Significantly higher hepatic copper concentrations in dogs compared to coyotes implicate excessive copper in most commercial dog foods

Sharon A. Center, DVM, DACVIM[1*]; Philip VanVranken, DVM[2]; John F. Randolph, DVM, DACVIM[1]; Cameron H. Friesen, DVM[3]; Ben G. Dowdy, BS[2]; McKenzie C. Hull[2]; Andrew D. Miller, DVM, DACVP[4]; Joseph J. Wakshlag, DVM, PhD, DACVIM, DACVSMR[1]; Raymond Glahn, PhD[5]; Jason A. Wiesinger, BS[5]

[1]Department of Clinical Sciences and [4]Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY

[2]Dickman Road Veterinary Hospital, Battle Creek, MI

[3]VS Imaging, St Albert, AB Canada

[5]Robert W. Holley Center for Agriculture and Health, United States Department of Agriculture, Ithaca, NY

*Corresponding author: Sharon A. Center (sac6@cornell.edu)

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ORCID

S. Center ***https://orcid.org/0000-0001-5598-2898***

ABSTRACT

OBJECTIVE: Compare liver copper (Cu) concentration, as a metric of dietary-Cu exposure, in senior dogs euthanized for geriatric health concerns against free-foraging coyotes.

METHODS: Liver samples (4/07/23-to-4/22/24) from 104 dogs and 88 coyotes were histologically evaluated, assigned rhodanine Cu-scores, and had liver-Cu quantified (µg/g dry weight liver [dwl]). Medical records, signalment, and dietary history were acquired for dogs. Water-Cu from relevant geolocations was analyzed. Dietary-Cu (manufacturer typical-analyses or inductively-coupled-plasma-atomic-emission-spectroscopy [ICP-AES, USDA-micronutrient-laboratory]) was normalized as mg Cu/100 kcal (ME was calculated using modified Atwater factorials). Dietary-Cu intake was categorized as Cu-restricted (0.15-0.24 mg Cu/100 kcal) or Cu-replete (0.31-0.39 and ≥0.40 mg Cu/100 kcal). Non-parametric statistics defined significant differences and associations.

RESULTS: There were 35/104 (34%) rhodanine-positive samples from dogs vs 0/88 from coyotes. Median (range; 95% CI) liver-Cu concentration (µg/g dwl) in dogs (248 [70-1,795; 267-369]) significantly exceeded coyotes (25 [5-114; 27-36]). Liver-Cu concentrations of 69 rhodanine-negative dog samples, including 13 dogs fed Cu-restricted diets, also were significantly higher than coyotes. Liver-Cu in all rhodanine-negative dogs was 201 (70-380; 182-218) µg/g dwl and restricted-Cu fed dogs was 190 (79-355;132-250) µg/g dwl.

CONCLUSIONS: Significantly greater liver-Cu concentration in geriatric dogs compared to free-foraging coyotes corroborates concern regarding higher dietary-Cu intake in dogs fed commercial dog foods. Findings do not infer, without further investigation, whether lower coyote liver-Cu is optimized for canid health and metabolism or merely reflects food-derived Cu-intake and interspecies differences.

CLINICAL RELEVANCE: Findings add case-based evidence supporting concern for excessive Cu in commercial dog foods.

Introduction

Copper (Cu) is an essential micronutrient for intermediary metabolism, functioning as a catalytic co-factor in redox homeostasis, mitochondrial respiration, and synthesis of numerous biological compounds (e.g., collagen, hemoglobin). Thus, homeostatic regulation of Cu is critical to cell function and survival.[1] Neutral Cu-balance is ordinarily achieved by self-regulatory adjustments involving enteric Cu-uptake, Cu-sequestration in non-reactive forms, and canalicular Cu-efflux in bile (primary Cu-elimination pathway). These governing mechanisms are influenced by a complex network of proteins including Cu-circulatory binding and distribution proteins, Cu-membrane transporters, intracellular Cu-chaperones, Cu-dependent enzymes, and transcriptional elements influencing genes controlling these variables.[1-3] Chronic Cu-intake exceeding an individual's tolerance negatively impacts the liver. Indeed, hepatocyte Cu-initiated apoptosis (cuproptosis) is recognized as an important mechanism of Cu-driven liver injury.[2,3]

Veterinary internists and pathologists with expertise in canine hepatology suspect that Cu-concentrations in commercial dog foods play a causal role in the increased prevalence of copper-associated hepatopathy (CuAH).[1] Progressive increase in liver-Cu in dogs since the late 1950s coordinates with commercialization of dog food and the practice of using Cu-containing premix supplements.[1] In fact, two peer-reviewed studies document significant increase in liver-Cu in dogs following the 1997 retirement of Cu-oxide that had been the standard food industry Cu-supplement.[4,5] Alternative Cu-supplements with higher bioavailability were subsequently subsituted.[6,7] Considering the dearth of longitudinal studies evaluating adequacy or toxicity of various dietary Cu-intakes among dogs, further studies are needed to guide regulatory recommendations.[8-13]

Genetic studies document evolution of dogs from gray wolves.[14] Liver-Cu concentrations (mean ± SD) in free-ranging gray (Croatian) wolves (n=15) and arctic (Yukon) wolves (n=21) are 61 ± 35 (range

8-212) µg/g dry weight liver (dwl) and 49 ± 13 µg/g dwl, respectively.[15,16] Mean liver-Cu concentrations from 410 wolves studied in Northwest Territories (Canada) ranged from 28 to 80 µg/g dwl (4 collections).[17] Closely related to wolves and dogs, the golden jackal is considered the European coyote.[18-20] Mean liver-Cu concentrations (µg/g dwl ± SD, range or 95% confidence interval [CI]) in 176 free-ranging golden jackals in four different European habitats are 58 (range 40-104; n=129), 80 ± 45 (range 18-188; n=34), 58 ± 7 (95% CI 50-65; n=10), and 58 ± 7 (range 51-65, n=3).[18-20] Interestingly, mean liver-Cu concentrations in wolves and golden jackals overlap concentrations in dogs before commercialization of dog foods and routine addition of Cu-containing micronutrient premixes.[1] Furthermore, a similar reference interval (15-55 µg/g dwl) exists for hepatic-Cu concentration in humans, a species with wide food diversity.[21,22] Dogs, wolves, coyotes, and golden jackals are all members of the phylogenetic wolf-like-clade, with enough genomic similarity to allow fertile dog hybrids.[23] Similar to wolves and golden jackals, coyotes are omnivorous mammals, carrion feeders, and opportunistic predators of small mammals. As such, they are viewed as nuisance predators and disease reservoirs for domestic pets. Consequently, multiple regions allow hunting of coyotes as a means of predator and disease control.

