



## Effect of pre-cardiac and adult stages of *Dirofilaria immitis* in pulmonary disease of cats: CBC, bronchial lavage cytology, serology, radiographs, CT images, bronchial reactivity, and histopathology



A. Ray Dillon<sup>a,\*</sup>, D.M. Tillson<sup>a</sup>, A. Wooldridge<sup>a</sup>, R. Cattley<sup>a</sup>, J. Hathcock<sup>a</sup>, W.R. Brawner<sup>a</sup>, R. Cole<sup>a</sup>, B. Welles<sup>a</sup>, P.W. Christopherson<sup>a</sup>, T. Lee-Fowler<sup>a</sup>, S. Bordelon<sup>a</sup>, S. Barney<sup>a</sup>, M. Sermersheim<sup>a</sup>, R. Garbarino<sup>a</sup>, S.Z. Wells<sup>a</sup>, E.B. Diffie<sup>a</sup>, E.R. Schachner<sup>b</sup>

<sup>a</sup> College of Veterinary Medicine, Auburn University, AL, United States

<sup>b</sup> College of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, United States

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### ABSTRACT

A controlled, blind study was conducted to define the initial inflammatory response and lung damage associated with the death of precardiac stages of *Dirofilaria immitis* in cats as compared to adult heartworm infections and normal cats. Three groups of six cats each were used: UU: uninfected untreated controls; PreS I: infected with 100 *D. immitis* L3 by subcutaneous injection and treated topically with selamectin 32 and 2 days pre-infection and once monthly for 8 months); IU: infected with 100 *D. immitis* L3 and left untreated. Peripheral blood, serum, bronchial lavage, and thoracic radiographic images were collected from all cats on Days 0, 70, 110, 168, and 240. CT images were acquired on Days 0, 110, and 240. Cats were euthanized, and necropsies were conducted on Day 240 to determine the presence of heartworms. Bronchial rings were collected for in vitro reactivity. Lung, heart, brain, kidney, and liver tissues were collected for histopathology. Results were compared for changes within each group. Pearson and Spearman correlations were performed for association between histologic, radiographic, serologic, hematologic and bronchoalveolar lavage (BAL) results. Infected cats treated with selamectin did not develop radiographically evident changes throughout the study, were heartworm antibody negative, and were free of adult heartworms and worm fragments at necropsy. Histologic lung scores and CT analysis were not significantly different between PreS I cats and UU controls. Subtle alveolar myofibrosis was noted in isolated areas of several PreS I cats and an eosinophilic BAL cytology was noted on Days 75 and 120. Bronchial ring reactivity was blunted in IU cats but was normal in PreS I and UU cats. The IU cats became antibody positive, and five cats developed adult heartworms. All cats with heartworms were antigen positive at one time point; but one cat was antibody positive, antigen negative, with viable adult females at necropsy. The

**Abbreviations:** BAL, bronchial alveolar lavage; CBC, complete blood count; HARD, heartworm associated respiratory disease; PI, post infection; SMA, smooth muscle actin.

\* Corresponding author at: Department of Clinical Sciences, College of Veterinary Medicine, Auburn University, AL 36830, United States. Tel.: +1 334 844 4690.

E-mail address: [dilloar@auburn.edu](mailto:dilloar@auburn.edu) (A. Ray Dillon).

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CT revealed early involvement of all pulmonary arteries and a random pattern of parenchymal disease with severe lesions immediately adjacent to normal areas. Analysis of CT 3D reconstruction and Hounsfield units demonstrated lung disease consistent with restrictive pulmonary fibrosis with an interstitial infiltrate, absence of air trapping, and decrease in total lung volume in Group IU as compared to Groups UU and PreS I. The clinical implications of this study are that cats pretreated with selamectin 1 month before *D. immitis* L3 infection did not become serologically positive and did not develop pulmonary arterial hypertrophy and myofibrosis.

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## 1. Introduction

By common definition, *Dirofilaria immitis* is discussed as having a 6-month life cycle (infection of host through development and sexual maturity) (Riley, 1922; McCall et al., 1992; Atkins et al., 2000; Bowman, 2009). The assumption that clinical disease does not develop until the parasite is a 6-month-old adult is incorrect. The initial arrival of immature adults in the small pulmonary vessels of the lungs in cats is associated with an intense eosinophilic pulmonary reaction, and clinical and radiographic signs may be present in this 3- to 6-month post-infection period (Dillon et al., 2007). This 3-month disease cycle precedes the production of microfilariae and circulating antigen by 2–3 months. Because of the difference in the host immune reaction (Dillon et al., 2008) and higher mortality of the immature worms in cats than dogs, the clinical signs, diagnosis, and effects of prophylaxis are different in cats than in dogs with heartworm infection (Lee and Atkins, 2010).

After a mosquito acquires the microfilariae, adequate exposure to warm temperatures is required for larval development. The infective larvae are deposited on the skin of an animal when the mosquito feeds again, and the L3 enters through the bite wound. The L3 stages molt to L4 and L5 (immature adults) and migrate to the pulmonary arteries arriving as immature adults (1–2 cm in length) approximately 70–90 days after infection in both dogs and cats.

Preventative medications are administered to prevent the adult infection by affecting the L4 stage of development (Campbell, 1987, 1989). The administration of selamectin beginning 1 month after L3 infection in cats has been shown to prevent mature adult heartworms, but resulted in positive heartworm antibody titers being present for several months after infection (Blagburn and Dillon, 2007; Dillon et al., 2007). However, the length of time the larvae may live and migration before death and the destination of larval product is unclear regardless of when preventative treatment is initiated. The goal of this study was to determine the response of the feline lung to early death of heartworm larvae in cats 8 months after infection. The hypothesis was that early death of immature larvae induced by pretreatment with preventative medication compared to responses in uninfected controls and infected untreated cats would not result in lung disease in cats, alter clinical diagnostic modalities, or alter bronchial ring reactivity. Many practicing veterinarians assume that when cats develop young heartworms in the pulmonary arteries and lungs, most cats will “self-cure” and the worms will die without any clinical consequence to the cat (Lee and Atkins, 2010). Research

has demonstrated that the early immature adult infection is associated with an intense inflammatory response (Dillon et al., 2007; Dillon, 2008; Holmes et al., 1992; Selcer et al., 2005). In addition, clinical experience and a prospective clinical study of cats with suspected heartworm disease (Dillon et al., 2000) has indicated that some cats will get the early infection, the worm(s) will die, but the cat will continue to develop chronic inflammatory lung disease (Robertson-Plouch et al., 2000), and lung pathology continue to be present after the infection (Browne et al., 2005).

## 2. Materials and methods

### 2.1. Experimental design

To determine the initial inflammatory response associated with pre-cardiac stages of heartworms, three groups of six age-matched, specific pathogen free, spayed female cats, 6 months old were used as uninfected untreated (UU), pretreated with selamectin-infected (PreS I), and infected untreated (IU) subjects. Group UU cats served as controls for the absence of any stages of heartworms. Cats in Groups PreS I and IU were infected with 100 *D. immitis* L3 (Duke strain) by subcutaneous injection into the flank.

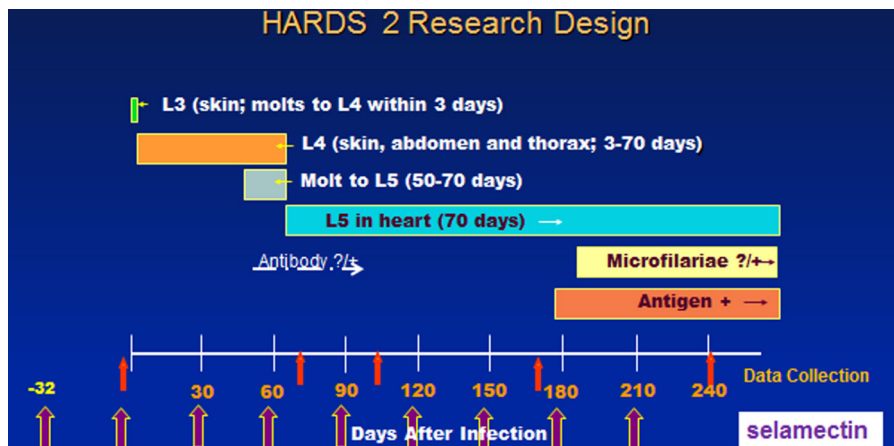
Cats in the PreS I group were treated topically with selamectin (Revolution<sup>®</sup>; Zoetis, Florham Park, NJ, USA) at the dosage based on body weight as indicated on the label 32 days and 2 days before the infection, and then once per month to kill immature larvae before they reach the heart (Fig. 1). Group IU cats were infected with L3 and left untreated; thus allowing larvae to mature into adult heartworms.

Cats were observed for a period of 8 months post infection (PI) and monitored daily. Physical examinations were performed weekly.

All groups of cats were housed as isolated groups in the indoor animal rooms of the Laboratory Animal Health Veterinary Research Building to prevent exposure to mosquitoes that could be carrying heartworm larvae. The protocol was approved by the Auburn University Institutional Animal Care and Use Committee and in an AAALAC accredited facility in an environmentally isolated facility.

### 2.2. Complete blood count, BAL cytology, and serum serology

Before infection (Day 0), and on Days 70, 110, 168, and 240 PI, peripheral blood for complete blood count (CBC)



**Fig. 1.** Study design. Design of *D. immitis* infection study in cats. The anticipated larval (L3, L4, L5) stages are noted on a time line of days after the L3 infection. Data collection points are red arrows. Selamectin administration times are brown arrows. Expected times for Antibody response, microfilaria production and antigen detection are above the time line.

and serum for serology was collected; blood samples were submitted for routine analysis.

