

# U.S. Fish & Wildlife Service

## California Nevada Fish Health Center FY2008 Technical Report

Health survey of Ash Meadows NWR Amargosa pupfish  
(*Cyprinodon nevadensis mionectes*).

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**Summary:** In 2006, moribund Devil's Hole Pupfish were diagnosed by histology with lymphosarcoma and nephrocalcinosis. These fish were reared at Willow Beach National Fish Hatchery and had previously been held at the Hoover Dam refugia. Additionally, lymphosarcoma was diagnosed in a pupfish and nephrocalcinosis in a second collected from Devil's Hole in 2007. This survey was conducted to determine if lymphosarcoma and nephrocalcinosis is a common occurrence in pupfish at Ash Meadows living in springs near Devil's Hole.

On April 1, 2008, 180 adult Ash Meadows Amargosa pupfish were sampled from five locations (Crystal springs, King's pool, Points of Rock, Fairbanks, and Roger's spring) at Ash Meadows National Wildlife refuge. Blood smears and histological sections did not demonstrate clinical signs of either lymphosarcoma or nephrocalcinosis in any collection group. Trematode infections were observed in 25% of the collection group from Crystal Springs however the infections were deemed insignificant to their health. The majority of pupfish at all locations contained lipoprotein pigments (presumptive lipofuscin) in their liver, kidney, thymus, and acinar cell foci (pancreatic tissue). Lipofuscin is the polymerized residue of peroxidized lipids and proteins. Fish were assayed for virus, bacteria, and external parasites. A mix of common aquatic bacteria were isolated from 7 – 40% of pupfish sampled from Crystal springs, Kings Pool, and Points of Rock however the fish showed no clinical signs of infection. No virus was isolated in cell culture nor were external parasites observed in gill wetmount preparations. The data suggests that Amargosa pupfish were generally healthy and Ash Meadow NWR spring water does not appear to be an obvious source of lymphosarcoma or nephrocalcinosis

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### **Introduction:**

In 2006, Devil's Hole Pupfish reared at Willows Beach National Fish Hatchery (NFH) were diagnosed with lymphosarcoma (B McConnel, Bozeman Fish Health Center report CHN 07-39 ). Lymphosarcoma was diagnosed from a mortality collected in Devil's Hole during the summer of 2007. Additionally, nephrocalcinosis was observed in the kidney tubules of pupfish from the Devil's Hole as well as Willow Beach NFH, Shark's Reef aquaria, and Hoover Dam refugia.

The primary objective of this survey was to determine the presence and severity of lymphosarcoma and nephrocalcinosis in adult Amargosa pupfish rearing in various springs in proximity to Devil's Hole spring. Additionally, these fish were screened for virus, bacterial, and parasitic infections.

### **Methods:**

On April 1, 2008, a health inspection was performed on Ash Meadow Amargosa pupfish collected from five locations at Ash Meadows NWR (Table 1). Larger individuals were selected for sampling to target older fish and obtain sufficient blood volume. Immediately after euthanization with MS222, weight (0.1g) and total length (mm) were measured, a condition factor calculated ( $KTL = (Wt/ TL^3) \times 10^5$ ), external signs recorded (hemorrhage on body, gill lesion or paleness {anemia}), and the caudal peduncle severed to collect blood into a heparinized microhematocrit tube. A blood smear was prepared and if there was sufficient sample a drop of blood was fixed in 100 $\mu$ L of Karnovsky's fixative (for transmission electron microscopy) and another drop frozen on dry ice within a 96 well plate (potential retrovirus RNA extraction). The fixed and frozen blood samples were archived to potentially confirm observations of lymphosarcoma in blood smears or histological sections. Tails were collected in absolute ethanol for genetic archiving. Specimens were placed into fixative within 2 minutes after euthanization to reduce post-mortem artifacts. The peritoneum and operculum was opened prior to fixation in Davidson's fixative. After 48 h, samples were transferred to 50% ethanol for storage. It was necessary to decalcify the specimens for 48h in Cal-Ex (cat.no. CS510, Fisher Scientific, [www.fishersci.com](http://www.fishersci.com)) prior to processing for 6 $\mu$ m paraffin sections stained with hematoxylin and eosin. A shallow (approximately 60 $\mu$ m deep) and deep (midline) sagittal section was obtained from each specimen in order to examine gill and thymus as well as kidney tissue. Sections were examined at 20 and 40x magnification for tissue abnormalities (e.g nephrocalcinosis, necrotic tissues, hepatocyte fat vacuoles) and parasitic infection in key organs (gill, thymus, liver, acinar cells, kidney, gonads, intestine, brain, and muscle).

