

2013

## Copper oxide wire particles used to control Haemonchus infections : efficacy in giraffe (*Giraffa camelopardalis*) at Busch Gardens Tampa and potential mechanism of action

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COPPER OXIDE WIRE PARTICLES USED TO CONTROL *HAEMONCHUS*  
INFECTIONS: EFFICACY IN GIRAFFE (*GIRAFFA CAMELOPARDALIS*) AT  
BUSCH GARDENS TAMPA AND POTENTIAL MECHANISM OF ACTION

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

Veterinary Medical Sciences

by  
Allyson Kinney Moscona  
B.S., Louisiana State University, 2003  
August 2013

## **ACKNOWLEDGEMENTS**

I would like to thank my major professor Dr. James E. Miller for his support and guidance throughout my graduate (and undergraduate) career. He has taught me more about being a parasitologist, about being a teacher, and about being a researcher than I could have ever imagined. He has helped me to be a better human being, and for that above all else I am supremely grateful.

I would also like to thank, very much, my other committee members, Dr. Christine Navarre and Dr. Kevin Macaluso, who have been patient and helpful throughout this entire process.

Thank you, Brooke Leibenguth Delcambre. From student worker to laboratory manager, you have tolerated my OCD and specificities without complaint, with a smile and a great attitude that I definitely did not deserve. Thank you, oh fearless leader, for everything.

I would like to say thanks to Leigh Ann Chafon, Javier Garza, Sarah Orlik, Sarah Verret Cothron, and Aimee Pitre Mulhearn for their support and help on these projects.

Special thanks to Ian Landry, for being my student worker and friend. I hope you realize how much I appreciate your support and patience and genuine friendship.

Lastly, I would like to acknowledge and thank my husband and children, who have sacrificed in every conceivable way for me to complete this document. I love you all more than words, more than 37, and I would never have made it this far without you. Thank you!

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## ABSTRACT

Gastrointestinal nematode (GIN) infections affect production systems and exotic hoofstock in zoos, particularly giraffe (*Giraffa camelopardalis*). Anthelmintic resistance (AR) prevalence is increasing in production systems and zoos. To combat the AR that compounds GIN problems, alternative control methods are used. One such alternative is copper oxide wire particles (COWP), which control the abomasal nematode *Haemonchus contortus*.

COWP was given to seven adult giraffe at Busch Gardens Tampa, at descending dosages: 25 g, 12.5 g, 6.3 g. Treatment administration time was determined by fecal egg count (FEC) being above 600 eggs per gram (EPG), and therefore varied with each animal. FEC following 25 g COWP treatment significantly ( $p < 0.05$ ) decreased between Week 0 and Weeks 2-5. FEC following 12.5 g COWP treatment significantly ( $p < 0.05$ ) decreased between Week 0 and Week 2. FEC following 6.3 g COWP treatment showed no reduction between Week 0 and all PT samples. A 25 g COWP treatment was an acceptable part of a GIN control program for treatment of *Haemonchus* in giraffe, a 12.5 g COWP treatment was minimally acceptable, and a 6.3 g COWP treatment was ineffective.

To determine the mechanism of action for COWP, 11 Suffolk lambs were abomasally fistulated and inoculated with *H. contortus* larvae. Abomasal contents, nematodes, feces, and blood were collected at 0, 12, 36, 60, and 84 hours post-treatment (HPT). pH was measured and nematodes were viewed using scanning (SEM) and transmission (TEM) electron microscopy and energy dispersive x-ray spectroscopy (EDS), and copper content analysis was performed. FEC and packed cell volume (PCV) were determined for each time period. pH was inconclusive. FEC decreased throughout the study and up to 33 days PT; no statistics were performed due to removal of nematodes. PCV had no substantial change, but the study time period was too short for a significant change. SEM showed no obvious changes between pre- and post-treatment. TEM showed cuticle damage present in PT samples. EDS showed copper present in 10 of 11 spots on PT samples. Copper content of nematodes was maximum at 60 HPT. Evidence suggests COWP has a direct effect on *H. contortus*.

## CHAPTER ONE

### INTRODUCTION AND REVIEW OF LITERATURE

Control of gastrointestinal nematode (GIN) infection, especially pathogenic species, is important to the successful management of ruminant hoofstock (Corwin, 1997). GIN infection contributes to economic losses for production systems in numerous ways, including the cost of prophylaxis or treatment, production and reproductive inefficiency, and mortality (Orlik, 2007). Economic losses associated with GIN infection include decreased quantity and/or quality meat due to poor weight gain or weight loss, often associated with decreased appetite (Bransby, 1997; Kelly et al, 2010); decreased milk production (Spence et al, 1996; Perri et al, 2011); decreased quantity and quality wool production (Liu et al, 2003; Kelly et al, 2010); decreased pregnancy rates (Loyacano et al, 2002; Kelly et al, 2010).

