# EFFECT OF ORAL COPPER SUPPLEMENTATION ON SUSCEPTIBILITY IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) TO CHRONIC WASTING DISEASE

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ABSTRACT: Chronic wasting disease (CWD) is an infectious disease, but reported associations suggest several metals—especially copper (Cu) and manganese—potentially play a role in this and other prion diseases. To assess the utility of dietary Cu supplementation in protecting white-tailed deer (*Odocoileus virginianus*) from CWD, we compared incidence and disease course among individuals naturally exposed to CWD while being maintained on sustained-release Cu boluses or unsupplemented (control). Oral Cu supplementation increased liver tissue Cu concentrations compared to controls but did not affect susceptibility to CWD or survival after natural exposure in the captive white-tailed deer we studied. Over the 27 mo study, 89% (8/9) of the Cu-supplemented deer and 86% (6/7) of control deer became CWD-infected. Survival to 27 mo postexposure did not differ between Cu-supplemented and control deer: model-averaged survival probabilities to 27 mo were 0.45–0.47 for all combinations of Cu treatment and *PRNP* gene haplotype presence. The *PRNP* gene haplotype influenced the probability of deer remaining biopsy negative for at least 17 mo but did not affect overall susceptibility.

*Key words:* Chronic wasting disease, copper, *Odocoileus virginianus*, prion, white-tailed deer.

## INTRODUCTION

Copper (Cu) plays a vital role in a myriad of physiological processes. Enzymes involved in a wide variety of functions—including integrity of the central nervous system, formation of red blood cells, and formation of collagen and elastin—require Cu for their activity (Pond et al. 1995). Copper is absorbed by the gastrointestinal tract, stored in the liver, and released as a Cu-containing enzyme or Cu-bound protein.

Deficiency (hypocuprosis) can arise from low Cu in the diet or from interference with Cu absorption caused by excess zinc, calcium, iron, molybdenum (Mo), or sulfates in feed and water. In ruminants, common signs of clinical Cu deficiency include diarrhea, poor weight gain, swollen painful joints, hind limb weakness, infertility, anemia, and decreased resistance to disease. Other, more subtle signs of Cu deficiency include unthrifty appearance and changes in hair coat such as hypopigmentation (Radostits et al. 2000). Copper status also has direct effects on immune function. Seasonal Cu deficiency has been reported in herbivores and appears to be most significant during late winter and spring. This period represents times of reduced forage availability and increased requirements from pregnancy and rapid growth. Although adequate Cu levels clearly are needed for optimal health and homeostasis in ruminant species, marginal deficiencies in populations are surprisingly difficult to assess.

Cellular prion protein (PrP$^C$), a surface-associated glycoprotein, is among numerous proteins that bind or interact with Cu and other metals (Brown 2017). The function of PrP$^C$ in the cell membrane remains a debated topic, but prevailing hypotheses include a role in cellular viability and resistance to oxidative stress, in Cu homeostasis, or in cycling at the synaptic level (Brown et al. 1998; Herms et al. 1999; Brown 2017). Prion diseases—including chronic wasting disease (CWD; Williams and Young 1980) of cervid species—result from the conversion of PrP$^C$ to an abnormal isoform and accumulation of this abnormal prion protein (PrP$^{Sc}$) in tissues of susceptible hosts (Prusiner 1998). Although CWD and some other prion

diseases are clearly infectious, reported associations with several metals, especially Cu and manganese (Mn), suggest a potential role in these diseases (Pattison and Jebbett 1971; Purdey 2000; Davies and Brown 2009; Brown 2017).

Following from these associations, the potential utility of dietary Cu supplementation as a tool for preventing prion diseases has been raised. Supplementing normal hamsters with Cu sulfate in drinking water increased cerebellar PrP$^C$ accumulation; moreover, scrapie-infected hamsters receiving Cu sulfate supplement showed delayed onset of clinical disease (Hijazi et al. 2003). In Cu-deficient sheep, intraruminal Cu oxide wires were effective in raising serum Cu for 10 mo (Leon et al. 2000). Here we evaluated whether dietary Cu supplementation could protect white-tailed deer (*Odocoileus virginianus*) from CWD by comparing incidence and the course of the disease among individuals naturally exposed to the CWD agent while being maintained at different levels of dietary Cu via use of a sustained-release Cu bolus.