Concentrations of small basophilic monomorphic lymphoid cells in several tissue types or locations (liver, peritoneum, red and white muscle) was the criteria for the presumptive diagnosis of lymphosarcoma (Bowser et al. 2002).

A second group of fish was sampled for gill parasites, systemic bacteria and virus. A wet mount of gill tissue was prepared from 5 fish per population and examined for external parasites by phase microscopy. Kidney tissue aseptically collected was inoculated onto Brain Heart Infusion (BHI) agar. The bacterial

samples were maintained at 10-25°C and colonies identified to genera by AFS/FHS Blue book (2007). A five fish pool containing the anterior third of the fish (including gill, kidney, and liver tissue) was collected, held at 10°C until processed at 30h for viral assays on 3 cell lines (EPC, CHSE214, and FHM). The EPC and CHSE214 cultures were held 18d at 15°C and the FHM cells at 30°C.

Table 1. Sample location and fish number collected for blood cells, histological specimens, viral and bacterial culture.

	Blood	Histol.	Viral / Bacte	Total
Crystal Springs	27	30	30	60
King's Pool	15	15	15	30
Point of Rocks	14	15	15	30
Fairbanks	13	15	15	30
Roger's Spring	7	15	15	30

### **Results and Discussion:**

*Morphometrics* - Size differences were observed between pupfish collected from the five locations (Table 2). The samples from Crystal and Roger's Springs contained the smallest fish with Points of Rock and Kings Pool having the largest fish ( $P < 0.001$ ,  $F = 9.861$ , 1-way ANOVA, Holm-Sidak multiple comparison method). Condition factor of the Crystal springs fish were significantly smaller than either Points of Rock or Fairbanks groups ( $P < 0.009$ ,  $F = 3.655$ , 1-way ANOVA, Holm-Sidak multiple comparison method). Gravid females had the largest condition factors and the undocumented percentage of gravid females in each sample likely influenced the condition factor statistics.

Table 2. Mean (standard deviation) total length, weight, and condition factor ( $KTL = (Wt/ TL^3) \times 10^5$ ) of adult pupfish sampled for blood and histological examination.

	Total Length (mm)	Weight (0.1g)	KTL
Crystal Springs	39 (4)	1.2 (0.4)	1.897 (0.234)
King's Pool	42 (3)	1.5 (0.3)	2.005 (0.159)
Point of Rocks	43 (2)	1.7 (0.3)	2.095 (0.140)
Fairbanks	40 (2)	1.4 (0.2)	2.087 (0.200)
Roger's Spring*	37 (3)	1.0 (0.2)	2.026 (0.197)

\* measurements from 10 fish

*Histological results* - Despite the decalcification step, it was difficult obtaining sections due to tearing artifacts from bone, scale, and sediment within the gastrointestinal tract. No clinical signs of either lymphosarcoma or nephrocalcinosis were observed in any pupfish population.

The histopathological signs of lymphosarcoma (proliferation of lymphoid cell type and invasion of tissues and organs) were observed in moribund Devil's Hole pupfish collected in 2006 from Willow Beach NFH (pers. comm. B. McConnell{CHN 07-39 Bozeman Fish Health Center report}). Presumptive retrovirus like particles were reported in the tissues of the affected fish. Lymphosarcoma has been described from northern pike, muskellunge, medaka, and Chinook salmon (Papas et al. 1977, Bowser et al. 2002, Harada et al. 1990, Kent and Dawe 1990). These findings were associated with characteristics of retrovirus infection (c-type viral particles, reverse transcriptase activity in the 1.16 g/mL fraction from a sucrose gradient). Retroviruses are a group of single stranded RNA viruses that characteristically contain a reverse transcriptase.

Nephrocalcinosis is a condition characterized by calcium salt precipitants in the kidney tubules and ureters (Roberts 1989). The tubules become necrotic followed by scar tissue (granuloma) in the surrounding kidney interstitium. It is often associated with metabolic acidosis due to elevated carbon dioxide levels in the water as well as high divalent ion content in the diet.