Serologic evaluation was performed on batched frozen serum (Antech Diagnostics, Irvine, CA) with DiroCHECK® (Symbiotics Co, Kansas City, KS; currently Zoetis) for heartworm antigen with ELISA microwell titer and ELISA for heartworm antibodies.

On Days 0, 110, 168, and 240 PI, bronchial alveolar lavage (BAL) with 10 ml of lactated Ringers solution was performed, and radiographic ventrodorsal and lateral thoracic images were acquired under before BAL sedation with an intramuscular dose of medetomidine (Dormitor®, Pfizer Animal Health, NY), butorphanol (Torbugesic®, Fort Dodge, IA), and ketamine (ketamine hydrochloride, Abbott Lab, Chicago, IL). BAL fluid was concentrated, and the cytology data determined at the Clinical Pathology Laboratory, Auburn University, College of Veterinary Medicine were recorded as subjective descriptive narrative, and cellular morphology was expressed as the percentage of cell types relative to the total nucleated cells observed.

### 2.3. Digital radiology

Thoracic radiographs were obtained on Days 0, 110, 168, and 240 and were graded for pulmonary artery size, tortuosity, blunting, bronchial pattern, interstitial pattern, and mixed bronchio-interstitial-alveolar pattern using a 0–3 system (0 = normal and 3 = severe lesions) (Brawner et al., 2000; Dillon et al., 2013). In addition, any abnormalities in cardiac, pleural, or diaphragm tissues were recorded. The observer, a board certified radiologist, was blinded to study groups. Following the BAL and radiograph procedures an intramuscular dose of atipamizole (Antisedan®, Pfizer Animal Health, NY) was administered to reverse the effects of sedation.

### 2.4. CT acquired images

On Days 0, 110, 168, and 240, pulmonary computed tomography (CT) images were acquired with a helical CT

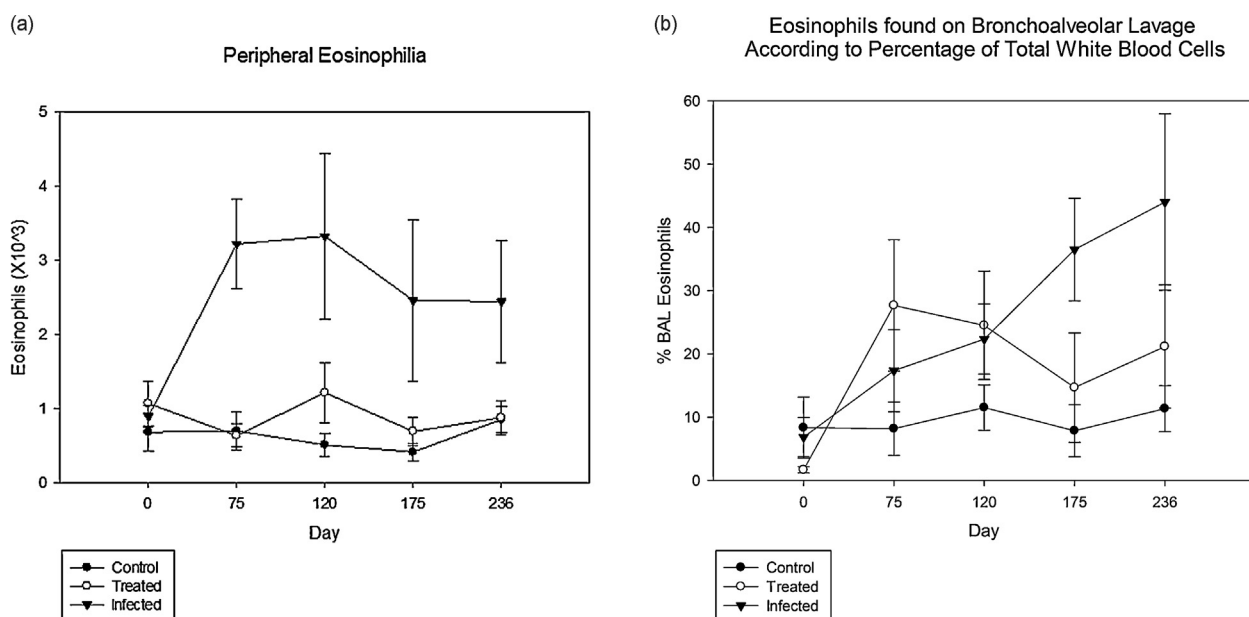
scanner (General Electric Co., Milwaukee, WI). Examinations were performed with cats in sternal recumbency under general isoflurane (0.5–0.75%) inhalational anesthesia (Isoflurane, USP, Piramal Healthcare, Ltd., Andhra Pradesh, India) using a breath-hold technique (airway pressure maintained at 14 cm H<sub>2</sub>O) (Reid et al., 2012). Images of the entire lung field were obtained, consisting of contiguous 5-mm collimated transverse images and thin-slice axial scans (1-mm collimated transverse images) spaced 10 mm apart. A non-iodinated IV contrast (Isovue-370 Iopamidol Injection 76%, Bracco Diagnostics Inc., Princeton, NJ) at a dose of 0.5 ml/lb was administered before contrast image acquisition. Transverse sequential CT images of the lung (1 mm) were acquired and reconstructed using a detail and bone algorithm. The CT scans were graded as previously described (0–3 scale) (Dillon et al., 2013) as adapted from human and canine studies (Johnson et al., 2004, 2005). For CT evaluation, images were viewed in a lung window (W 1500, L 600). The grading scale was based on the number of affected lung lobes, degree of increased opacity, and the location of the region of the lung.

#### 2.4.1. Lung density by Hounsfield units

The DICOM images of the CT scans were imported into the 3D Slicer software ([www.slicer.org](http://www.slicer.org), version 2.8). Density mask analysis of the lung parenchyma was performed based on the contiguous 1-mm thin-section images. In the software, automatic extraction of the lung is achieved by performing threshold holding using the Otsu Method (Otsu, 1979). The CT slices were exported into the COPD-Emphysema module, and lung density histograms were obtained ranging from 300 to –1000 in Hounsfield units (HU) (Dillon et al., 2013). Distribution curves were generated for each and the frequencies of HU at –600, –856, –910, –925, and –950 were calculated.

#### 2.4.2. Lung volume and colorization of CT reconstructions

Total lung volume was acquired at fixed inspiratory pressure of 14 cm H<sub>2</sub>O and volume calculated via 3D Slicer software ([www.slicer.org](http://www.slicer.org), version 2.8). For color



**Fig. 2.** (A) Peripheral eosinophil counts for groups of cats, including uninfected controls, *Dirofilaria immitis* infected cats pretreated with selamectin, and infected untreated cats. (B) Percentage of eosinophils in bronchoalveolar lavage. A. Absolute eosinophil count as mean  $\pm$  SD error bars at each sampling day after infection. The Infected cats (IU) were significantly different ( $p < 0.05$ ) that both Pre-treated selamectin cats and control cats on Days 75, 120, 175, and 236. B. Percent eosinophils on BAL expressed as percent of total nucleated cells. Treated cats were significantly different ( $p < 0.05$ ) from control cats on Day 75 and day 120. Infected cats were significantly different from both treated and control groups on Days 175 and day 236.

illustration of lung lesions, each structure of the anatomy was mapped with manual identification of discrete areas of lung lesions on individual CT slices and Avizo 7.0 software. Three-dimensional reconstructions were rendered as has been described (Schachner et al., 2013, 2014).

## 2.5. Echocardiogram

Echocardiograms were performed by a board-certified cardiologist on cats in the PreS I and IU groups on Days 0, 110, 168, and 240. Images were acquired with a Philips S12-4 probe on a Philips iE33 ultrasound system (Philips Healthcare, Andover, MA).

## 2.6. Necropsy

On Day 245, cats were humanely euthanized under sedation, using pentobarbital sodium and phenytoin sodium solution (Euthasol<sup>®</sup>, Virbac AH, Fort Worth, TX) 1 ml/10 lb intra-peritoneally, and complete necropsies were conducted with collection of lung, heart, kidney, and liver for histopathology studies. Right caudal lung lobes were fixed by perfusion with 10% formalin via the bronchi to a pressure of 14 cm H<sub>2</sub>O. Pathologists and radiologists were blinded to the treatment groups to which cats were assigned, creating a controlled, blind study format.

## 2.7. In vitro bronchial ring reactivity

From the left caudal lung lobe, bronchial rings were collected for bronchial ring reactivity via in vitro challenge. Isometric force of epithelium-intact third- to fourth-generation intra-parenchymal bronchioles (IPB) (internal

diameter, 300–500  $\mu$ m) from IU and UU cats was performed as described (Wooldridge et al., 2012). Force data were recorded digitally (PowerLab, ADInstruments, Colorado Springs, CO). Rings were equilibrated for 30 min with  $<1$  mN resting tension and then contracted with 80 mM KCl-substituted Krebs' solution to determine optimum resting tension. All subsequent experiments were performed at this resting length. Rings were contracted three times with  $10^{-5}$  M acetylcholine (ACh) followed by rinsing until stable contractions were observed. Cumulative concentration-response curves to administration of ACh,  $10^{-9}$  to  $10^{-5}$  M, 5-hydroxytryptamine (5-HT,  $10^{-9}$  to  $10^{-5}$  M), histamine (HIS,  $10^{-9}$  to  $10^{-5}$  M), isoproterenol (ISO,  $10^{-9}$  to  $10^{-5}$  M), substance P (SubP,  $10^{-9}$  to  $10^{-5}$  M), and sodium nitroprusside (SNP,  $10^{-10}$  to  $10^{-5}$  M) were performed. Rings were first contracted by administration of  $10^{-5}$  M 5-HT before the response curves were generated for ISO, SubP, and SNP. At the end of the study, bronchial rings were blotted and weighed and circumference and length were measured under a dissecting microscope.