Capitalizing on the close phylogenic relationship and physiological similarities between domestic dogs and coyotes and considering that pet dogs are chronically fed Association of American Feed Control Officials (AAFCO) compliant commercial foods while free-ranging coyotes consume an opportunistically diverse natural diet (without Food and Drug Administration [FDA]/AAFCO designated micronutrient supplements), we hypothesized that coyotes would have significantly lower liver-Cu concentrations than pet dogs but similar values to other free foraging wolf-like-clade canids - reflecting dietary-Cu influence on liver-Cu concentration.

Methods

Prospective Study

Dietary data collection and histological analyses of liver were completed before liver-Cu quantification and rhodanine stain interpretation. All tissue samples were blinded as to their origin to obviate observer bias.

Animals

Dogs- Postmortem liver samples were sequentially collected with guardian consent from geriatric dogs euthanized for age-related health concerns over a 1-year period (4/07/23-to-4/22/24) at a large primary-care Midwest veterinary hospital (Dickman Road Veterinary Hospital, Battle Creek, MI). Samples were not actively recruited from dogs with known liver disease, but rather reflected natural distribution of health problems. A demographically representative sample of senior dogs within the United States was sought, not skewed by disorders targeting specialty veterinary experts or pathology centers. Medical records for dogs were reviewed with pertinent information transcribed including breed, age, body weight (BW), and sex.

Coyotes- Liver samples were opportunistically collected from carcasses made available by predator control agents up to 30 hours after animal death. Most Michigan samples were derived from an annual predator hunt authorized by the Michigan Department of Natural Resources. Ambient temperature during collection ranged between ~~30-40^{o} F~~ 4.4 to -1.0^{o} C ; no tissues were frozen before or after collection. Death was confirmed prior to liver sample collection by a licensed Michigan veterinarian (PV) who did not participate in the hunt. Additional coyote liver samples were similarly collected from predator control agents in Alberta, Canada. Sex, estimated maturity (based on dental wear), and geographic location of sample acquisition were recorded for coyotes. Blood from post-mortem cardiac aspiration was collected from 10 Michigan coyotes for testing of *Dirofilaria immitus*, *Borrelia burgdorferi, Ehrlichia canis/ewingii,* and *Anaplasma phagocytophilum/platys* (IDEXX in-house SNAP 4Dx Test, IDEXX Laboratories, Inc., Westbrook, ME, USA) to elucidate the status of regional coyotes as canid vectors for these disorders.

Dietary Copper Analyses for Dogs

Dietary histories, derived from pet caretaker interviews and medical record reviews, included specified brand-name(s), specific diet label(s), duration of feeding, food type and flavor[s], and any additional commercial or home-cooked toppers and/or treats with amount and frequency specified. Some owners fluctuated flavors of a specific brand, estimating relative frequency. This was integrated calculation of average dietary-Cu intake. Dietary Cu-content (mg/kg dry matter [DM]) was normalized by expression as mg Cu /100 kcal with metabolizable energy (ME) determined using modified Atwater factorials. Copper-content of commercial dog foods, dog food toppers, and treats was derived from manufacturer typical analyses. For foods where this information was unavailable, Cu-concentration was determined using validated inductively-coupled-plasma-atomic-emission-spectroscopy (ICP-AES; Robert W. Holley Center for Agriculture and Health, United States Department of Agriculture, Ithaca, NY). Food samples were homogenized then lyophilized for analysis, as described.[24] Copper-content of human foods provided as treats or supplements (e.g., cheese, cottage cheese, vegetables, specified meats) and any home-cooked rations were derived using the USDA's comprehensive source of food composition data.[25]

Liver Histopathology, Rhodanine Scoring, Liver Copper Analyses

Biopsies from 2-4 liver lobes, immediately fixed in 10% neutral-buffered formalin, had samples grouped for embedding in a single paraffin block. Sections (7 µM) were stained with hematoxylin and eosin (H&E) and rhodanine (Cu-specific stain). Histological features in H&E-stained sections were independently assessed by two of the authors with expertise in hepatopathology (SAC, ADM). Using an organizational spread sheet, histological features were scored as present or absent, with severity subjectively qualified as mild, moderate, or severe for glycogen-type hepatocyte vacuolation, micro- or macro-vesicular lipid hepatocyte vacuolation, centrilobular hepatocyte or macrophage lipofuscin accumulation, eosinophil focal aggregates, extramedullary hematopoiesis, reactive hepatitis (non-specific inflammatory infiltrates [lymphocytes, macrophages, occasional neutrophils and plasma cells] reflecting primary non-hepatic systemic disorders), non-suppurative hepatitis (any zone), suppurative hepatitis (any zone), copper-associated hepatitis, biliary hyperplasia, bile duct distention with mucinous debris, canalicular cholestasis, nodular hyperplasia, and regenerative nodules. Histological features graded only as present or absent included cirrhosis, portovenous hypoperfusion, neoplasia, and ductal plate malformation. After independent histological interpretations, rare divergent findings were reconciled into a collaborative opinion.

