sacrifice to determine gross pathology. This is the best combination of approaches that can all be filmed in a single day, as each of the behavioural tests requires differing periods of survival.
- 4) and 5) These are no longer highlighted.

- **Results:** *Please limit/avoid the subheadings within this section.*

**Authors’ response:** Subheadings have been removed from the results.

- **Discussion:** *JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to*

existing methods, 4) future applications and 5) critical steps within the protocol.

**Authors' response:** The discussion has been expanded slightly to address reviewer comments. We believe we have done this in accordance with the guidance above.

- **Figure/Table Legends:** *Mention statistical test used and sample size. Define all error bars.*

**Authors' response:** This has been done.

- **References:** *Please spell out journal names.*

**Authors' response:** The journals appeared in the way that automatically resulted from the JoVE EndNote style, but this has been edited.

- **Table of Materials:** *Please include animal strain.*

**Authors' response:** Ferrets are outbred with no specific strain. This has been added to the Table of Materials.

- *Please define all abbreviations at first use (e.g. LPS)*

**Authors' response:** This has been done.

### Comments from Peer-Reviewers:

#### Reviewer #1:

*Manuscript Summary:*

*In this study, the authors established a ferret model of inflammation-sensitized late preterm hypoxic-ischemic brain injury to mimic infants with the neurological sequela of prematurity. The objective of this study is clear and the results are interesting. However, there are some concerns:*

*Major Concerns:*

*The authors stated "intermittent iatrogenic hyperoxia is commonplace in the neonatal intensive care unit (NICU). These combined factors are thought to contribute to developmental and physiologic vulnerability of the brain, and result in or exacerbate the encephalopathy associated with poor developmental outcomes in preterm infants". As we known, chorioamnionitis and perinatal insults such as hypoxia-ischemia are the major causes of neonatal brain injury. Moreover, intermittent iatrogenic hyperoxia is not commonly used in NICU in many countries. Most of animal models of neonatal brain insult were conducted using inflammation and/or ischemia-hypoxia methods, but combining with hyperoxia process wasn't very common. I wondered whether only inflammation and/or ischemia-hypoxia alone are enough to perform this model.*

**Authors' response:** We agree with the reviewer that the background information could be improved, and we have changed the first paragraph of the introduction, and added a sentence to the discussion to discuss processes of injury more specifically than the exposures themselves. Hyperoxia was introduced due to the fact that oxidative stress is believed to be central to both susceptibility of and injury to the preterm brain. This was added as part of an iterative model development process that has been described in a previous publication (Wood *et al.*, Developmental

Neuroscience, 2019).

*At the step of sequential hypoxia, hyperoxia, and hypoxia, why the hypoxia process was divided into two period? Furthermore, the authors said "If intra-hypoxic mortality is seen, the second hypoxia should be terminated early". Was there a criterion to decide when to terminate hypoxia? It would be better to specify it.*

**Authors' response:** As mentioned above, the second hypoxia period was added as part of a previous iterative model development process – a single hypoxia period resulted either in death or survival with no significant injury. As ferrets are able to tolerate long periods of acute hypoxia or bilateral carotid artery ligation without showing significant brain injury, our current hypothesis is that the period of hyperoxia results in elevated metabolism and vasodilatation that facilitates brain ischemia during the second hypoxia period. These details have been added to the discussion.

*Behavioral test data is presented only from surviving animals with visible gross injury. Was there no difference between injured animals without visible gross injury and controls? Or the behavioral assessments were not conducted in animals without visible gross injury?*

**Authors' response:** This is an important point. We initially focussed on the animals with gross visible injury because these animals are part of a therapeutic study that will be published separately. However, we appreciate that this then raises multiple questions about behavioural testing in animals without visible gross injury. All injured animals display behavioural deficits, therefore we have expanded the results and associated figures to include all injured animals.

*What is the standard of the behavioral testing of ferrets?*

**Authors' response:** The behavioral tests described were largely developed within the laboratory, with some basis in reflex tests previously described in the developing ferret, with catwalk and open field tests adapted from adult rodents to be used in juvenile ferrets. Other groups have also described open field, maze, and gait testing in adult ferrets after traumatic brain injury, as well as the effect of in utero inflammation on social interaction in adult ferrets. This information has been added to the discussion.