## 2.8. Histopathology

Multiple sections of fixed-perfused right caudal lung were stained with hematoxylin and eosin and  $\alpha$ -smooth muscle actin (HSRL Inc, Mount Jackson, VA). Sections were scored on a scale of 0–3 individually for interstitium, bronchus, bronchioles, pulmonary arterioles, and major pulmonary artery areas (Browne et al., 2005; Dillon et al., 2013). The right caudal lung at the mid-lobar cross-section, corresponding to a plane and location of CT slice, was used for section for evaluation.

**Table 1**Number of cats in each group<sup>a</sup> within each grade for percentage of eosinophils on bronchiole alveolar lavage fluid cytology.

Grade <sup>b</sup>	Day 0			Day 75			Day 120			Day 168			Day 236		
	UU	PreS I	IU	UU	PreS I	IU	UU	PreS I	IU	UU	PreS I	IU	UU	PreS I	IU
0	6	6	6	5	2	3	5	1	0	6	3	1	5	4	1
1	1	0	0	1	1	3	1	3	5	0	2	1	1	1	1
2	0	0	0	0	1	0	0	2	1	0	1	4	0	0	2
3	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1

<sup>a</sup> Treatment groups: UU, uninfected untreated; PreS I, pretreated with selamectin infected; IU, infected untreated.<sup>b</sup> Grade key: 0 = 0% ≤ 16%; 1 = 17–35%; 2 = 36–60%; 4 = >60

### 2.9. Correlation of diagnostic findings

All statistical analyses of data were performed with Systat Software (Sigma Plot 12, Systat Software Inc, San Jose, CA). Data points across time within each group were evaluated by paired Student's *t*-test for changes between collection dates. Data between groups were evaluated by ANOVA using a Holm–Sidak test. Pearson and Spearman rank correlations were performed to evaluate associations between histologic, CT, radiographic, serologic, hematologic, and BAL results.

Data reported in this paper include results from thoracic radiographs, CBC, serology, BAL cytology, CT findings, bronchial ring reactivity, and lung histopathology.

## 3. Results

### 3.1. Peripheral complete blood count

None of the cats developed any significant changes in neutrophils, monocytes, or lymphocytes during the observation period. Peripheral eosinophilia (>1500/μL, range 1500–5100/μL) was noted in all IU cats on Days 70, 120, 175, and 236 (Fig. 2A).

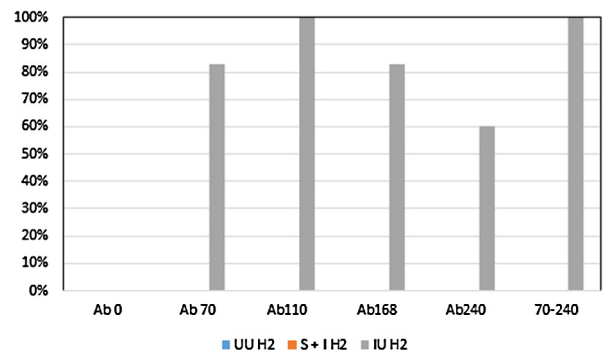
### 3.2. BAL cytology

On Days 75 and 120, PreS I cats had a significant eosinophilic cytology ( $p < 0.05$ ) than UU cats. On Days 175 and 236, eosinophilic cytology for the IU group was significantly different ( $p < 0.05$ ) from the PreS I and the UU group (Fig. 2B, Table 1). The degree of increased BAL eosinophilic cytology was not consistent across time points in individual cats.

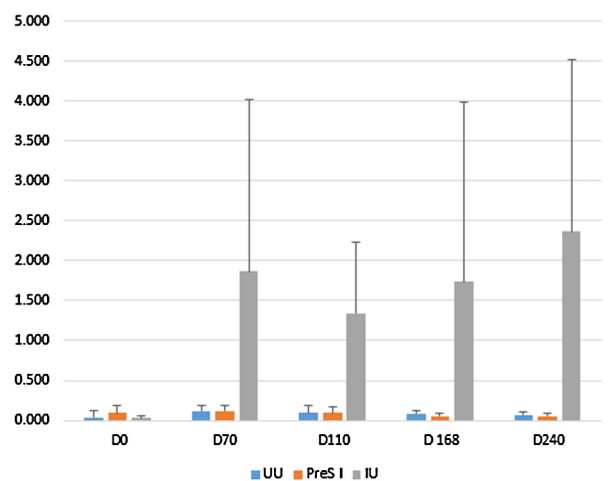
Analysis of the presence of an elevated peripheral eosinophilia (>1500/μL) and cytological BAL eosinophilic response (eosinophils >16%) did not demonstrate a significant correlation (Pearson correlation,  $r = 0.457$ ) within groups or within data from all groups combined. In individual cats, increased eosinophilic cytology (>16%) on BAL cytology was noted with normal peripheral eosinophil counts. Elevated eosinophilic BAL cytology (>60%) was present in cats with modest eosinophilia (<2500/μL), and cats with elevated peripheral eosinophilia (>3000/μL) were associated with 16–35% eosinophilic BAL cytology.

### 3.3. Necropsy and serology

None of the cats in the UU or PreS I groups developed a positive antibody titer at any time (Fig. 3). In the IU group, the heartworm antibody optical density (OD) varied



**Fig. 3.** Percentage of cats positive for *Dirofilaria immitis* antibodies. Treatment groups: UU, uninfected untreated; S+I, pretreated with selamectin infected; IU, infected untreated. D 70–240 represents the percent of cats which were positive at any one time point.



**Fig. 4.** Heartworm antibody optical density. Treatment groups: UU, uninfected untreated; PreS I, pretreated with selamectin infected; IU, infected untreated. The mean OD (+SD) result from each treatment group at Days after L3 infection. For Day 70–240, Group IU was significantly different ( $p = 0.001$ ) at each time point from Group UU and PreS I. Positive titer OD > 0.4 for D 70–240.



**Table 2**

Number of heartworms recovered Day 245 and serologic results from infected untreated (IU) cats on Day 240.

Cat ID	No. of heartworms (F, M)	Degenerated fragments	Heartworm antibodies	Heartworm antigen
16532	0	0	Neg	Neg
16533 <sup>a</sup>	13 (9F, 4M)	2	Neg	Pos
16534	3 (1F, 2M)	0	Pos	Neg
16535 <sup>b</sup>	1 dead (M)	3	Pos	Neg
16536	1 (F)	1	Pos	Pos
16537 <sup>c</sup>	8 (4F,4M)	11	Pos	Pos

<sup>a</sup> Cat 16533 was also antibody negative Day 168.

<sup>b</sup> Cat 16535 died Day 168; serology data is shown for that day.

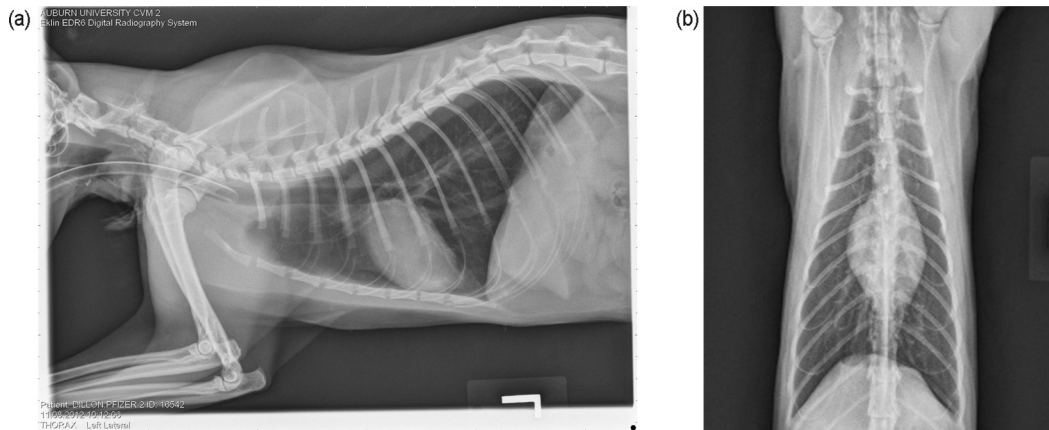
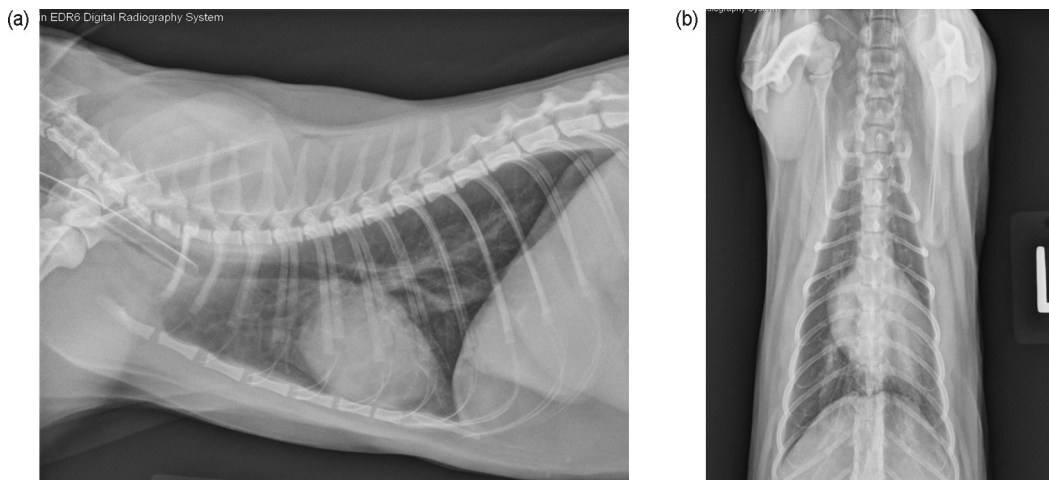
<sup>c</sup> Cat 16537 also was antigen positive Days 110 and 168. F, female; M, male.