## MATERIALS AND METHODS

Our study plan received Colorado Division of Parks and Wildlife (Fort Collins, Colorado, USA) institutional animal care and use review and approval prior to commencing (file no. 1-2003). We used 16 white-tailed deer housed at the Foothills Wildlife Research Facility (40°34′54″N, 105°08′49″W; Supplementary Material Table S1). Fawns were born on-site ($n$=3) or acquired from the wild ($n$=13), bottle-raised, and housed together in a 1,000 m$^2$ paddock that had not held CWD-positive deer. Fawns were screened for *PRNP* gene haplotype (US Department of Agriculture, Pullman, Washington, USA). Haplotypes were identified by the deduced amino acids glutamine or histidine encoded at position 95, glycine or serine at position 96, alanine or glycine at position 116, and S or asparagine at position 138 in each *PRNP* gene allele, abbreviated as the encoded amino acids occur in order of position. We stratified by reported haplotype and randomly assigned individuals within each haplotype to copper supplemented (Cu-sup) or unsupplemented (control) groups such that group composition was balanced by haplotype to the extent possible (Table S2).

We began the study when fawns were about 8 mo old. For the Cu-sup treatment we used Cu oxide needles administered orally in a slow-release capsule (Copasure®, Animax Veterinary Technology, Columbus, Ohio, USA). The initial dose (study month 0) was 12.5 g, then the dose was increased to 25 g (as the deer grew in size) when given at 9, 17.5, and 24 mo after the initial dose. All deer were maintained on alfalfa hay and a pelleted diet (Ranch-Way Feeds, Fort Collins, Colorado, USA) low in Cu (<10 ppm; Table S3).

Beginning immediately after the first treatment, we exposed all subject deer to the CWD prion by housing them in a 2,300 m$^2$ paddock used to hold infected white-tailed deer ($n$=11). Prion exposure came from the environment (more than 2,367 infected-deer days over the previous 10 mo) and from live, infected deer (467 infected deer-days during study), where an infected deer-day was a day's exposure from a known-infected deer living in the paddock. Subject deer infected during the course of the study provided additional sources of prion exposure.

Because the timing of individual infection varied with natural exposure, we used tonsil biopsy for detection of preclinical infection and as an adjunct to interpreting clinical observations and determining study endpoints. Tonsil biopsies were performed at the beginning of the study (0 mo, prior to exposure) and again at the same time as the Cu bolus supplementation at 9, 17.5, and 24 mo. We did not collect additional antemortem biopsies from an animal after it yielded a positive biopsy. Tonsil biopsy techniques were previously described (Wolfe et al. 2002). For sampling and treatment, we immobilized deer with a combination of 10 mg medetomidine (50 mg/mL) and 200 mg ketamine (200 mg/mL; Wildlife Pharmaceuticals, Fort Collins, Colorado, USA) delivered remotely. A mouth gate was placed in the mouth and a small flashlight used to illuminate tonsillar crypts. A 30-cm-long, 6-mm rectal forceps (Sontec Instruments, Centennial, Colorado, USA) was used to biopsy tonsillar tissue. We cleaned and sonicated biopsy instruments and other reused implements for 10 min and manually cleaned biopsy cups. We then cleaned and soaked mouth gates and biopsy instruments in LpH disinfectant (Environ LpH Steris Corporation, St. Louis, Missouri, USA; Race and Raymond 2004) for at least 30 min and autoclaved them for 60 min (121 C, 220 kPa). Extracted tonsillar tissue was preserved in 10% neutral buffered formalin for histological evaluation and examined by immunohistochemistry (IHC; Colorado State University Veterinary Diagnostic Laboratory [CSUVDL], Fort Collins, Colorado, USA) with a mouse monoclonal antibody (MAb F99/97.6.1; VMRD Inc., Pullman, Washington, USA) against PrP$^{CWD}$ (Spraker et al. 2002); the IHC techniques were as

described by Spraker et al. (2002) and Miller and Williams (2002). Biopsy results were interpreted as positive or not detected (ND).

Animals were observed daily for apparent health. In addition, we subjectively scored (0=not shown, 1=subtle, 2=obvious) each deer for signs of CWD monthly using each of the following criteria: loss of body condition, changes in behavior, ataxia, and signs of polyuria-polydypsia. Animals with a score summing to ≥4 were considered clinical and were euthanized. This relatively conservative endpoint—chosen for humane reasons—truncated the period of clinical disease, and we therefore report survival to clinical endpoint as survival, acknowledging that most infected individuals would have lived longer to end-stage clinical disease had we not intervened. In cases of irreparable trauma ($n=1$) or poor recovery from immobilization ($n=2$), we euthanized animals before they reached the clinical endpoint based on scoring.