*Minor Concerns:*

*P17 kits considered to be equivalent to an infant of 32-36 weeks' gestation. I think it would be better to delete "and term infants" at the part of short abstract.*

**Authors' response:** That phrasing has been removed.

*At the step of sequential hypoxia, hyperoxia, and hypoxia, animals were placed in the chamber before the concentration of oxygen reached 9% or 80%. Considering that the size of chambers or equipments used for modeling may be varied among different labs, thus the time cost in adjusting the concentration of oxygen from normal oxygen to 9% or 9% to 80% varied. It might be more rigorous if a predetermined break time was suggested, or putting the animals in the chamber after the aiming concentration of oxygen has reached.*

**Authors' response:** With the equipment commonly used to expose neonatal animals to hypoxia, placing the animals in the chamber *after* it has reached the

required oxygen concentration is not possible. However, we do appreciate that this is an important factor, and have added a sentence in the first step saying that the required flow rate should be calculated based on chamber size to ensure that the target oxygen concentration is achieved in 8-10 minutes, which is what we saw with our equipment.

*It seems that the damages in both hemispheres were the same in figure 1. Did the damage in left hemispheres was equal to the right?*

**Authors' response:** No difference was seen in injury between the right and left hemispheres. A sentence has been added to the results to say this explicitly.

*In recent years, mild to moderate brain injury is more common than severe brain injury in neonates. It is important to test putative neuroprotective therapies for brain injury in neonates with mild and moderate brain injury. In this protocol, animals without significant gross injury may have mild to moderate injury which can be detected using MRI or histopathology. Verifying the degree of brain damage is suggested in the further study.*

*Gender-related difference in vulnerability to brain injury exists in humans. Is this difference investigated in ferrets?*

**Authors' response:** We completely agree with the reviewer with regards to injury severity, assessment with MRI, and sex differences in outcomes. We have previously published sex-based differences in neuropathology in the ferret (Wood *et al.*, Developmental Neuroscience, 2019), and all of those procedures will be included in upcoming therapeutic studies using the model. This has been added to the final sentence of the discussion.

## **Reviewer #2:**

### *Manuscript Summary:*

*This article described a ferret model of LPS-sensitized hypoxic-ischemic brain injury at late preterm (P17) that is relevant to study and therapy of human encephalopathy of prematurity caused by maternal infection combined with hypoxia-ischemia. The description is clear and the text provided enough detailed protocol for other scientists to follow.*

### *Major Concerns:*

*1. It would be helpful if the authors provide a timeline of the experimental procedure starting from P17 LPS injection to the ages of various behavioral tests till animal sacrifice at the end.*

**Authors' response:** This is an excellent suggestion. A new Figure 1 has been added with a timeline for both the insult and follow-up period.

*2. In discussion, the authors stated that "each litter of animals should be exposed to hypoxia within their own chamber", what is the average litter size of ferret and how many animals were placed in one same chamber during the sequential hypoxia-hyperoxia-hypoxia period? This is important because if too many animals were placed in one chamber, some of them might be overheated and die during hypoxia or hyperoxia, and also increase variability of injury.*

**Authors' response:** Ferrets are born in litters of 4-12; however, we purchased sex-balanced litters of 8 to minimize variability, as suggested. The following has been added to the discussion: "To minimize variability in the model, we used pre-ordered sex-balanced litters of 8 ferrets that arrived in our facility on P15. In each litter, 6-7 animals underwent surgery followed by hypoxia within a single chamber."

*3. Are there any changes in the height, length, width and weight of the brains following the procedure as the authors made these measurements in 12.2-12.5?*

**Authors' response:** Yes, significant differences are seen in all of these parameters. The following has been added to the results: "Median (IQR) brain weight was 8.1g (7.9-9.7g, n=6) in control animals, and 7.0g (6.5-7.7g) in injured animals (n=26, p=0.005). In control animals, median (IQR) brain length was 28.9mm (27.8-29.6mm, n=6) compared to 27.5mm (25.5-38.0mm, n=26) in injured animals (p=0.007). Similar patterns are seen across the brain, with median width and height 5-7% smaller in injured animals."