Metacercaria of an unidentified trematode(s) was observed in 25% of the pupfish collected from Crystal Springs and one fish at Kings Pool (Table 3). They were encysted in muscle and within the peritoneum (Fig. 1). Fish are often an intermediate host in many trematode lifecycles that include a snail and the final mammal or bird host (Roberts 1989). Low number of parasites and lack of associated tissue damage indicate that these parasites did impair the fish's health. The iteroparous nature of pupfish was portrayed by the range of gamete maturity stages observed in a single ovary (Fig. 2) or testes (Fig. 3). The presence of ovaries was not recorded during histological processing which prevents the determination each sample group's sex ratio.

Lipopigments were observed in the kidney, liver, and thymus of the majority of fish at all locations (Fig. 4). The qualitative rating of these pigment foci within the kidney and liver was generally considered minor (#1 score) and this finding is not viewed as a health impairment (Table 3). The endogenous pigments, lipofuscin / ceroid {referred here collectively as lipopigments} and potentially hemosiderin, were observed in several tissues. The pigments were most often found in large cytoplasmic vacuoles of mononuclear cells that formed discrete aggregates in the kidney, liver, and thymus. Wolke (1992) reported that endogenous pigments are commonly found in macrophage aggregates of many species of fish. Lipopigments, such as lipofuscin and ceroid, are polymerized residues of peroxidized lipids and proteins. These membrane bound pigments are usually within secondary lysosomes of the cell (Sohol and Brunk 1989). These authors consider the lipopigment ceroid, to be an early "immature" form of lipofuscin. The insoluble yellow-brown pigment, lipofuscin is often referred to as "aging pigment" (Cotran et al. 1989). Harmon (1989) states that isolated lipofuscin contains 10 –

25 % phospholipid, 1 –2 % metal, and the remainder protein. This author differentiated the two related lipopigments by stating that ceroid is found in both mitotic and post-mitotic cells while lipofuscin is only found in post-mitotic cells. While macrophage aggregates with lipoproteins are a normal feature of the kidney, spleen, and liver of many teleosts its presence is also a biomarker for oxidative stress.

Hemosiderin is a golden-brown pigment composed of aggregates of ferritin micelles and occurs when there is an excess of iron in tissue (Cotran et al 1989). Hemosiderosis in fish is usually associated with either hemolytic anemia or excess dietary / environmental iron intake (Thiyagarajah et al.1998). Low quantities of hemosiderin is typically concentrated in the reticuloendothelial cells of the spleen and kidney as they recycle iron from degraded erythrocytes. No iron specific stains (e.g. Prussian blue) was applied to the sections for differentiation of lipoproteins from hemosiderin.

Table 3. Histological examination results reported as number of sections showing a feature / total sections with that tissue (%) for ovary, testes, metacercaria (metacer), hepatocyte lipofuscin (liver LPF), kidney interstitium lipofuscin (kidney LPF), acinar cell lipofuscin and melanin (acinar LPF/MM), and thymus lipofuscin (thymus LPF). The quantity of lipofuscin pigment is rated as minor (1) or multi-focal (2) in the kidney and liver. Not all tissues were present in all fish sections.

	Crystal Spg N= 28	King's Pool N= 15	Point of Rocks N= 15	Fairbanks N=15	Roger's spg N= 15
Ovary	4 / 28 (14)	3/ 15 (20)	1/ 15 (7)	3/15 (20)	1/15 (7)
Testes	7/28 (25)	6 / 15 (40)	3/15 (20)	3/15(20)	1/15 (7)
Metacer.	7 / 28 (25)*	1 / 15 (7)	0 /15	0 / 15	0/15
Liver LPF					
1	17/23 (74)	4 / 10 (40)	8 / 10 (80)	10 /11(91)	10/12 (83)
2	2/23 (9)	2 / 10 (20)	0 / 10	1/11 (9)	2/12(17)
Kidney LPF					
1	18/26 (69)	8 / 14 (57)	15/15 (100)	10/13(77)	14/14 (100)
2	7/26 (27)	5 / 14 (36)	0 / 15	3/13 (23)	0/14
Acinar LPF/MM	10/27 (37)	7/14 (50)	4/14(29)	6/13 (46)	8/15 (53)
Thymus LPF	3 / 8 (38)	6 / 8 (75)	2/6 (33)	7/12(58)	3/60 (60)

\* cestode observed in one fish with metacercaria.