We terminated the study after 27 mo exposure. At that time only six individuals remained (three Cu-sup and three controls). All three Cu-sup and one control were already IHC-positive on tonsil biopsy. One control had reached the clinical endpoint, and two deer—one control and one Cu-sup—showed some clinical signs. Lacking any evidence of a treatment effect on CWD incidence and because the experiment had been compromised when two controls inadvertently received Cu treatment at the 24 mo handling, we elected to euthanize all six remaining subject deer.

At postmortem examination, we collected and screened retropharyngeal lymph node tissue (RLN) for evidence of prion infection via enzyme-linked immunosorbent assay (ELISA; CSUVDL; Hibler et al. 2003) and IHC. We collected liver tissue from all subject animals for measurement of select metal concentrations (Cu, Mo, Mn) via atomic absorption spectrophotometry (CSUVDL; Association of Official Analytical Chemists 1990). Concentrations were reported as ppm dry weight of tissue. We also collected brain tissue from 11 (seven Cu-sup and four control) deer to measure Cu concentrations (dry weight). As a further reference for comparing metal values, we analyzed liver tissue collected opportunistically from 15 free-ranging white-tailed deer, all CWD-ND, that had been culled or killed in collisions with vehicles in northeastern Colorado.

In retrospect, we learned that the original PRNP haplotypes reported for eight of our subject deer included a nonfunctional pseudogene sequence (denoted as haplotype QGAN in Table S1; O'Rourke et al. 2004). Consequently, we used the occurrence of at least one QSAS allele for analyses to account for potential genetic influence on disease progression associated with the deduced presence of S rather than G at codon 96, the only

potential polymorphism discernable from available data (Johnson et al. 2003, 2011; Wolfe et al. 2007).

We used R (R Core Team 2013) for all analyses. We used Fisher's exact test to assess the effect of Cu supplementation on survival to 27 mo; the null hypothesis was that survival to 27 mo was independent of Cu supplementation (i.e., the proportion surviving to 27 mo for the treatment group was the same as the proportion surviving for the control group; Agresti 2013). To evaluate whether the presence of a QSAS allele influenced survival to 27 mo, as well as the probability of remaining biopsy-negative at 17 mo, we constructed five logistic regression models that included treatment, presence of at least one QSAS allele, additive and multiplicative combinations of treatment and QSAS, and a null model (i.e., no treatment or QSAS effect; all deer have the same probabilities). To compare competing models, we used Akaike's information criterion corrected for small sample size (AICc) and compared models using both ΔAICc (Lebreton et al. 1992; Burnham and Anderson 2002) and normalized AICc weights ($w_i$; Burnham and Anderson 2002). We used the model set to generate model-averaged survival probabilities to 27 mo and probabilities of being biopsy negative at 17 mo.

We used Student's $t$ test to compare tissue metal values. Data from the two control deer mistakenly given Cu supplement at 24 mo were excluded from comparisons of liver tissue metals. However, because the error occurred just 3 mo before study termination these two animals were included in incidence and survival comparisons. We used $\alpha=0.05$ to ascribe differences between treatment and control.

## RESULTS

All deer were classified as tonsil biopsy ND by IHC when the study began. Over the 27 mo study, all but two became infected. Eight of the nine (89%) Cu-sup deer and six of the seven (86%) controls had evidence of PrP[Sc] accumulation in RLN tissue tested postmortem by ELISA and IHC (Table S1). The two ND animals were confirmed or suspected hemorrhagic disease cases: one was a control deer that died at 6 mo postexposure, the other was a Cu-sup deer euthanized at 17.5 mo postexposure after poor recovery from immobilization. Ten of 14 RLN-positive deer also were IHC-positive on tonsil biopsy prior to

death. Three of the four biopsy-ND deer carried at least one QSAS allele (Table S1).

At least half of the Cu-sup (4/8) and control (4/6) deer surviving greater than 12 mo reached the clinical endpoint or died from CWD-related causes during the 27 mo study. Two additional animals that were alive at the end of the study also showed some clinical signs. The most common clinical sign observed was loss of body condition. We observed changes in ambulation (e.g., ataxia, abnormal gate) less frequently. Changes in behavior were subtle. One animal showed signs of teeth grinding.

Survival to 27 mo postexposure did not differ between Cu-sup and controls ($P$=1; Fig. 1). A null model best explained survival ($w_i$=0.64; Table S4), and total model weigh for models without a treatment effect was 4.0× that for models containing a treatment effect. Model-averaged survival probabilities to 27 mo were 0.45–0.47 for all combinations of Cu treatment and QSAS haplotype presence (Fig. 2A). In addition, model selection detected no effect of Cu treatment on the probability of deer remaining biopsy-negative at 17 mo; the $\Delta$AIC$_C$ was $\geq$3.47 for models including Cu treatment and total model weight for models without a treatment effect was 4.6 greater than that for models containing a treatment effect (Table S5).