(OD > 0.4 positive, range 0.40–5.00, with 5.00 as maximum of assay) (Fig. 4). One cat with no heartworms at necropsy was antibody positive on Days 70, 120 and 168. One cat with adult heartworms at necropsy was antigen positive on Days 168 and 240, antibody positive on Days 120 and 168, but was antibody negative on Day 240 (Table 2). Only cats in the IU group were antigen positive.

No live heartworms or heartworm fragments were found in any of the controls (Group UU) or cats pretreated

with selamectin (Group PreS I) at necropsy, and no cat of either group were heartworm-antibody or -antigen positive (Fig. 3).

One cat in Group IU that died of respiratory distress Day 168 had one dead female worm and was antibody positive but antigen negative on that date. Of the remaining five cats in the IU group, one had no heartworms at necropsy, was antigen negative on Day 168, and antibody positive on Days 75, 110, 168 but was negative on Days 0 and 240

**Fig. 5.** Pre-selamectin infected cat on Day 240 with no difference from D 0.**Fig. 6.** (A–C) Enlarged pulmonary arteries and interstitial lung infiltrates evident in IU cat on Day 240.

(Table 2). The other four cats in this group had heartworms at necropsy, and one of these four cats that had 13 heartworms was antibody negative and antigen positive on Day 240. Another cat was antibody positive but antigen negative with one female and two male heartworms on Day 240 (Table 2). All IU cats were antibody positive at some time during the study. The cats were not examined for ectopic heartworm infection.

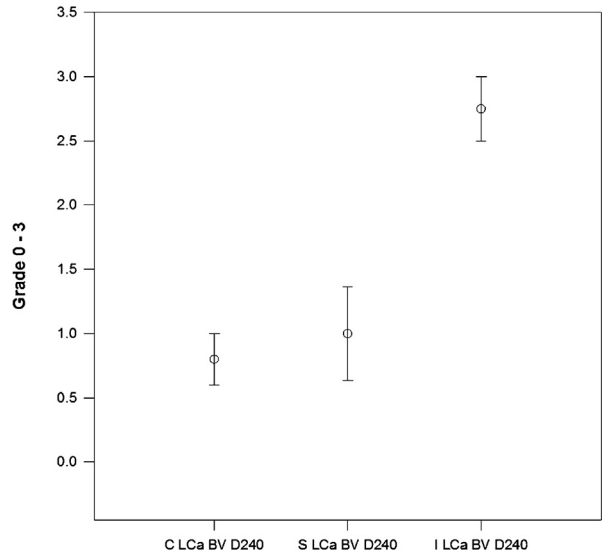
### 3.4. Echocardiography

Adult heartworms were visualized in three of six IU cats on Day 168 and in two of five cats in this group on Day 240. The right ventricle had an increased right ventricular end diastolic volume in two of the five cats on Day 240. Control cats (UU) and cats pretreated with selamectin had normal cardiac echocardiograms.

### 3.5. Radiographs

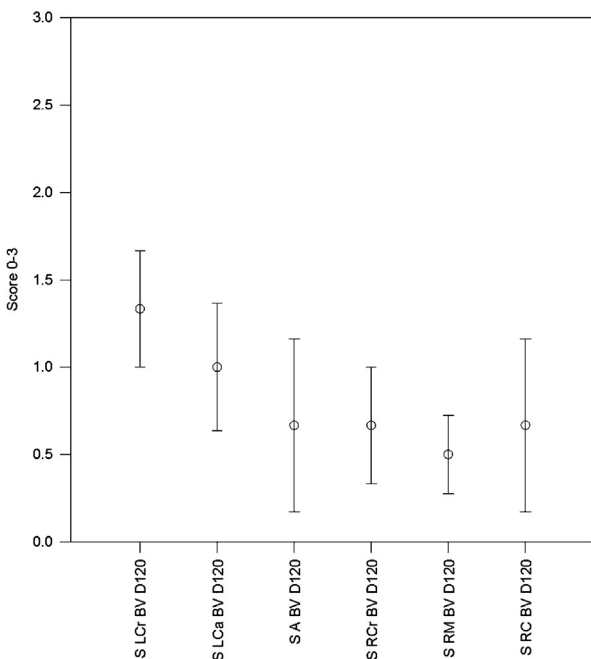
Radiographs were normal on Days 0, 75, 110, 168, and 240 for UU and PreS I cats. Infected untreated cats had radiographic increased interstitial density on Day 110, 168, and 240. Enlarged pulmonary arteries were evident in three of five IU cats on Day 240 (Figs. 5 and 6). The interstitial lung pattern for this group was intense on Day 168 and became more organized into a peribronchial pattern on Day 240.

**D. immitis L3 Infected Cats  
Left Caudal Peri-vascular/bronchial**

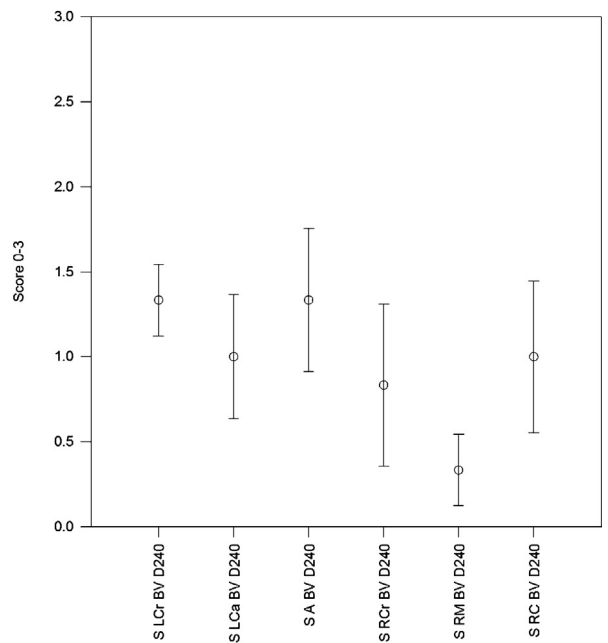


**Fig. 7.** Computed tomography scores for groups of cats on Day 240. Treatment groups: C = UU uninfected untreated, S = preselamectin treated infected, I = infected untreated. I cats are significantly different ( $p = 0.002$ ) than both C and S cats. Lobes of the lung: LCr = Left cranial, LCa = left caudal, A = accessory, RCr = right cranial, RM = right middle, RC = right caudal.

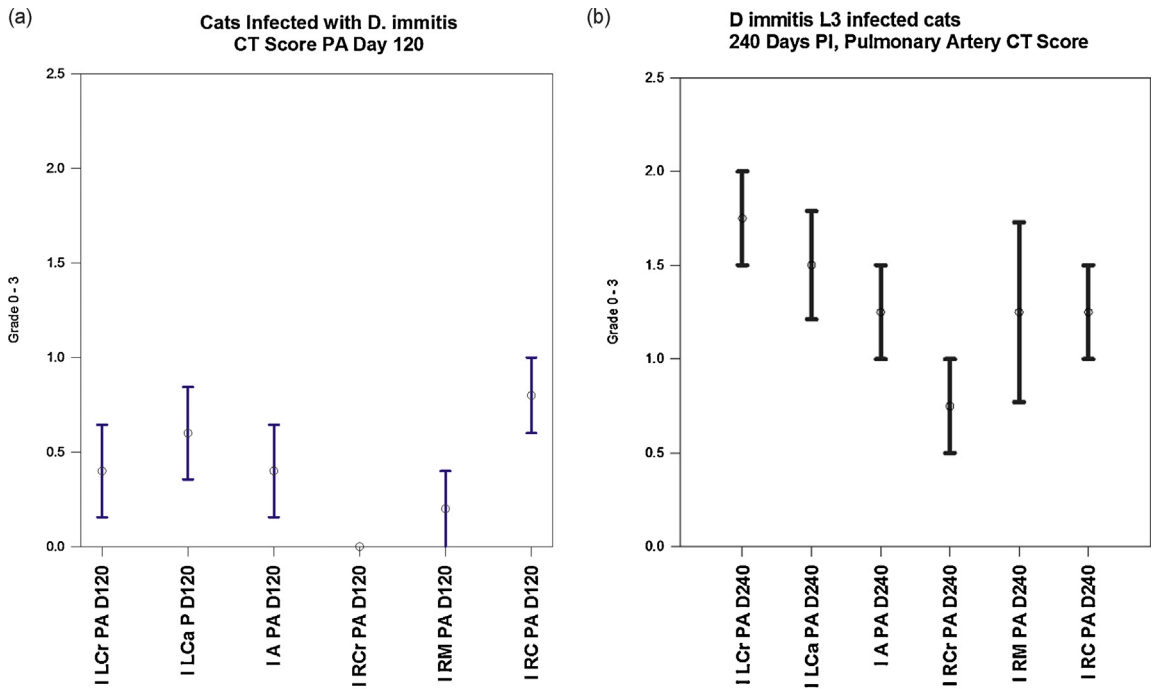
**(a) Cats Infected with Heartworms  
Peri-bronchial/vascular area Day 120 pi**



**(b) Cats with D. immitis Infection  
Peri-bronchial/vascular area**



**Fig. 8.** Computed tomography mean lung scores for *Dirofilaria immitis* infected (untreated) cats on Day 120 (A) and Day 240 (B). Peri-bronchial/vascular increases in hazy density on CT images of cats. Subjective scoring of the midlobe peri-bronchial/vascular areas of the lung in cats. A. Mean score and SE of density in the accessory lung lobe of uninfected untreated controls (C), pre-selamectin treated infected cats (S), and infected untreated cats (I). B. For each graph, Lobes of the lung: LCr = Left cranial, LCa = left caudal, A = accessory, RCr = right cranial, RM = right middle, RC = right caudal. Represents the mean and SD of subtle hazy density in pre-treated selamectin infected cats (S).