Rhodanine-stained sections were assigned a Rhodanine Score (RS) of 0-5, as previously described.[26] Digital-Cu analysis was completed for all rhodanine-positive samples (College of Veterinary Medicine, Cornell University). Flame-atomic-absorption-spectroscopy (FAAS; Veterinary Diagnostic Laboratory, Fort Collins, CO) measured liver-Cu concentrations in all samples with RS ≤1. Seven liver samples were analyzed in triplicate and 10 samples were dually analyzed by FAAS and inductively-coupled-plasma-mass-spectrometry (ICP-MS; Veterinary Diagnostic Laboratory, Michigan State University, Lansing, MI) to verify FAAS results. Liver-Cu concentration was expressed as µg/g dwl. The Cu-content of formalin for specimen preservation was measured by ICP-MS to assess potential for tissue Cu contamination during fixation.

Water Copper Concentration

Analysis of 17 water samples from geolocations in Michigan shared by dogs and coyotes were obtained from county water analyses. Samples had been collected from free-flowing tap water, public water fountains, well water, and regional terrain/ground water streams and lakes. Water Cu concentrations were measured (Alliance Analytical Laboratories, Coopersville, MI) using ICP-AES with 3-

replicates. Copper plumbing in homes was denied by pet caretakers. Samples of terrain/ground water streams, rivers, and lakes from geolocations relevant to Canadian coyotes were acquired from surveillance reports from the Alberta province.[27] Water Cu concentrations were measured by ICP-MS with replicates. High Cu concentrations in water were declared by applying the EPA standard of ≤ 1.3 µg/L.[28]

Statistical Analysis

Descriptive statistics for metrics defining the pet dog population (breed, age, sex, BW, duration of diet ingestion and categorical dietary-Cu-intake [mg Cu/100 kcal and ingested dietary-Cu mg/kg BW]) and associations between these metrics and liver-Cu concentrations are reported in a companion manuscript.[8] Some metrics are repeated herein as needed for study objectives. Distribution of descriptive statistics including liver-Cu concentrations was non-parametric (histograms, Shapiro-Wilk test) warranting data expression as median, 95% confidence interval (CI), and range when relevant. Natural data breaks stratifying dietary groups as low-risk (0.15-0.24 mg Cu/100 kcal) vs high-risk (0.31-0.39 and ≥0.40 mg Cu/100 kcal) were justified using R-segmented-breakpoint linear regression.[8] Distribution of male vs female dogs and coyotes, and sex distribution these species was examined using 2 x 2 tables and Fisher exact test. Michigan water-Cu concentrations are reported as median (95% CI), whereas Alberta, Canada water-Cu concentrations are reported as median, 95% quartiles, and maximal Cu-concentration.[27]

Comparisons of liver-Cu concentrations between Michigan and Canada coyotes, and between dogs and coyotes, were completed using Wilcoxon rank sum test; two-tailed $P \leq 0.05$. Upon confirming no significant difference between Michigan and Canadian coyotes, all coyotes were combined into a single group. To avoid over-interpretation of liver-Cu assessments between species, independent comparisons of only rhodanine-negative dogs to coyotes and only low risk 0.15-0.24 mg Cu/100 kcal diet dogs to coyotes were performed; these comparisons were repeated censoring coyotes with liver-Cu ≤ 10 µg/g dwl that might indicate Cu-insufficiency.[10,27,28,29,30] Liver-Cu concentrations in dogs and coyotes are illustrated as dot plot scatter graphics demonstrating range, median, and 95% CI. Number of dogs and coyotes with rhodanine positivity were enumerated. Percentage of dogs consuming Cu-replete diets, the only canine diets affiliated with rhodanine-positive samples, was calculated to represent dogs intolerant to Cu-intake. Dogs with liver-Cu concentration ≥400 µg/g dwl and ≥600 µg/g dwl were enumerated as actionable clinical thresholds guiding recommendations for dietary-Cu restriction or dietary-Cu restriction and d-penicillamine Cu-chelation, respectively.[1] Association between RS and liver-Cu concentrations was examined using Spearman rank correlation. Associations designated by rho (correlation coefficient) ≥ 0.65 were considered strong, 0.45-0.64 moderate, and < 0.45 weak with significance determined using $P \leq 0.05$.

Statistical computations (descriptive statistics, two-by-two tables, Fisher exact test, Wilcoxon Rank Sum test, Spearman correlations) were completed using Statistix (Version 10, Analytical Software, Tallahassee FL, www.statistix.com). Dot plot graphics were constructed using GraphPad Prism (Version 10.0.0 for Windows, GraphPad Software, Boston, MA, www.graphpad.com). Segmented-linear regression was completed using R Statistics (segmented.package).[31], R Core Team (2024). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Results

The 104 recruited dogs included 51-males, 53-females, and 32-breeds (13-Pit Bull-type dogs, 9-Labrador Retrievers, 5-Chihuahuas, 5-Rottweilers, 4-Golden Retrievers, 4-Shih Tzus, 3-Cocker Spaniels, 3-

Miniature Dachshunds, 3-Pμgs, 3-Yorkshire Terriers, 2-Pomeranians, and 1-each of Australian Cattle Dog, Bluetick Coonhound, Boxer, Catahoula Leopard Dog, Doberman Pinscher, French Bulldog, Foxhound, German Shepherd,-Great Dane, Great Pyrenees, Havanese, Irish Wolfhound, Old English Sheepdog, Pekingese, Pembroke Welsh Corgi, Shar Pei, Siberian Husky, Springer Spaniel, Tibetan Mastiff, Toy Poodle, and Wheaten Terrier) with the remaining 29-dogs of mixed-breeding. Median age, weight, and diet duration were 11.6 (95% CI 10.6-12.1) years, 23.0 (95% CI 20.1-25.4) kg, and 8.0 (95% CI 7.0-8.7) years, respectively.[8] There were no significant differences between male and female dogs in these metrics nor in the sex distribution.