A model including QSAS presence or absence was among the two best competing models explaining the probability of remaining biopsy-ND at 17 mo ($w_i$=0.57; Table S5), although a null model was weaker but still somewhat competitive in explaining the observed patterns ($\Delta$AIC$_C$=1.65; $w_i$=0.25; Table S5). Total model weight for models with a QSAS effect was 2.2 greater than that of models without QSAS effect. The model-averaged estimated probability of remaining biopsy-ND at 17 mo among deer with at least one copy of QSAS was approximately 5 times higher than deer without (Fig. 2B).

Metal analysis data were not available for one control deer. In addition to the two deer inadvertently treated, tissue metal data from one Cu-sup deer euthanized less than 1 mo after supplementation also were censored.



FIGURE 1. Survival curves of captive copper-supplemented (Cu-sup; solid line) and unsupplemented (control; dotted line) white-tailed deer (*Odocoileus virginianus*) naturally exposed to chronic wasting disease. Five remaining animals were euthanized at 27 mo postexposure (three Cu-sup and two controls). Gray arrows denote time points when Cu-sup deer received sustained-release Cu boluses.

Mean liver tissue Cu concentration in Cu-sup deer was higher than in either controls ($P$=0.031) or in free-ranging deer ($P$=0.011; Table 1). Mean liver tissue Mo concentration was lower in Cu-sup deer than in either reference group (control: $P$=0.020; free-ranging: $P$=0.002), but Mn concentrations were similar among groups (control: $P$=0.200; free-ranging: $P$=0.0995; Table 1). Copper concentrations in brain tissue from Cu-sup deer did not differ from controls ($P$=0.299; Table 1).

## DISCUSSION

Oral Cu supplementation increased liver Cu concentrations compared to controls but did not affect susceptibility to CWD or survival after natural exposure in the captive white-tailed deer we studied. This outcome is similar to outcomes of prior experimental attempts to prevent CWD in mule deer (*Odocoileus hemionus*) using pentosan polysulfate, tannic acid, or tetracycline hydrochloride as a prophylactic therapy (Wolfe et al. 2012). Prevention of CWD via practical vaccination approaches has proven equally unrewarding (Pilon et al. 2013; Wood et al. 2018).
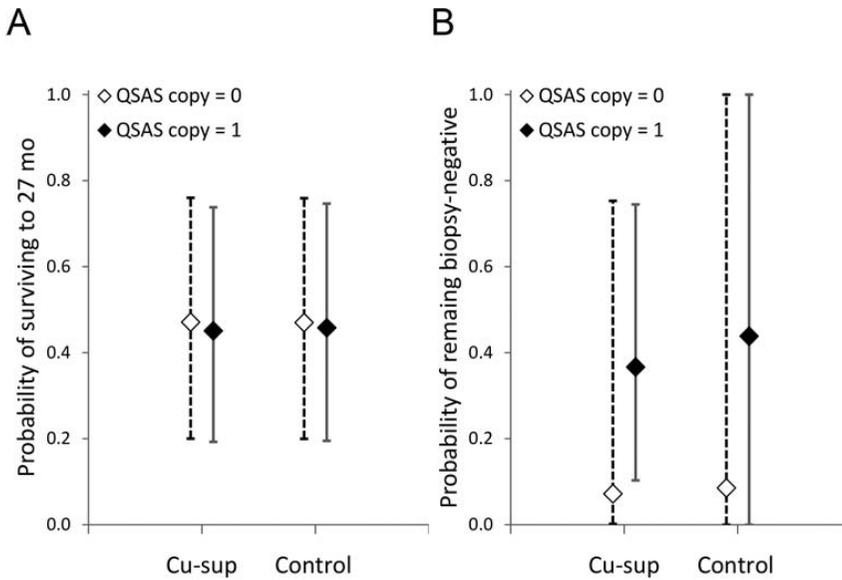
FIGURE 2.    Effects of copper (Cu) supplementation and *PRNP* gene haplotype on A. the probability of survival to 27 mo postexposure and B. the probability of a negative tonsil biopsy at 17 mo postexposure among captive white-tailed deer (*Odocoileus virginianus*) naturally exposed to chronic wasting disease. Individual deer were either copper-supplemented (Cu-sup) or unsupplemented (control) and were determined to have either no (0; open diamonds) or at least one (1; solid diamonds) copy of the QSAS haplotype identified by the deduced amino acids glutamine (Q), serine (S), alanine (A), and S encoded at positions 95, 96, 116, and 138, respectively (i.e., animals heterozygous or homozygous for S at PRNP codon 96; Table S1). Vertical bars represent ±95% confidence intervals for model-averaged estimates of the respective probabilities.