**Fig. 9.** Computed tomography mean pulmonary artery scores for *Dirofilaria immitis* infected (untreated) cats on Day 120 (A) and Day 240 (B). For each graph, Lobes of the lung: LCr = Left cranial, LCa = left caudal, A = accessory, RCr = right cranial, RM= right middle, RC= right caudal. The subjective scores reflect mean and SD for infected untreated (I) group. A. Pulmonary arterial scores for each lobe on Day 120. B. Pulmonary arterial scores for each lobe on Day 240.

### 3.6. CT subjective evaluation

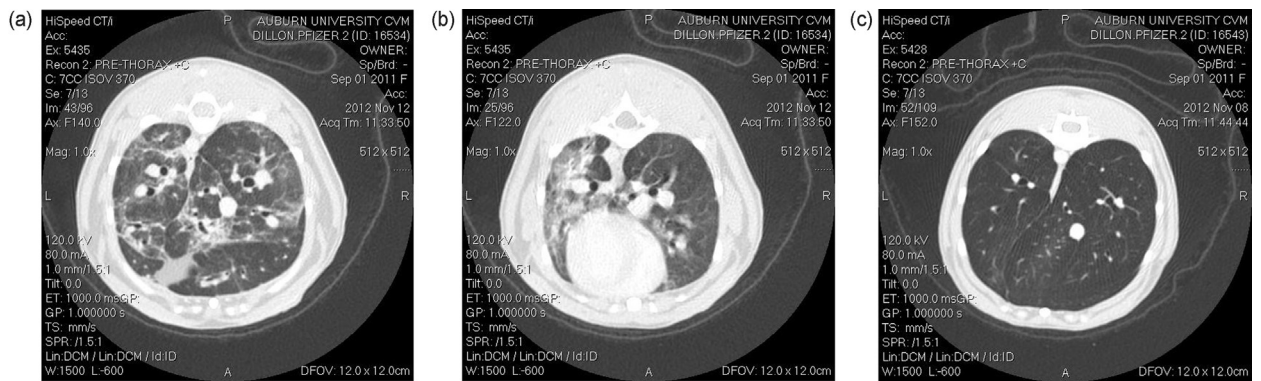
CT images were normal for UU cats during the observation period with scores <1 for hazy opacity in isolated lung lobes. Infected cats pretreated with selamectin had mean total lung scores of 1.8 for increased opacity on Day 120 and 2 on Day 240 (Fig. 7).

Infected untreated cats had mean lung scores of 2 on Day 120 and 3 on Day 240. The location of the increased densities were more noted in the peribronchial/vascular mid-lobe area on Day 120 in these IU cats than on Day 240, when significant increases in pulmonary arterial segments were noted (Figs. 8 and 9). The pulmonary arteries of the left caudal, cranial, and accessory lobes also were

consistently more severely affected than the right caudal, cranial and middle lung lobes in the IU cats. Relatively normal lung slices (5 mm) were observed immediately adjacent to regions of severe disease, and within the same slice, a high degree of variability in abnormal densities was observed (Fig. 10). Color illustrations of degrees of opacity on CT of IU cats were used to demonstrate the diversity of the distribution and variability of opacities, which was not evident on radiographic examination (Fig. 11).

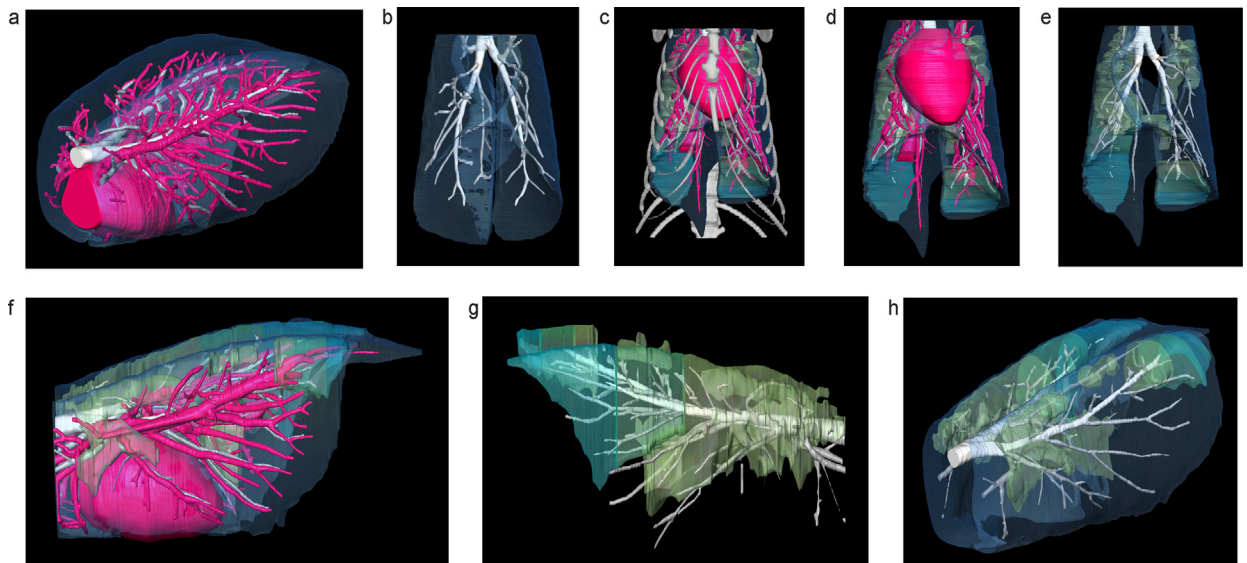
### 3.7. CT evaluation of HU densities and lung volume

Evaluation of the distribution of lung densities at Days 0, 120, and 240 using HU analysis demonstrated that there



**Fig. 10.** (A–C) CT images of IU (infected untreated) and PreS I (pre-selamectin treated infected) Cats on Day 240. A. IU cat just caudal to apex of the heart, area associated with mid-caudal lobe sections for histopathology. Diffuse interstitial infiltrates and pulmonary arterial enlargement in both left and right caudal lobes. B. IU cat cranial to image A, at mid-cardiac area with significantly more severe left lobe disease. C. PreS I cat with normal CT image.





**Fig. 11.** (A–H) Color illustrations showing degrees of opacity in lung slices from infected untreated cat. 3D reconstruction of color segments of CT images in at cat 16537 Infected untreated at Day 0 (A, B) and Day 240 (C–H). White = major airways, Grey = secondary bronchi, Red = cardiovascular, lung area with no color = normal lung, light green = mild densities, darker green = moderate densities, blue = intense densities. A. Left craniolateral view of lungs and cardiovascular Day 0. B. Ventral dorsal view of normal lungs with cardiovascular removed Day 0. C. Ventral dorsal view with skeletal structures included Day 240. D. Ventral dorsal view with bone densities removed Day 240. E. Ventral dorsal view with cardiopulmonary structures removed showing only lung and airways Day 240. F. Right lateral view with cardiopulmonary structures included Day 240. G. Right lateral view with cardiac densities removed Day 240. H. Left craniolateral view of pulmonary structures Day 240. The variety of regional lesion between and within lobes highlight the difficulty in radiography to identify segmental disease and illustrate the unpredictable results of histopathology based on the area sectioned.

was no change in the  $-600$  HU densities associated with increased tissue densities in the UU and PreS I groups but a shift to toward the  $-600$  region was identified both at Day 120 and 240 in the IU group which was significantly different (Fig. 12A) ( $p < 0.01$ ). The total lung volume on Day 210 compared with that on Day 0 in each cat was significantly decreased ( $p = 0.001$ ) in the IU Group compared with that for cats in the UU and PreS I groups, which were not different from each other (Fig. 12B and Fig. 13).

### 3.8. Bronchial ring reactivity

Resting tension was 2.8 mN for all in vitro studies. Responses of IU cats' IPB to three cycles of  $1 \times 10^{-5}$  M ACh were significantly decreased compared with responses for cats in the PreS I and UU groups (Fig. 14). IPB from infected (PreS I), and control (UU) cats had concentration-dependent contractile responses to ACh and to 5-HT, and IU cats had a significantly diminished response to both agonists at the highest concentrations compared with responses by cats in PreS I and UU groups (Fig. 15A and B). Responses to ACh and 5-HT were not different between PreS I and UU control groups. There was no difference between any groups in response to histamine (Fig. 15C).