Common hepatic histological features among dogs included glycogen-type vacuolation (n=61), centrilobular macrophage and/or hepatocyte lipofuscin accumulation (n=38), reactive hepatitis (n=20), and neoplasia (n=18); the entirety of classified histological features and estimated severities is provided in a companion manuscript.[8] Only a single dog (Doberman Pinscher) had severe CuAH, and another dog displayed hepatic centrilobular parenchymal collapse associated with marked Cu accumulation but only minor inflammatory infiltrates. Rhodanine-staining was positive in 35/104 (34%) dogs with RS distributions shown in **Table 1**. Qualitative RS ≥ 1 had a significant ($P < 0.0001$) strong association (rho= 0.90) with liver-Cu concentration. Sixteen dogs with RS ≥ 2 had significantly ($P < 0.0001$) higher median liver-Cu concentration compared to dogs with RS of ≤ 1 (650 [95% CI 620-989, range 509-1,795 μg/g dwl vs 225 [95% CI 210-249, range 70-479] μg/g dwl, respectively). Breeds of dogs with RS ≥ 2 included 4 Labrador Retrievers, 2 Pit Bull-type dogs, 2 Pomeranians, and 1 each Catahoula Leopard, Chihuahua, Cocker Spaniel, Doberman Pinscher, Golden Retriever, Pekingese, Pug, and mixed-breed (Goldendoodle). Formalin Cu-concentration was < 2 μg/mL discounting tissue-Cu contamination during fixation.

Among 88 coyotes (70 Michigan, 18 Canada) were 46-males, 42-females, with 2-immature animals. As there was no significant difference in liver-Cu concentrations between Michigan and Canada coyotes, liver-Cu concentrations for all coyotes were considered together. There was no significant difference in sex among coyotes nor in liver-Cu concentrations between male and female coyotes.

Histological features in coyote liver included glycogen-type vacuolar hepatopathy (n=2), lymphocytic focal hepatitis (n=7, Canada coyotes only), and multifocal eosinophilic granulomas or portal tract eosinophilic infiltrates (n=25). No coyote liver had rhodanine-stainable Cu (**Table 1**). Among Michigan coyotes, partial profiles of sinusoidal microfilaria were suspected in numerous animals but could not be definitively confirmed. However, abundant cardiac nematodes presumed to be *Dirofilaria immitis* were observed in the hearts of several deceased animals. Because it is recognized that coyotes in this Midwest region serve as host reservoirs for heartworm and tick-borne disorders, ~~SNAP 4DX testing on~~ 10 representative deceased adult coyotes (7 male, 3 female) ~~was~~ were ~~queried~~ tested (SNAP 4DX; Idexx Laboratories, Inc.),revealing positive results for *Dirofilaria immitis* in 5/10, *Borrelia burgdorferi* in 10/10, and *Anaplasma* spp. in 5/10; testing for *Ehrlichia* spp was uniformly negative.~~.~~

Median liver-Cu concentration was significantly ($P < 0.001$) higher for dogs (248 µg/g dwl), compared with coyotes (25 µg/g dwl; **Figure 1**). ~~Dogs had significantly ($P < 0.0001$) higher liver-Cu (248 [95% CI 267-369, range 70-1,795] µg/g dwl) compared to coyotes 25 [95% CI 27-36, range 5-114] µg/g dwl);~~ ~~**Figure 1.**~~ Thirteen dogs consuming low-risk restricted 0.15-0.24 mg Cu/100 kcal diets [8] also had significantly ($P < 0.0001$) higher liver-Cu concentration than coyotes (190 [95% CI 132-250, range 79-355] µg/g dwl vs 25 [95% CI 27-36, range 5-114] µg/g dwl, respectively). No dog consuming low-risk Cu-diets evidenced signs of Cu-insufficiency or had liver-Cu concentration ≤ 10 µg/g dwl.[8,29,30] Among dogs fed low-risk Cu-diets were 6/13 (46%) with liver-Cu ≤ 114 µg/g dwl (as illustrated in **Figure 2**). Among coyotes, 16 had liver-Cu concentrations ≤ 10 µg/g dwl (**Table 1**). ~~Comparison of 69 dogs lacking rhodanine-stainable Cu (including the 13 dogs consuming low-risk restricted Cu-diets) still had significantly (*P* < 0.0001) higher liver-Cu concentrations (201 [95% CI 182-218; range 70-380] µg/g dwl) than coyotes, even after censoring the 16 coyotes with liver-Cu ≤ 10 µg/g dwl (**Figure 2**).~~ Median liver-Cu concentration was significantly (*P* <0.001) higher for dogs lacking rhodanine-stainable Cu (201 µg/g dwl; n = 69, including the 13 dogs consuming low-risk restricted Cu-diets), compared with coyotes (29 µg/g dwl; n = 72, after censoring the 16 coyotes with liver-Cu ≤ 10 µg/g dwl; **Figure 2, Table 1**).

Ground Water Copper Concentration

Median Michigan water Cu-concentration was 0.12 (95% CI 0.08-0.17) mg/L (ppm) and median Alberta, Canada water Cu-concentration was 0.006 mg/L (95% quartile 0.07 mg/L, maximal value 0.12 mg/L).[27] All water Cu-concentrations were below the upper tolerance level of 1.3 mg/L recommended by the EPA.[28]

Discussion

We and others detail rising liver-Cu concentrations in pet dogs since modification of Cu-premix supplements in 1997.[1,4,5] Using coyotes as canine surrogates consuming a diverse natural diet, we document significantly lower liver-Cu concentrations in coyotes than pet dogs routinely fed AAFCO approved diets. Liver-Cu concentrations in coyotes are not uniquely low, but rather reconcile with values published for other wolf-like-clade canids.[15-20] Intriguingly, liver-Cu concentrations among free-ranging wolf-like-canids overlap with values reported for healthy humans (another mammalian species consuming a varied diet).[21,22] Liver-Cu concentration >100 µg/g dwl raises alarm for environmental contamination in wolf-like canids and Wilson's Disease in humans.[16-22] Lastly, liver-Cu concentrations in coyotes and other wolf-like-clade canids overlap with values in healthy dogs before commercialization of dog foods (mid-1950s to early 1970s).[1] Findings herein absolve regional water as a critical source of liver-Cu in studied regions, and also dismiss contribution of Cu-plumbing among studied dogs.