The most common and most pathogenic GIN parasites for ruminant hoofstock are trichostrongylids, which include species of *Haemonchus*, *Ostertagia/Teladorsagia*, *Trichostrongylus*, *Cooperia*, *Oesophagostomum*, and *Nematodirus* (Urquhart et al, 1987).

The GIN of most concern for small ruminants and some exotic hoofstock species is *Haemonchus contortus*, commonly called the Barber Pole Worm because of the striking contrast in adult females, where white ovaries and red intestines are entwined spirally. *Haemonchus contortus* is classified as: Phylum Nematoda, Class Rhabditea, Order Strongylida, Superfamily Trichostrongyloidea, and Family Trichostrongylidae (Roberts and Janovy, 2005a). *Haemonchus contortus* are highly pathogenic, abomasal blood-feeding nematodes that reproduce sexually. The life cycle is direct, and adult females lay between 5,000 and 15,000 eggs per day (Gordon, 1967), which are released into the environment in the feces of the host. When environmental conditions are appropriate (moist, warm) the eggs hatch into the first larval stage (L1), which feed on organic material in the feces and develop to the second larval stage (L2). L2s feed on organic material and develop to the infective third larval stage (L3), maintaining the cuticle of the L2 as a sheath. Development from L1 to infective L3 can occur in as little as five days or may be delayed for weeks in unfavorable environments. The ensheathed L3s migrate from the feces when a moisture medium is present: rain, heavy dew, flooding (van Dijk and Morgan, 2011; Santos et al, 2012). They move up vegetation with the dew in the morning, and are ingested by grazing animals. Under optimal conditions (frequent measurable rainfall, high relative humidity,

high temperature), L3s can migrate higher than 20 cm along a blade of grass (Santos et al, 2012). Once ingested, L3s exsheath in the rumen before moving with ingesta to the abomasum where they penetrate and migrate into the mucosa to molt to the fourth larval stage (L4; Urquhart et al, 1987). L4s then return to the lumen of the abomasum where they start bloodfeeding and molt to the fifth larval stage (L5) and mature to the final adult stage, or they may enter a period of arrested development (Georgi and Georgi, 1990) or hypobiosis. In a hypobiotic state, the L4s remains quiescent in the abomasal mucosa for an extended period of time. This occurs when environmental conditions are unfavorable for development and survival of the free-living larval stages (Urquhart et al, 1987). Adult worms feed on blood by using the lancet in their buccal cavity to slash the mucosa to allow blood flow from disrupted capillaries (Urquhart et al, 1987).

Clinical signs of haemonchosis, the disease state associated with *Haemonchus* spp. infection, include anemia, weight loss, edema under the jaw (called bottlejaw), wool loss, dark feces, and weakness. Diarrhea is not commonly associated with this parasite unless other pathogenic species are present (Urquhart et al, 1987; Georgi and Georgi, 1990). In heavy infections, *H. contortus* adults and larvae can remove up to one-fifth of the circulating red blood cells by volume per day. Mortality occurs when erythrocytes are removed at a higher rate than the animal's ability to replenish them (Georgi and Georgi, 1990).

Other *Haemonchus* species of note are *H. placei* and *H. similis*, which infect cattle and have similar pathogenic capabilities as *H. contortus*. However, cattle tend to develop immunity to these species better than sheep develop immunity to *H. contortus* (Urquhart, 1987). *H. similis* also infects deer species. *H. bedfordi* infects African buffalo species and gazelles. *H. dinniki* infects gazelles. *H. krugeri* infects impalas. *H. lawrenci* infects duikers. *H. longistipes* infects camels. *H. mitchelli* infects elands, gazelles, and oryx species. *H. vegliai* infects oryx and antelope species (Gosling, 2005).

Other important trichostrongylid species for ruminant hoofstock are *Ostertagia* and *Teladorsagia*. These are typically indistinguishable, but accepted nomenclature is that *Ostertagia* infect cattle and *Teladorsagia* infect small ruminants, specifically sheep and goats (Bowman, 2009; Georgi and Georgi, 1990). For exotic hoofstock, the parasites are typically classified as *Ostertagia*, though there is some variation for individual animal species (Lichtenfels and Hoberg, 1993).

*Ostertagia/Teladorsagia*, commonly called the Brown stomach worms, is a mucosal-feeding abomasal nematode, but can ingest blood if present (Roberts and Janovy, 2005a). They are also classified in the Family Trichostrongylidae, and have a life cycle similar to that of *Haemonchus*. Also similar to *Haemonchus*, if environmental conditions are less than ideal for survival of free-living stages, development will arrest (hypobiosis) at the early L4 for up to six months. Another mechanism of survival is that some L3s can survive in the soil for up to one year before migrating up the herbage to continue the life cycle. Mucosal migrating larvae destroy chief and parietal cells which produce pepsinogen and HCl, respectively. Such destruction compromises digestion that leads to reduced weight gain or weight loss which is a significant economic problem (Urquhart et al, 1987).