Figure 1. Trematode metacercaria encysted in muscle of Amargosa pupfish (Crystal Springs). Note lack of inflammation or necrosis. H& E stain.

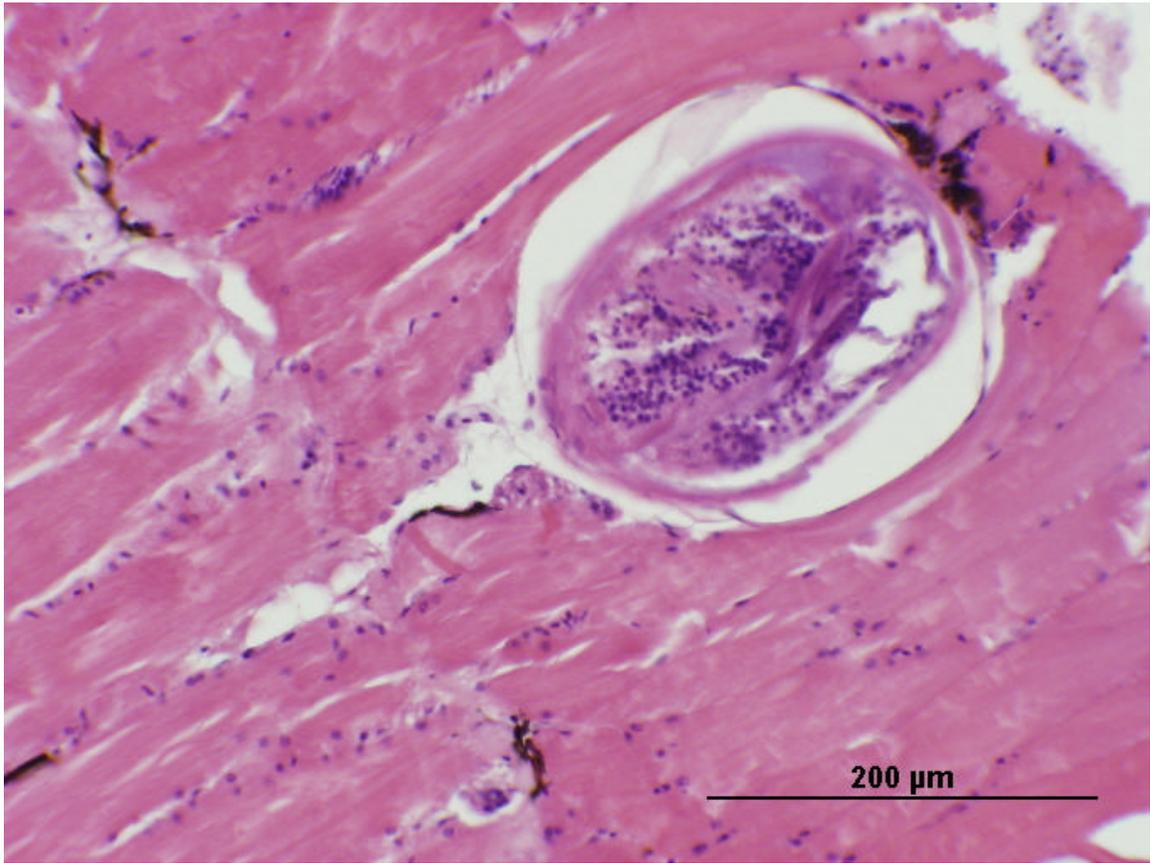


Figure 2. Ovary of Amargosa pupfish (King's Pool) with primary (\*) and secondary (arrow) oocytes. H&E stain.