Exactly what liver-Cu concentration signifies Cu-insufficiency remains controversial in most mammals. In humans and mature dogs, it is estimated at < 10 µg/g dwl.[10,29,30] Yet, low liver-Cu concentrations in free-foraging wolf-like-clade species range between 8-15 µg/g dwl.[15,17,20] It is uncertain if these low extremes represent individual variation or tissue Cu-insufficiency. However, out of an abundance of caution to avoid data over-interpretation, we compared liver-Cu between rhodanine-negative dogs and coyotes (all rhodanine negative) while additionally censoring coyotes with questionably insufficient liver-Cu concentrations (≤ 10 µg/g dwl). This analytic strategy did not change significant findings, supporting either Cu over-supplementation in pet dogs or unique species differences influencing Cu-tolerance in dogs.

Case-based study of dogs fed low-risk 0.15-0.24 mg Cu/100 kcal diet (lacking Cu-premix supplements) demonstrated protection against liver-Cu accumulation compared to diets with ≥ 0.31 mg Cu/100 kcal.[8] Among dogs fed low-risk Cu-diets were 6/13 (46%) with liver-Cu ≤ 114 µg/g dwl (as illustrated in **Figure 2**). Nevertheless, these 13 dogs still had median liver-Cu concentration significantly exceeding that of coyotes. A pivotal variable likely influencing liver-Cu concentrations in this study is the limited diet diversity in dogs (repetitious feeding of formulated commercial foods) compared to humans and free-ranging wolf-like-canids. It remains unclear if dogs are uniquely intolerant to dietary-Cu-intake compared to other monogastric species. We acknowledge that genomic variability may play a role in dietary-Cu tolerance among dogs. However, it seems doubtful that simultaneous genetic mutations across all dog breeds since 1997 explain the increased prevalence of CuAH recognized by a cadre of veterinary specialists.[1,4,5] Current genetic tests estimating risk for Cu-associated liver injury have dubious value for most breeds except Bedlington Terriers.[32~~1~~] We cannot discount evolutionary differences occurring as dogs phylogenetically diverged from the other wolf-like-clade canids. However, because coyotes and other wolf-like-clade canids are free-foraging omnivores consuming a diversity of plants and animals, it remains compelling to consider that dogs have increased risk for liver-Cu accumulation secondary to our well-intentioned feeding practices.

Several ~~variables~~ limitations in the current study~~,~~ deserve mention. The Cu-content of the coyote diet is an unquantifiable study covariate (i.e., prey vulnerability might associate with less-than-optimal nutritional status and dietary-Cu-intake). Yet, finding no differences between liver-Cu concentrations in Michigan and Canadian coyotes and among values reported for other wolf-like-clade canids suggest lower liver-Cu concentrations appear to be a universal characteristic. Higher activity level (e.g., hunting, scavenging) and lower energy intake in coyotes sharply contrast with the relatively sedentary and often overfed status of most pet dogs, potentially increasing risk for liver-Cu accrual in the pet dog population.[8] Geriatric age of dogs in the present study also differed from studied coyotes, and reported wolf-like-clade canids.[8,15-20] It has been amply demonstrated among different species that chronic Cu-intake exceeding neutral-Cu balance leads to gradual liver-Cu accrual over time. Thus, the older age of dogs compared to ~~vs~~ coyotes~~,~~ would increase risk for Cu accumulation in dogs chronically fed dietary Cu exceeding their ability to maintain neutral balance. However, study of humans refutes propensity for liver Cu accumulation merely associated with advanced age.[33] ~~a~~ ~~dogs risk for liver-Cu accrual over time.~~ Presently, with few exceptions, differences in total Cu concentrations among batches of a given dog food are not scrutinized. Thus, the dietary Cu intake for geriatric dogs reported herein does not reflect this variability. Ideally, total dietary Cu for each batch of food would be quantified and available for pet caretakers.

Pioneering dietary-Cu studies (early 1960s) reported a growth-promoting benefit of supplemental dietary-Cu (Cu-sulfate up to 250 mg/kg diet) in weanling pigs.[342-364] These and additional studies in swine, poultry, and ruminants confirmed a causal association between chronic dietary-Cu supplements and liver-Cu accrual.[353,364] Furthermore, slow (weeks to months) decline in liver-Cu concentration has been demonstrated upon cessation of Cu-supplementation in experimental models as well as pet dogs with increased liver-Cu accumulation.[353,375] Profitability of growth promotion with dietary-Cu supplements in the food animal industry sustained this husbandry practice. In the 1990s, Cu-oxide, a common food grade Cu-supplement, was realized to have variable and generally low bioavailability in production animals and puppies. By 1997, Cu-oxide was retired as a feed additive being replaced by more bioavailable options.[1,12,38,3936,37] During the decades of Cu-oxide use, most dietary-Cu in commercial dog foods was seemingly derived from native or foundational ingredients. Yet, apparently sufficient dietary-Cu was available in those ingredients as Cu-insufficient dogs were neither published nor recognized, to our knowledge.[1]

Additional mitigating factors in commercial dog food formulations during Cu-oxide use are difficult to deduce as there are no public databases detailing native ingredient Cu-concentrations or bioavailability of supplemental micronutrients. Unfortunately, bioavailability of Cu-supplements has not been comprehensively studied in dogs.[386-4038] Rather, assumptions extrapolated from other monogastric species (e.g., swine, chickens) are used that may not be reliable.[4139,4240] Increasing complexity of dietary ingredients in commercial dog foods due to recent marketing strategies and pet caretaker preferences (i.e., inclusion of higher protein concentrations, fresh rather than extruded products, novel ingredients) has altered the nutritional landscape of commercial dog foods, further confounding the diversity of Cu-bioavailability.[39~~37~~] This issue is further complicated by lack of US labeling requirements disclosing total Cu-content in commercial dog foods or treats (foundational nutrient Cu, supplemented premix Cu). ~~.~~ Even when ~~such~~ information regarding dietary Cu is provided, expression on an 'as fed' or 'dry weight basis' confuses the average consumer. Normalizing Cu-content against energy (mg Cu per ~~/~~100 kcal) as done herein assists product comparisons as most foods are fed to individualized energy provision. Distressingly, some companies consider Cu-content proprietary and will not disclose information, while others seem uninformed. Because of such hindrances, we undertook mineral analyses of diets when Cu-details were unavailable.