To evaluate relaxation responses, all tissues were submaximally contracted with the ED<sub>80</sub> dose of  $10^{-5}$  M 5-HT, and then increasing concentrations of relaxation agonists ISO), SubP, and SNP were added to the baths. Responses to ISO demonstrated a typical sigmoidal dose response curve that reached 100% relaxation. Rings from PreS I cats were significantly ( $p < 0.05$ ) more sensitive to ISO than cats in

the IU or UU groups at the lower concentrations (Fig. 15D). Rings from PreS I cats were significantly ( $p < 0.05$ ) less sensitive to SubP-induced relaxation than rings from IU or UU cats (Fig. 15E). There was no difference between any groups in response to SNP (Fig. 15F).

**Table 3**

Comparisons of histopathology scores 8 months after *Dirofilaria immitis* infection.

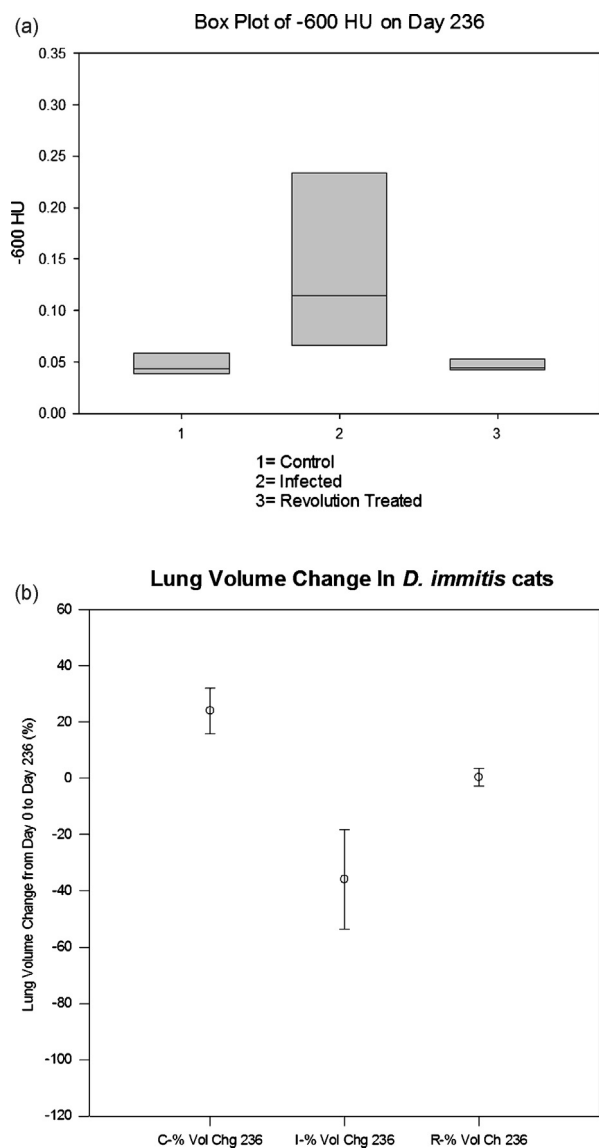
Lung structure	Group <sup>a</sup>	Mean score $\pm$ SD <sup>b</sup>	$p$ -Value <sup>c</sup>
Bronchus	UU	0.00 $\pm$ 0.00	0.001
	PreS I	0.00 $\pm$ 0.00	
	IU	1.83 $\pm$ 1.17	
Bronchiole	UU	0.00 $\pm$ 0.00	0.001
	PreS I	0.00 $\pm$ 0.00	
	IU	1.83 $\pm$ 0.98	
Alveolus/interstitial	UU	0.17 $\pm$ 0.41	0.05 <sup>d</sup>
	PreS I	0.67 $\pm$ 0.52	
	IU	2.33 $\pm$ 0.82	
Arteriole	UU	0.00 $\pm$ 0.00	0.003
	PreS I	0.67 $\pm$ 0.82	
	IU	1.33 $\pm$ 0.52	
Pulmonary artery	UU	0.00 $\pm$ 0.00	0.007
	PreS I	0.33 $\pm$ 0.52	
	IU	1.00 $\pm$ 0.63	

<sup>a</sup> Treatment groups: UU, uninfected untreated; PreS I, pretreated with selamectin infected; IU, infected untreated.

<sup>b</sup> Score (0–3) is from fixed pressure perfused mid-caudal lung lobe.

<sup>c</sup>  $p$  values are for differences (tested by Holm–Sidak method) for IU group compared with UU and PreS I groups.

<sup>d</sup>  $p$  value for comparison with UU group.



**Fig. 12.** Lung density using HU analysis Day 236. A. Box plot of cats demonstrating the statistically significant ( $p > 0.05$ ) in  $-600$  densities in infected untreated cats (2) compared to uninfected untreated control (1) and revolution pretreated infected cats (3). B. Mean and SD of percent change in total lung volume at fixed inflation pressure of 14 cm H<sub>2</sub>O for CT scan at Day 240 compared to each cats Day 0 values. Cats in infected untreated (I) were significantly decreased ( $p < 0.05$ ) compared to either uninfected untreated controls (C) or pretreated selamectin infected cats (R). The C cats had over 20% increase in volume over time and the R cats had no change but the difference was not significant.

### 3.9. Lung histopathology

Cats in the IU group had statistically significant higher scores for bronchus, bronchiole, pulmonary arteries, arterioles, and alveolar/interstitial than cats in PreS I and UU groups ( $p < 0.01$  or  $p < 0.001$ ) (Table 3 and Fig. 16). Randomly distributed areas of subtle (smooth muscle actin-positive) myofibrocytes in the alveolar-interstitial areas (score range 0–1) in four of the six PreS I cats were different than in UU cats. The airways of the PreS I cats

were generally considered normal. Based on specific areas of increased densities noted on CT examination of IU cats, additional histopathologic sections of targeted areas noted more severe focal areas of uneven interstitial myofibrosis.

### 3.10. General necropsy results

Data from histopathology of other organs are not included in this paper. Gross serial sections of the brain did not suggest the presence of aberrant sites of heartworms.

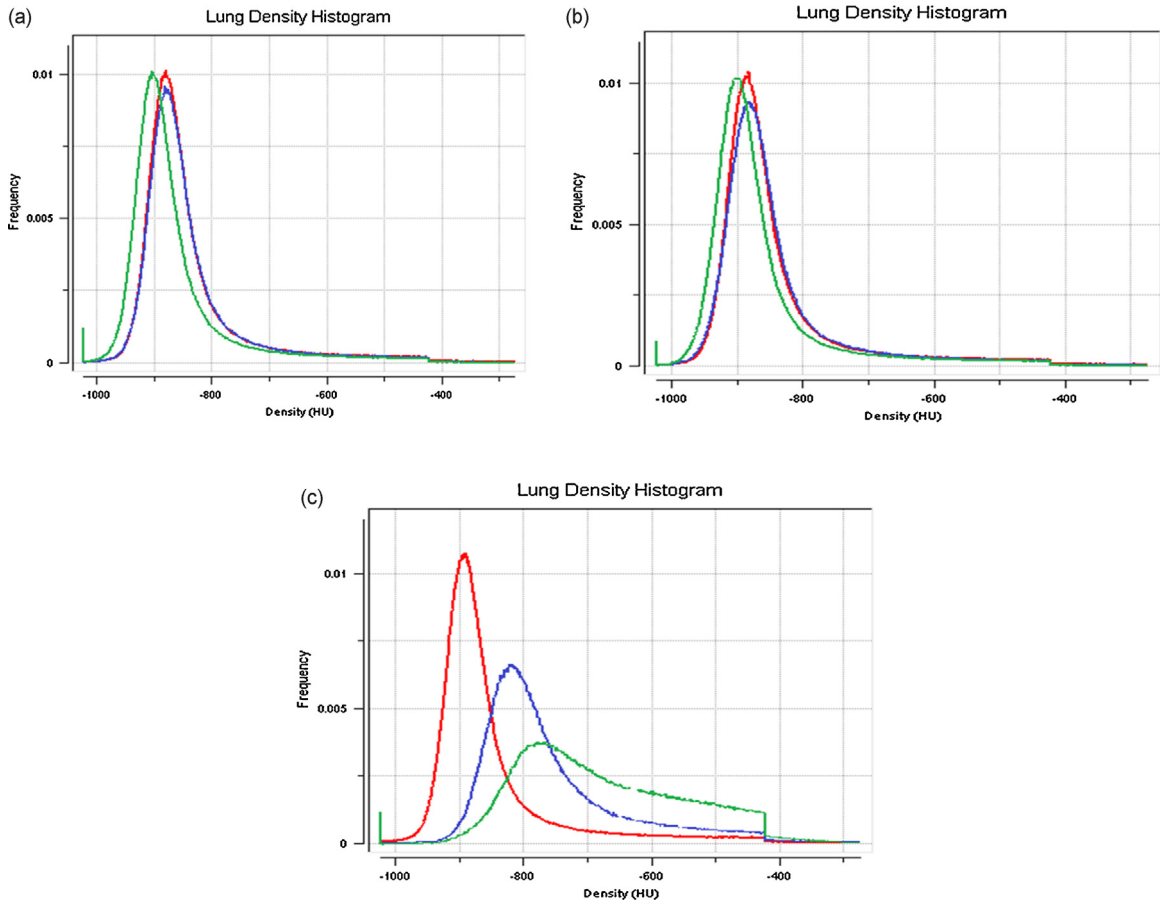
## 4. Discussion

Using this same 8-month model, the abbreviation of immature adult heartworms with oral ivermectin treatment initiated 72 days PI prevented the development of adult heartworms (Blagburn and Dillon, 2007; Dillon et al., 2007) but was associated with heartworm-associated respiratory disease (HARD). The HARD in treated (immature abbreviated L5) cats at 8 months PI was not significantly different from untreated (IU) cats with viable heartworms at necropsy. In the previous study, one group of cats was infected with L3 heartworms and treatment with monthly selamectin was initiated 28 days PI (Dillon et al., 2007). In that study, the selamectin-treated group did not develop heartworms but seroconverted to a serum antibody-positive status, and alveolar and pulmonary arterial abnormalities were noted in histologic evaluation of selamectin-treated cats. Similar lesions have been noted in random-source cats that were heartworm antibody negative. (Browne et al., 2005) The purpose of the current study was to determine whether a different result would be associated with treating with selamectin 1 month before, rather than 28 days after a L3 *D. immitis* infection.