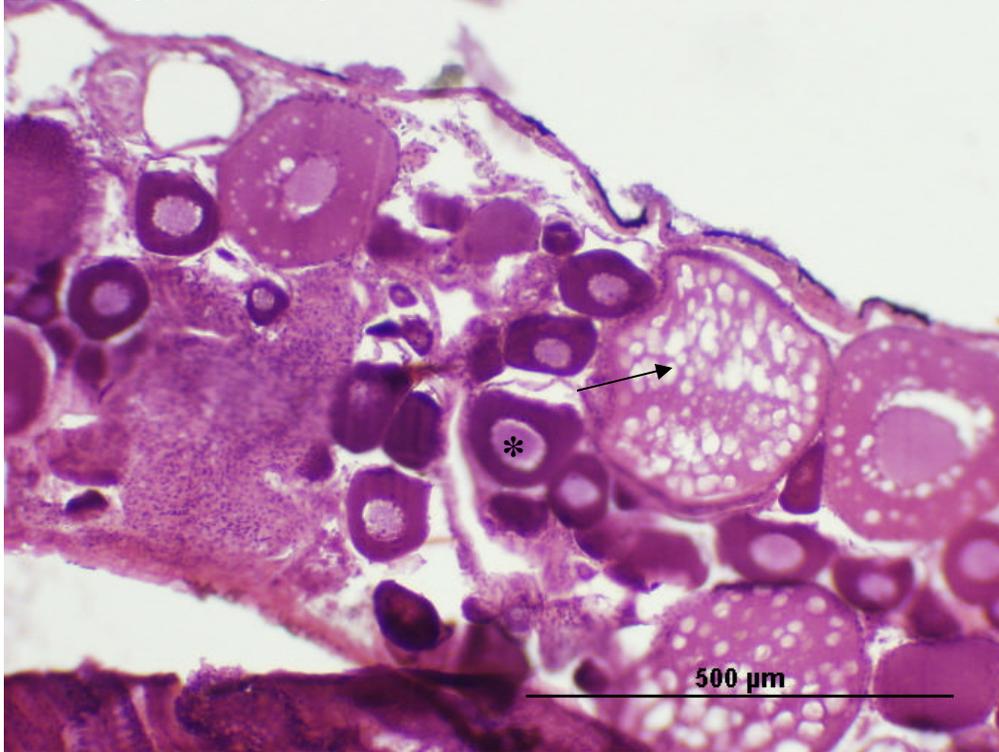


Figure 3. Testes of Amargosa pupfish (King's Pool) with various stages of development (spermatogonium {\*} , primary (arrow) and secondary spermatocytes). H&E stain.