At least under the conditions of our experiment and with the limited number of animals exposed, Cu supplementation did not seem to influence prion disease patterns in captive white-tailed deer. As noted by others (Leach et al. 2007; Brown 2017), Cu and other metals bind PrP$^C$, but there is no clear causative association between trace minerals and prion diseases. In reviewing the role of Cu in prion diseases, Varela-Nallar et al. (2006) concluded that Cu has been shown both to promote prion misfolding and to interfere with PrP$^{Sc}$ propagation. Ragnarsdottir et al. (2006) found that soils from scrapie-prone areas in Iceland have low bioavailable Cu and high bioavailable Mn; however, no clear correlation could be made between scrapie status and Cu or Mn in sheep rations. Similarly, Wolfe et al. (2010) did not find a relationship between select tissue metal con-

TABLE 1.    Select liver and brain tissue metal concentrations in captive white-tailed deer (*Odocoileus virginianus*) supplemented with copper, unsupplemented (control), and free-ranging. Values reported as mean (SE) concentrations of copper, molybdenum, or manganese measured in parts per million (ppm) dry weight.

| Group | | Mineral concentration (ppm [SE]) in liver | | | | | Mineral concentration (ppm [SE]) in brain | |
|---|---|---|---|---|---|---|---|---|
| | *n* | Copper | *n* | Molybdenum | *n* | Manganese | *n* | Copper |
| Copper-supplemented | 8 | 121 (7) | 7 | 1.1 (0.1) | 7 | 7.3 (1.5) | 7 | 11.2 (1.6) |
| Control | 4 | 77 (15) | 4 | 1.7 (0.2) | 3 | 8.9 (0.9) | 4 | 9.8 (2.1) |
| Free-ranging | 15 | 84 (13) | 15 | 1.9 (0.2) | 15 | 9.5 (0.5) | NA[a] | Not done |

[a] NA = not applicable.

centrations and CWD status in free-ranging mule deer. Although Hijazi et al. (2003) delayed scrapie onset in hamsters by supplementing Cu, in other studies lower dietary Cu—accomplished via Cu chelation or by establishing a high magnesium:Cu ratio—seemed associated with slower prion disease progression (Sigurdsson et al. 2003; Nichols et al. 2016). It is noteworthy that the three studies reporting disparate effects involved artificial laboratory models of prion disease. In contrast to Cu supplementation, the occurrence of at least one QSAS allele (i.e., animals heterozygous or homozygous for S at *PRNP* codon 96) appeared to influence disease progression in experimental subjects. Our findings reaffirmed prior observations that the presence of S rather than G at *PRNP* codon 96 likely affected PrP$^{Sc}$ deposition (Wolfe et al. 2007; Keane et al. 2009; Johnson et al. 2011). Use of ELISA on postmortem samples also may have enhanced detection of cases with relatively scant PrP$^{Sc}$ deposition (Hibler et al. 2003; Wolfe et al. 2014). This outcome underscored the importance of considering key *PRNP* attributes of respective host species in the design of studies and in interpreting antemortem test results (Wolfe et al. 2007; Keane et al. 2009).

Cattle receiving low dietary Cu with or without high dietary Mn did not show differences in PrP$^{c}$ functionality although the concentration of brain tissue Cu was decreased by 26% (Legleiter et al. 2008). As in our study, Legleiter et al. (2008) concluded that these changes in dietary minerals had very little effect on the prion proteins. Although liver Cu was increased with supplementation in deer studied here, there was no elevation in brain Cu detected.

Zimmerman et al. (2008) evaluated liver samples from 42 white-tailed deer in South Dakota and found that the mean (SE) Cu concentration was 62.19 (4.81) ppm, similar to the controls in our study and slightly lower than in the free-ranging deer we sampled. Overall, Cu concentrations in liver tissues from our supplemented deer were about 1.5 times the concentrations in controls and free-ranging individuals. However, two animals

that died greater than 1 mo after Cu supplementation had liver tissue Cu concentrations greater than 900 ppm. Although neither death was attributed to Cu intoxication, these findings suggest that uptake of Cu within the first few weeks after treatment with the slow release boluses we used may have been higher than anticipated. Where indicated, alternative approaches for supplementing dietary Cu in white-tailed deer may yield a more sustained and better modulated treatment.

## SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2019-10-260.

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