As reported by AAFCO in 2022, average total dietary-Cu of commercial dog foods ranges between 20-30 mg Cu/kg DM (equivalent to ≥ 0.5-0.8 mg Cu/100 kcal, assuming energy density of 3,600-4,000 kcal/kg DM).[40~~38~~] This level of Cu-intake exceeds minimum daily Cu-allowances (0.15 and 0.18 mg Cu/100 kcal set by the National Research Council (NRC) and AAFCO, respectively) by ≥ 3-5 fold and is at odds with Cu-intake (0.15-0.24 mg/100 kcal) that we show protective against liver-Cu accrual in a companion publication.[7,8,40~~38~~] To our knowledge, no maximum dietary-Cu threshold is declared for dog food by NRC or AAFCO, warranting concern that unregulated inclusion of over-formulated Cu-bearing premixes have amplified dietary-Cu intake for dogs.[7,41~~39~~] Although the European Union established a dietary maximum Cu-limit of 28 mg/kg DM due to concerns regarding environmental Cu pollution, we predict this threshold will not prevent liver-Cu accrual in some dogs.[38~~36~~] Assuming 3600-4000 kcal/kg DM diet energy provision, 28 mg Cu/kg DM approximates 0.70-0.77 mg Cu/100 kcal, well above the low-risk 0.15-0.24 mg Cu/100 kcal diet shown protective against liver-Cu accrual in dogs.[8,38,~~36,~~40,42] Case-based data also predict that the FDA-CVM and AAFCO considered Cu-threshold of 15 mg Cu/kg diet DM for a 'Cu-limited diet' (i.e., equivalent to no more than 0.375 mg Cu/100 kcal, 4,000 kcal/kg diet) also will not protect against liver-Cu accrual in some dogs.[8,~~38,~~40,42]

Intriguingly, toxicological assessment of Cu-limits recommended for humans in the United States and Europe sets acceptable maximum daily Cu-intake at 0.04-0.07 mg Cu/kg BW with no liver-Cu retention expected.[43-451-43] Extrapolating these limits to dogs predicts safe Cu-intake for 10 kg or 30 kg adult dogs as 0.4-0.7 mg/day to 1.2-2.1 mg/day, respectively. Estimating daily energy (kcal ME) for an average senior dog using 1.4(70 x kg $BW^{0.75}$), acknowledging that optimal energy requirement varies among dogs, predicts these thresholds are easily exceeded by diets delivering > 0.24 mg Cu/100 kcal (common for commercial dog foods).[8,38,40,42] Although the low-risk 0.15-0.24 mg Cu/100 kcal diet should help safeguard dogs predisposed to liver-Cu accumulation, we and others have also shown protective efficacy with 0.10-0.12 mg Cu/100 kcal diets against liver-Cu accrual in some CuAH affected dogs.[3735,4240] Dietary-Cu-intake at this level slowly reduced liver-Cu by 36% over 8-16 months in Labrador Retrievers with CuAH.[3735] We also have concern that long-term excessive dietary-Cu-intake in dogs has probably increased the current liver-Cu reference limit for dogs. This brings into question the validity of using this reference interval as justification of a benign impact of Cu-fortified commercial dog foods.[46 440mit44]

Finding rhodanine-stainable Cu is abnormal in adult mammals.[1,47-5145-49] Because this Cu-specific stain reveals lysosomal and protein-bound hepatocellular Cu, positive staining implies an advanced stage of Cu-accumulation.[48,49 50,51] Safe dietary-Cu-intake should not affiliate with positive rhodanine-staining in the liver. Among the geriatric population studied herein, 34% dogs fed diets ≥ 0.31 mg Cu/100 kcal displayed rhodanine-stainable Cu whereas neither dogs fed 0.15-0.24 mg Cu/100 kcal nor coyotes showed rhodanine-positivity. The contention that stainable liver-Cu without concurrent evidence of hepatocellular injury (biochemical or histological) is innocuous is misleading, disregarding the malicious potential of this transition metal. Experimental studies amply document the detrimental impact of excessive hepatocyte Cu on redox status. Indeed, additional to independently provoked hepatocyte injury, Cu-accrual can provoke a 'second-hit' phenomenon, augmenting centrilobular liver injury initiated by other conditions (e.g., drug-induced, hypoxia [severe anemia, hypovolemia, reperfusion phenomenon], surgical or anesthetic complications, sepsis).[1] Copper accumulation in hepatocytes also can evoke cuproptosis (form of apoptotic cell death).[2,3] Experimental models confirm that chronic dietary-Cu exceeding homeostatic-Cu tolerance provokes inflammatory transcriptional responses before clinical, biochemical, or histological evidence of liver damage.[52][50]

Findings in this study should not be interpreted as a directive for targeting reduction of liver-Cu concentrations in dogs to ranges found in free-foraging wolf-like-clade canids. This conclusion is presently unwarranted and may lead to undesirable nutritional consequences. An apparent sensitivity of domestic dogs to dietary-Cu intolerance remains enigmatic. However, adherence of the pet food industry to NRC regulatory guidelines has cultivated acceptance of original Cu-threshold estimates that may be too generous. These values derive from factorial estimates based on study of puppies as well as lactating and pregnant dogs, life stages with higher Cu-requirements than non-reproductively active adult dogs.[53-55][51-53] Those estimates, foundational to AAFCO and NRC guidelines, require knowledge of Cu-bioavailability as well as Cu-turnover and retention (not described in regulatory document references).[7,41][39],[54,55][52,53] Additionally, Cu-bioavailability considered in those factorial estimates (~30%) is no longer relevant. Recent incorporation of novel molecular Cu-complexes as feed additives have further complicated the dietary-Cu conundrum. To our knowledge, Cu-bioavailability from numerous premix additives have not been specifically investigated in dogs.[1,38-40][36-38] The lack of a maximum limit for dietary-Cu in dog food in the US has permissively escalated use of over-formulated micronutrient supplements, rationalized to avoid (the unlikely) development of Cu-insufficiency.