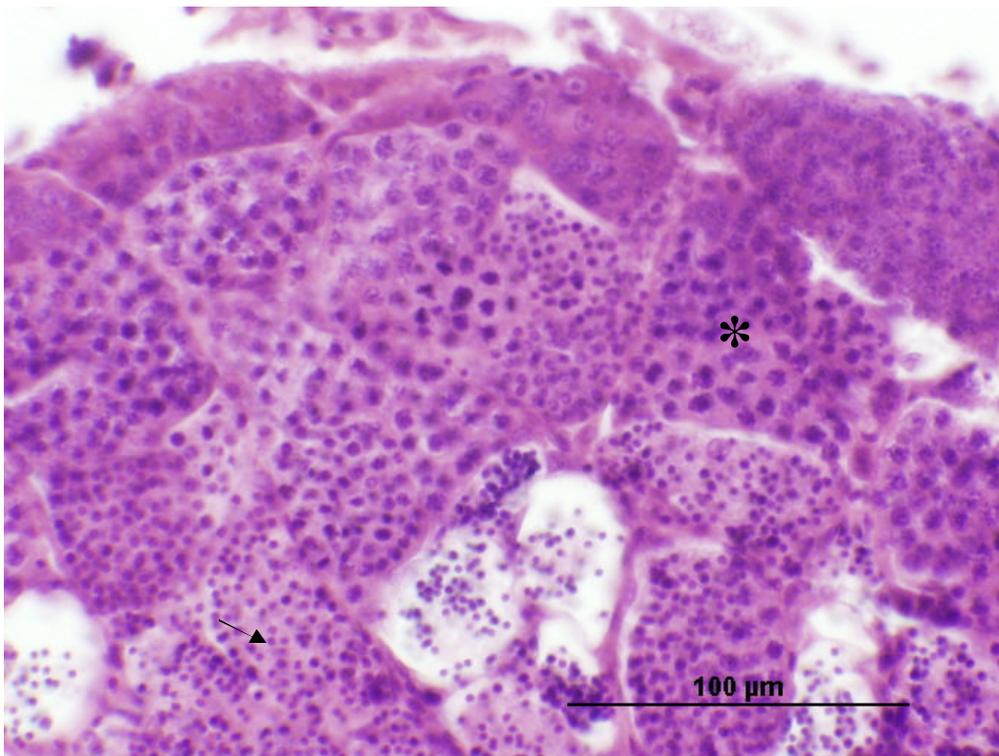
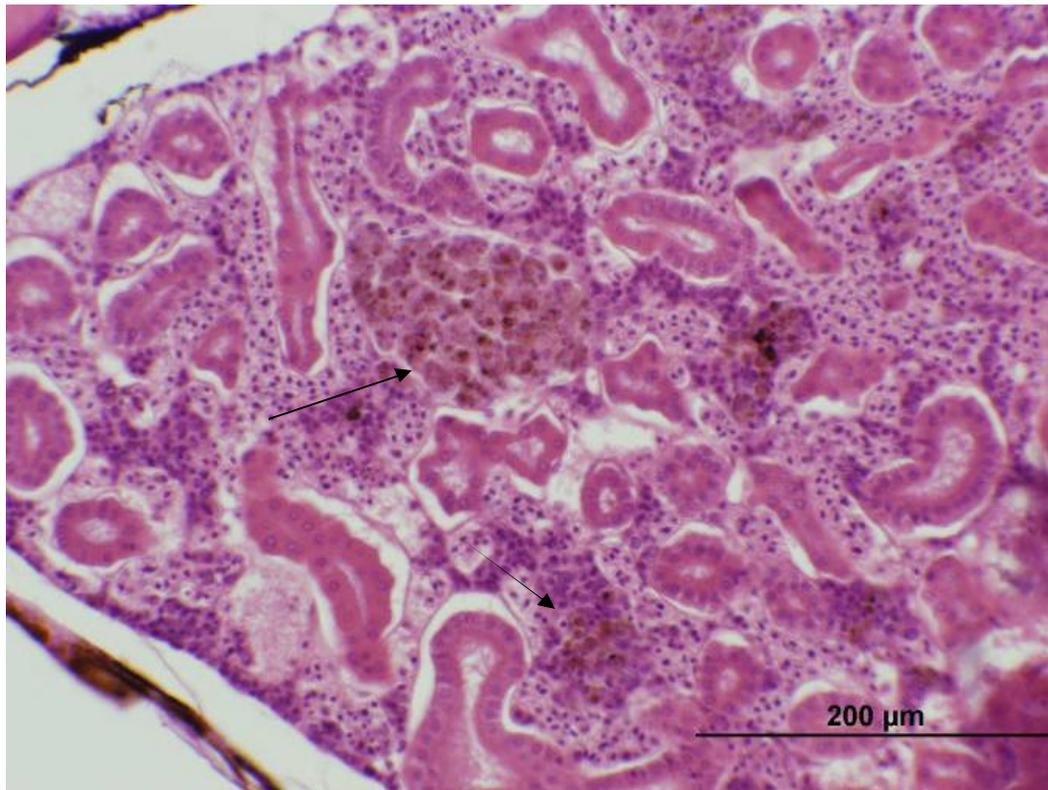


Figure 4. Lipofuscin pigment foci in kidney (arrow) of Amargosa pupfish (Fairbanks). H&E stain.



*Bacterial, parasitic, and viral agents-* No parasites were observed in any gill sample examined by phase microscopy. No virus was detected in cultures from the three cell lines. Mixed colonies of common aquatic bacteria (*Aeromonas hydrophilia*, *Pseudomonas* sp., *Staphylococcus* sp.) were isolated from 27 – 40% of the pupfish from Crystal Springs (9 of 30, 30%), King's Pool (6 of 15, 40%), and Point of Rocks (4 of 15, 27%). No bacteria were isolated from the 15 samples collected from Fairbanks and Roger's Spring pupfish. The lack of clinical signs of infections, low colony numbers, and cosmopolitan nature of the bacteria isolated indicate some of these isolates were likely sample contaminants and were unlikely to be associated with impaired health.

*Blood smear examination-* Given the lack of clinical signs for lymphosarcoma seen in the histological sections, only 5 blood smears per population were microscopically examined. Few leukocytes of any kind were seen in over 80 fields (80f) examined at 600x magnification. The number of lymphocytes ranged from 0 to 9 per 80f with thrombocytes being slightly more numerous (0 – 43 per 80f). No leukocytes were seen in four blood smears. Low numbers of neutrophils and eosinophils were seen in only 8 of the 40 smears. Differential leukocyte counts were not performed because of the low leukocyte

counts. The relative absence of lymphocytes supports the histological finding of no lymphosarcoma or any obvious inflammation.

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