In the current study, the infective L3 successfully developed to adult heartworms in the IU cats. The results of CBC, BAL cytology, serologic, radiographic, and histology are consistent with the previous 8-month study.

Pretreatment with selamectin 32 and 2 days before the infection was not associated with the subtle lung pathology of the post-infection selamectin group of the previous study, and were not significantly different from uninfected untreated controls in the present study. In previous studies, cats had been treated 28 days after L3 infection, and subtle abnormalities were noted at both 8 months and 18 months in individual cats within these groups. Increased smooth muscle of the pulmonary arterioles and increases in interstitial myofibrocytes was particularly concerning in the continued presence of the histologic lesions in cats 18 months PI, which at that point had been heartworm antibody negative for more than 6 months.

Although there is absence of statistically significant lung pathology in the PreS I group compared with the UU group, the precardiac stages in the current study did initiate a subtle increase in interstitial opacities on CT images on Days 120 and 240 in one cat, a consistent increase in eosinophil percentage of BAL cytology, and subtle increase in myofibrocytes of the lung interstitium. None of the cats in the PreS I group seroconverted to antibody positive, which does suggest early death of developing larvae, but

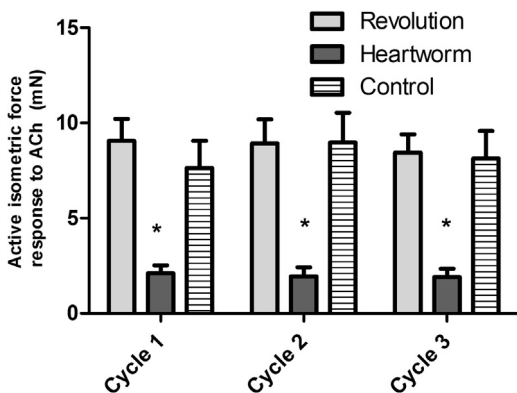


**Fig. 13.** (A–C) Hounsfield Unit distribution on CT images in cats. Distribution of lung densities. For each graph, red= Day 0, blue= Day 120, green= Day 240. Treatment groups: UU = uninfected untreated, PreSI = pretreated selamectin infected, IU = Infected untreated. A. UU control cat demonstrating no change in distribution over time. B. PreSI selamectin cat demonstrating no change in distribution over time. C. IU infected cat demonstrating a shift in the distribution curve to the right over time with a decrease in the air density (–900 range) and toward the tissue density (–600 range). Air trapping would be associated with a shift in the curve towards the right.

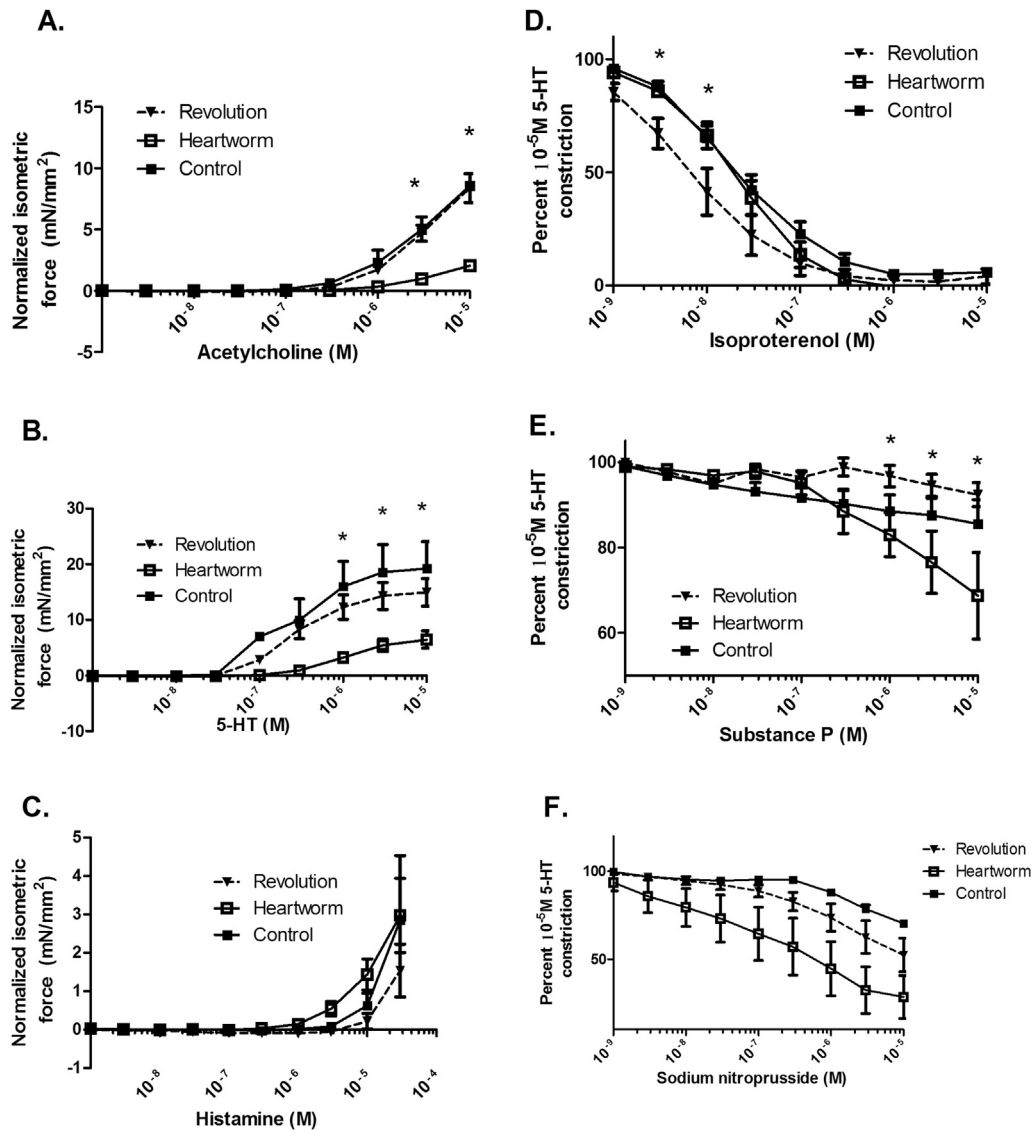
the above observations suggest some immunologic surveillance. In that cats uniquely have pulmonary intravascular macrophages compared with dogs (Dillon et al., 2008), the phagocytic ability of the lung to identify precardiac

larval byproducts derived from subcutaneous tissues can be a consideration. The location and time of death of L4 after selamectin is undetermined in cats or dogs. The arrival and early death of small “sick” immature adults within the lungs must also be a consideration based on CT results. The clinical concerns over these subtle changes are moot, but this could cloud diagnostic testing when an increased eosinophilic BAL cytology is inadvertently associated with successful heartworm prevention in a client’s cat.

In the current study, none of the cats in the pretreated selamectin group (PreS I) developed a positive heartworm antibody titer; however, in other studies, when selamectin was administered after the infection, 100% of cats developed positive titers. Because selamectin treatment was administered 32 and 2 days before the infection in the current study, it is open to speculation as to whether cats pretreated 2 weeks before infection would seroconvert. Treatment after infection does prevent adult heartworms, but to a practicing veterinarian, the clinical aggravation of client cats on heartworm prevention having a positive heartworm antibody result could be avoided by initiating heartworm prevention several months before the



**Fig. 14.** Responses of cats’ intra-parenchymal bronchioles to three cycles of  $1 \times 10^{-5}$  M acetylcholine (Revolution = pretreated with selamectin infected; Heartworm = infected untreated; Control = uninfected untreated). \* indicates  $p < 0.05$ .