There is a paucity of canine-specific longitudinal studies investigating Cu-insufficiency, adequacy, or excess and none (to our knowledge) in reproductively inactive adult dogs.[9-13] Four studies of juvenile dogs explored dietary-Cu insufficiency, sufficiency, or excess.[9-13] Details of Cu-insufficiency in developing puppies has been elaboratively defined.[9,10] Study of Cu-oxide vs Cu-sulfate bioavailability in puppies confirmed Cu-sufficiency at Cu-sulfate intake overlapping with the low risk Cu-diet (0.15-0.24 mg Cu/100 kcal) reported in companion publication.[8,12] A year-long pharmaceutical study of ~64 research dogs (unspecified young age; 4 groups: 6-8 males, 6-8 females) explored adequacy and toxicity of daily Cu-intake of 0.42, 2.1, and 8.4 mg Cu/kg BW.[11] Unfortunately, study details are obscured by loss of the master research report with incomplete data surviving in abridged summaries. A last study of 2-groups of weaned puppies (5-each) fed either Cu-deficient (0.021 mg/100 kcal) or Cu-sufficient (0.25 mg/100 kcal) diets up to 5-months of age established overt features of Cu-insufficiency with the 0.021 mg/100 kcal diet within several months.[13] Liver-Cu concentrations (mean $\pm$ SD) at 6-months were 19 $\pm$ 4 µg/g dwl in Cu-deficient and 246 $\pm$ 48 µg/g dwl in Cu-sufficient puppies.[13]

A tolerable upper limit for dietary-Cu in dogs should represent the highest daily intake likely to pose no adverse health risk; this includes positive rhodanine staining in the liver.[56~~54~~] Case-based canine studies predict that average Cu-content of commercial canine diets (reported by AAFCO as 20-30 mg Cu/kg DM) exceeds the upper-tolerance for many dogs.[1,8,37,40,42 ~~35,38,40~~] We hope regulatory experts will use this case-based information in re-visiting Cu-allowances for commercial dog foods and recommend labelling mandates improve transparency of product Cu-concentrations.

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Figure 1. Copper concentrations in liver biopsies (µg/g dry weight liver [dwl]) sampled between 4/07/23 and 4/22/24 in 104 geriatric pet dogs fed commercial canine diets and 88 free-foraging coyotes. Dot-plot scatter shows individual animals (blue circles = dogs, yellow circles = coyotes). Wide black horizontal line = median, and whiskers = 95% Confidence Interval; bar with *P*-value designates significant difference between groups.

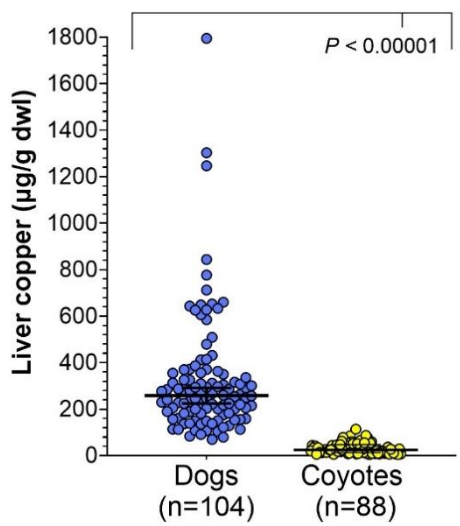


Figure 2. Liver copper concentrations (µg/g dry weight liver [dwl]) in 69 geriatric pet dogs with negative rhodanine staining of liver (green circles = 13 dogs fed low-risk 0.15-0.24 mg Cu/100 kcal diets, blue circles = dogs fed ≥ 0.31 mg Cu/100 kcal diets) and 72 coyotes with liver copper concentrations > 10 µg/g dwl (yellow circles) from animals described in Figure 1. Wide black horizontal line = median, and whiskers = 95% Confidence Interval; bar with *P*-value designates significant difference between groups.