*4. In 13.1, according to the text, the width of the longitudinal fissure was measured and used for the gross injury scoring, while in Fig. 2D legend, it appears that the length of the longitudinal fissure was presented (shortening of the longitudinal fissure, also on page 12, second paragraph), please clarify.*

**Authors' response:** This is correct. The width of the longitudinal fissure is visually examined in the pathology score, and the length is measured and presented separately as a quantitative test. Step 13 and associated sub-steps have been re-written for clarity.

*5. It seems that reversal of right carotid ligation did not make any differences between left and right hemisphere in regards to the gross injury (Figure 2B, 2C, 2E, 2F), but maybe more tissue loss on the right side (Fig. 1), is this a necessary procedure?*

**Authors' response:** The right carotid artery ligation reversal was initially used in order to increase reperfusion injury; however, the injury is uniformly bilateral. We are unsure whether permanent bilateral carotid artery ligation would be feasible from a long-term survival standpoint, but this is planned for future iterations of the model. A sentence has been added to the discussion saying this.

Minor Concerns:

Page 9: under 11. Fixation-perfusion, the sub-steps should be changed to 11.1-11.10, accordingly, in 13.1, first line, change 10.9 to 11.9 and brain measurements from step 12

**Authors' response:** These have been adjusted based on the revised steps.

### **Reviewer #3:**

*Manuscript Summary:*

*In this study, the authors established a ferret model of inflammation-sensitized late preterm hypoxic-ischemic (HI) brain injury. Postnatal day 17 ferrets underwent lipopolysaccharide administration followed by bilateral cerebral ischemia, hypoxia and hyperoxia. Injured animals displayed a range of gross injury severity, reflex development, slower and more variable speed of locomotion on the catwalk and*

*decreased exploration in open field tests.*

*This manuscript describes a novel ferret model which provides a platform to test putative therapies for infants with neonatal encephalopathy associated with inflammation and HI and to study mechanisms of injury that affect neocortical development. I only have a couple of comments that will only require minor revisions by the authors.*

*Major Concerns:*  
*None.*

*Minor Concerns:*

*1. In the Protocol, the authors mention a procedure that combines hypoxia, hyperoxia and then hypoxia again. Please provide the rationale for including the hyperoxia and second hypoxia exposure. Is this a clinically relevant and if so, when?*

**Authors' response:** We agree that these are important questions regarding model development and clinical relevance. As with any preclinical model, the goal was not to accurately reproduce the exposures encountered by preterm infants clinically, but to provide a confluence of the mechanistic factors thought to be involved in premature brain injury. These include inflammation, hypoxia, and oxidative stress. Hyperoxia was introduced due to the fact that oxidative stress is believed to be central to both susceptibility of and injury to the preterm brain. This was added as part of an iterative model development process that has been described in a previous publication (Wood *et al.*, Developmental Neuroscience, 2019). The second hypoxia period was also added as part of a previous iterative model development process – a single hypoxia period resulted either in death or survival with no significant injury. As ferrets are able to tolerate long periods of acute hypoxia or bilateral carotid artery ligation without showing significant brain injury, our current hypothesis is that the period of hyperoxia results in elevated metabolism and vasodilatation that facilitates brain ischemia during the second hypoxia period. These details have been added to the discussion.

*2. The second hypoxia was terminated early if intra-hypoxic mortality was seen. Please describe more objective measures that other users can implement to establish when to terminate the hypoxia, for example, measures of heart rate, blood oxygenation, carbon dioxide levels, cerebral blood flow, etc. Also, please indicate whether resuscitation was performed.*

**Authors' response:** Monitoring in the chamber is performed visually, with particular attention paid to respiratory rate. If any intra-hypoxic mortality is seen (as suggested by respiratory arrest), the second hypoxia period is terminated. These details have been added to step 5.4.. Previous attempts at resuscitation (oxygen, chest compressions) have not been successful in the ferret model, so they are not routinely performed.

*3. In the Results where the behavioral assessments were described the authors only included the animals with visible gross injury in the injured group. It would be useful to provide a strong explanation for excluding those animals without visible gross injury and to perhaps provide the results that would be obtained were those animals not excluded.*



**Authors' response:** This is an important point. We initially focussed on the animals with gross visible injury because these animals are part of a therapeutic study that will be published separately. However, we appreciate that this then raises multiple questions about behavioural testing in animals without visible gross injury. All injured animals display behavioural deficits, therefore we have expanded the results and associated figures to include all injured animals.