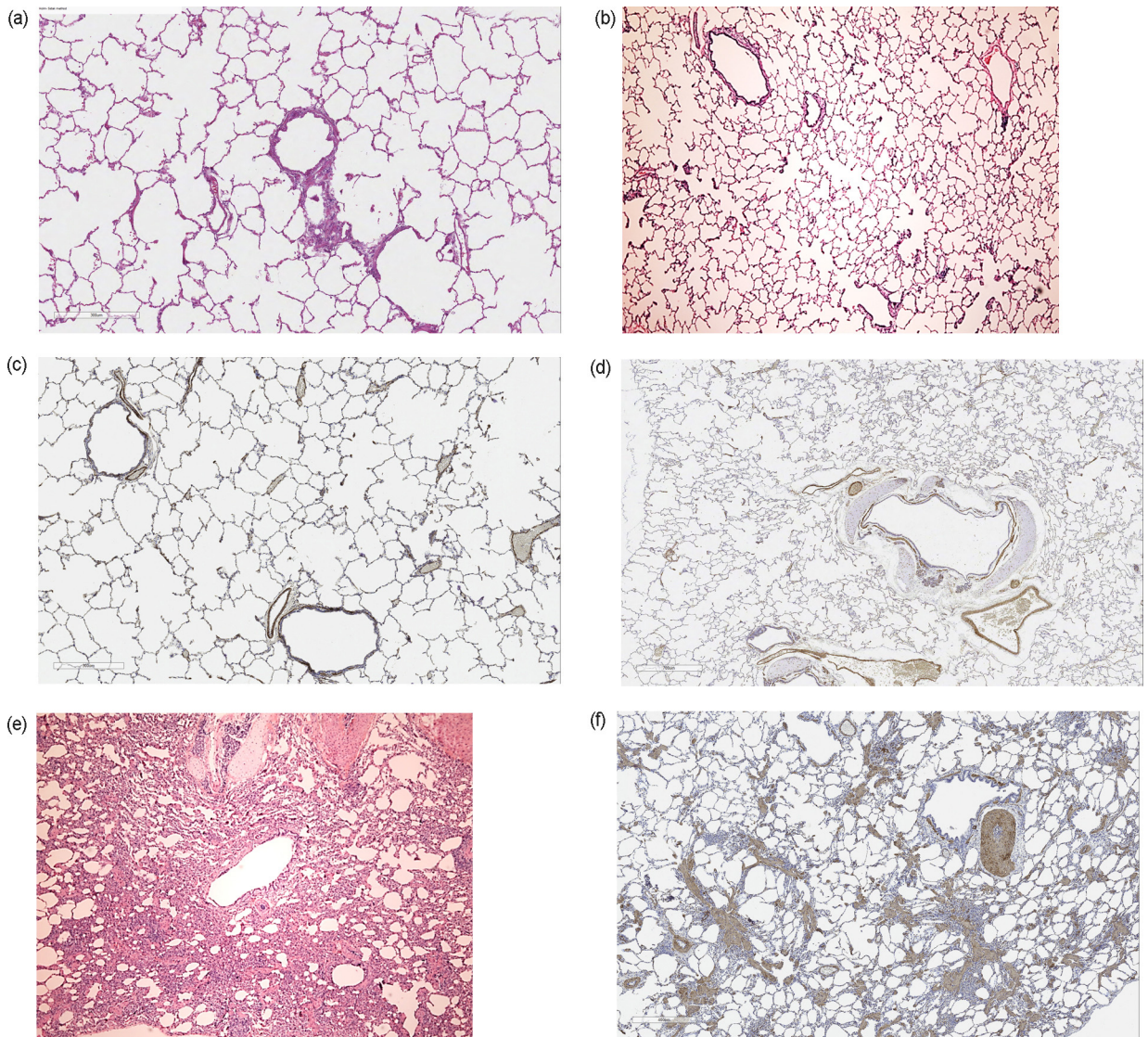
**Fig. 15.** Effect of treatment in heartworm infection on contractile and relaxation responses of feline intrapulmonary bronchioles (IPB). All contractile responses are expressed as normalized isometric force which is calculated as the active force (maximum-baseline) divided by the cross sectional area of the ring and all relaxation responses are expressed as a percentage of the force elicited by 5-hydroxytryptamine (5-HT). A,B: IPB from HW (IU) cats have a diminished response to acetylcholine and 5-HT compared to control (UU) and revolution (PreS I) treated cats. Responses of revolution (PreS I) treated cats were not different from control (UU). C. Histamine responses are similar between all groups. D. IPB from REV (PreS I) cats were significantly more sensitive to ISO induced relaxation. E. IPB from REV (PreS I) treated cats were less sensitive to SubP induced relaxation than IPB from HW (IU) infected cats. F. There was no difference between groups for SNP induced relaxation. Acetylcholine (ACH), histamine (HIS), isoproterenol (ISO), sodium nitroprusside (SNP), substance P (SubP). \*indicates  $p < 0.05$ .

mosquito season or by recommending year-round prevention. Although cats in groups with selamectin treatment before or after L3 infection did not develop radiographic lung changes, the concern of accumulation of lesions from repeated precardiac stages after repeated infections in endemic areas was a strong consideration in proposing the current study. Further, the eosinophilic BAL cytology, even in pretreated infected cats, is consistent with an immunologic pulmonary response to precardiac stages of *D. immitis*.

In the current study, bronchial ring contractility reaction of the PreS I group was not significantly different

from that of uninfected controls (UU). The PreS I group did not demonstrate an increased sensitivity or blunted response to constriction agonist. Consistent with a previous study, (Wooldridge et al., 2012), IU cats in the current study with adult heartworms had blunted contractility to methacholine and 5-HT, and no hypersensitivity to histamine. Although there is marked peribronchial thickening in cats with adult heartworms as well as in cats with HARD, the majority of the increase in peribronchial wall is muscle mixture of smooth muscle and myofibrosis. Cats in a *Toxocara cati* study demonstrated a similar but less significant histologic peribronchial involvement and had normal





**Fig. 16.** (A–F) Lung histopathology results. A. PreS I Cat H&E stain with no lesions on section. B. PreS I cat H&E stain with mild increase in interstitium. C. PreS I cat SMA stain with very subtle increases in myofibrocytes. D. UU cat SMA stain with no lesions. E. IU cat H&E stain with severe perbronchial infiltration, corresponds to Fig. 10B right lobe. F. IU cat SMA stain with myofibrocytes and pulmonary arterial hypertrophy but minimal bronchial disease. Corresponds to Fig. 10A right lobe. A. PreS I Cat H&E strain with no lesions on section. B. PreS I cat H&E strain with mild increase in interstitium. C. PreS I cat SMA stain with very subtle increases in myofibrocytes. D. UU cat SMA stain with no lesions. E. IU cat H&E stain with severe perbronchial infiltration, corresponds to Fig. 10B right lobe. F. IU cat SMA stain with myofibrosis and pulmonary arterial hypertrophy but minimal bronchial disease. Corresponds to Fig. 10A right lobe.

response to contraction agonist and an increased sensitivity to high concentrations of histamine.

The CT results in the heartworm-infected cats are consistent with restrictive lung disease as demonstrated by an increase in interstitial densities, a decrease in total lung volume, and the absence of air trapping. Although CT evaluations were not performed on HARD cats in prior studies, based on the similarity of radiographic and histologic findings, it can be assumed that HARD also had the same pattern of restrictive lung disease.

Combined with the bronchial ring reactivity, a hyper-response asthma-like syndrome would seem to be an unlikely clinical syndrome in heartworm infected cats. Of

clinical importance is that both *D. immitis* and *T. cati* infections are associated with a similar interstitial bronchial radiographic pattern, enlarged pulmonary arteries, and markedly high eosinophilic cytology on BAL (Dillon et al., 2013) but have distinctly different reactions to bronchial constrictors and relaxation agonist and antagonist.

A research concern is the sporadic distribution of uneven lung lesions demonstrated on CT images. The echocardiographic and radiographic changes are consistent with previous description of cats with mature adult heartworm infections (Atkins et al., 2000; DeFrancesco et al., 2001; Selcer et al., 2005). The colorized illustration of grades of lung densities into 3D images provides insight



into the unpredictability of the disease pattern as were also reported in *T. cati* infections (Dillon et al., 2013). Further, the pattern of the early disease on Day 120 PI included interstitial lung and pulmonary arterial involvement of all lung lobes, and by Day 240, the diseased pulmonary arteries included all lobes and was not consistently more severe in the caudal lobes. The left anterior and left caudal pulmonary arteries consistently had higher scores than other lung lobes. Lung lobes, which classically do not harbor mature adult heartworms, had interstitial and pulmonary arterial changes that may be associated with byproducts of heartworms rather than the physical presence of adults or the consequence of death of early immature adults. The validity of histopathology grading of randomly selected histopathology sections becomes a concern. In the present study, normal lung was identified and graded, but based on CT images, targeted selection of lung sections revealed a very different grading score.

## 5. Conclusion

Pretreatment with selamectin 32 and 2 days before L3 infection was not associated with radiographic cardiopulmonary changes or seroconversion to heartworm antibody positive status. Compared with normal (uninfected) controls, some of the cats pretreated with selamectin had subtle increases in lung interstitial myofibrocytes and an increased haze on CT images, but no appearance of airway disease. Blunted bronchial ring contractility and relaxation were identified in cats with adult heartworms even with a marked eosinophilic BAL cytology. The bronchial reactivity of infected cats pretreated with selamectin was normal. The current study, combined with the previous studies, suggests that precardiac stages of heartworms in cats has a role in lung injury. The clinical implication of the study is that heartworm prevention should be initiated at least 1 month before the first risk of infection.

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## Conflict of interest statement

In the past five years, author Dillon has received reimbursement, honorarium, or research support from Pfizer Animal Health (now Zoetis), manufacturers of *D. immitis* antigen tests and heartworm preventives, Bayer Healthcare LLC, Idexx Laboratories, Elanco Animal Health, and Medtronic Inc., Medical Device Division. Other authors have no competing financial interests to disclose.

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