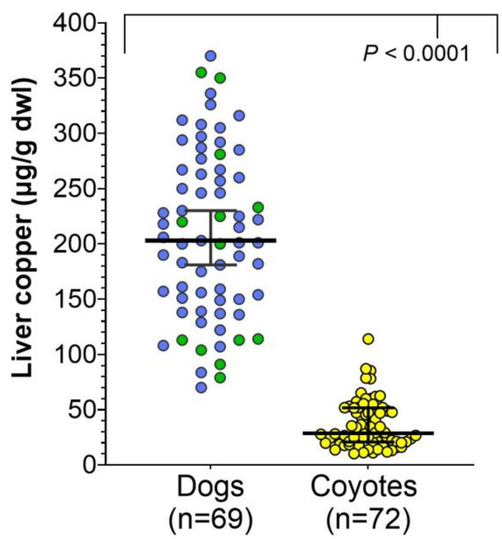


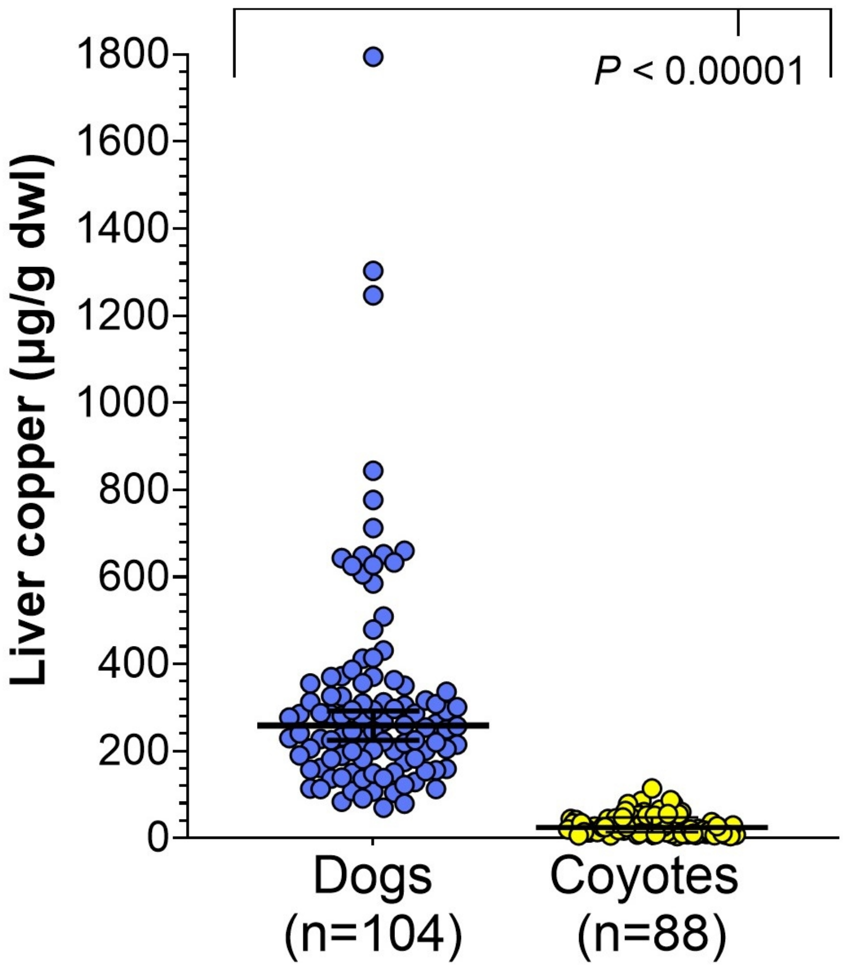
Table 1. Median, mean, 95% Confidence Interval [CI], and range of copper concentrations in liver biopsies (µg/g dry weight liver [dwl]) sampled between 4/07/23 and 4/22/24 in 104 geriatric dogs fed commercial canine diets, 88 free-foraging coyotes, and a coyote subset with liver copper concentrations > 10 µg/g dwl (n=72) cross tabulated against liver rhodanine scores (0-5). n = number of animals, na= not applicable.

	Score = 0	**Score = 1**	**Score = 2**	**Score = 3**	**Score = 4**	**Score = 5**
	Liver copper concentration µg/g dwl					
Dogs: n	69	19	10	4	1	1
Median	201	325	635	811	1,303	1,795
Mean	200	338	627	875	1,303	1,795
95% CI	182-218	305-371	589-665	458-1,294	na	na
Range	70-370	225-479	509-712	633-1,247	na	na

Coyotes: n	88	0	0	0	0	0
median	25	0	0	0	0	0
mean	31	0	0	0	0	0
95% CI	27-36	na	na	na	na	na
range	5-114	na	na	na	na	na

Liver copper > 10 µg/g dwl

Coyotes: n	72	0	0	0	0	0
median	29	0	0	0	0	0
mean	37	0	0	0	0	0
95% CI	32-42	na	na	na	na	na
range	11-114	na	na	na	na	na



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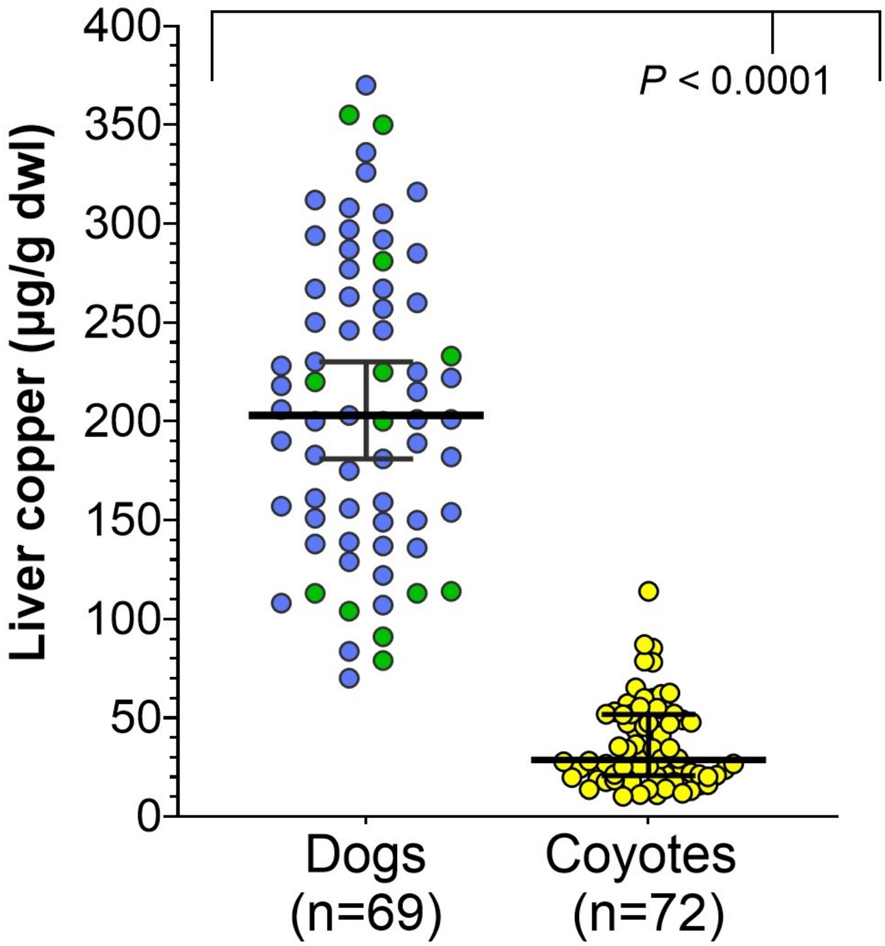
391x387mm (72 x 72 DPI)



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387x389mm (72 x 72 DPI)