*Trichostrongylus*, commonly called the Bankrupt worm, is a ubiquitous trichostrongylid parasite, and infects most ruminants, equids, suids, leporids, and some fowl (Georgi and Georgi, 1990; Urquhart et al, 1987). They are mucosal-feeders and the host organ varies with the host species. In ruminants, they are either abomasal or small intestinal worms. *T. axei* is found in the abomasum of ruminants or the stomach of horses and pigs; other species are small intestinal with a higher order of host specificity (Georgi and Georgi, 1990). The life cycle is similar to that of *Haemonchus*, and intestinal species can also migrate and molt in the abomasal mucosa before moving to the intestines to feed and reproduce. Free-living larval stages are very resilient, and can survive a cold environment and dessication from dehydration. They also can undergo hypobiosis as L3s if necessary. *Trichostrongylus* spp are not typically highly pathogenic, but in large numbers they can lead to rapid weight loss and watery diarrhea. In smaller numbers, clinical signs, if present at all, may be difficult to distinguish from malnutrition (Urquhart et al, 1987).

*Cooperia* and *Oesophagostomum* are trichostrongylid parasites that have a typical trichostrongylid life cycle, with the final niche being the small intestines for *Cooperia* and colon for *Oesophagostomum*. These nematodes do not feed on blood, but can cause minor pathogenesis. In large numbers, diarrhea, anorexia, decreased weight gain, weight loss, and submandibular edema similar to bottlejaw associated with haemonchosis can occur (Urquhart et al, 1987). Ulcerative damage due to *Oesophagostomum* larval migration and adult feeding activity can result in bleeding and anemia.

*Nematodirus* is also a small intestinal trichostrongylid, but the life cycle is different from the majority of other trichostrongylids. The L3s develop within the egg and hatching may take several weeks or more, depending on the species and the environmental temperature.

*Nematodirus* are typically temperate species, and require cooler temperatures than *Haemonchus*. Pathogenesis associated with nematodiriasis is mainly due to larval stages. Development through L4 to L5 after ingestion of the L3 from the pasture takes approximately 10-12 days, during which time intestinal mucosal functions including nutrient absorption and fluid exchange is severely limited. When diarrhea begins, rapid dehydration is common (Urquhart et al, 1987).

With the exception of *Nematodirus*, trichostrongylid nematodes have very similar egg types, commonly referred to as “trichostrongyle-type” or more generally “strongyle-type” eggs. These smooth, oval eggs are indistinguishable between species, and results of fecal examinations are commonly reported only as a strongyle/trichostrongyle count (Georgi and Georgi, 1990). *Nematodirus* eggs are larger and football shaped. (Sloss and Kemp, 1978).

Overall, the pathogenicity of these trichostrongylid GINs is associated with disruption of normal GI function, loss of appetite, decreased feed conversion efficiency, decreased growth rates, dull coats, significant diarrhea, and frequently high prevalence of morbidity (Urquhart et al, 1987).

Prevention of GIN infection in grazing animals is impossible (Yazwinski et al, 2009); however, prevention of disease associated with GIN parasitism is a goal of production systems and animal managers. Control of parasites is the definitive method of disease prevention. The most popular method of parasite control for the past 50-60 years has been broad spectrum chemical anthelmintics, which are readily available on the global market (Ostlind et al, 2006). Broad-spectrum anthelmintics have activity against most pathogenic GIN, and are divided into classes according to their mechanism of action. Benzimidazoles (thiabendazole, fenbendazole, albendazole, and oxfendazole) are tubulin-binding compounds which bind to tubulin proteins in the parasitic intestinal cells, leading to starvation. Imidazoles (levamisole and morantel tartrate) are ganglion-blocking compounds which cause muscle contraction and spastic paralysis, leading to expulsion of paralyzed nematodes. Macrocyclic lactones (ivermectin, doramectin, and moxidectin) are GABA inhibitors and increase membrane permeability, affecting feeding, motility, and reproduction of susceptible nematodes (Sargison, 2012; Orlik, 2010). *H. contortus*

resistance to anthelmintics in all 3 classes, individually or in combinations, is common worldwide (Sargison, 2012).

Resistance to any anthelmintic can be induced by repeated exposure to sub-therapeutic concentrations (Sargison, 2012). Also, increased frequency of anthelmintic administration even at appropriate dosing levels increases the rate of resistance development (Coles, 1986). The introduction of resistant genes to a production system through importation of animals infected with nematodes with resistance alleles, or by selection of existent resistant alleles by eliminating susceptible alleles via anthelmintic administration, can lead to system-wide anthelmintic failure (Sargison, 2012; Yazwinski et al, 2009; Kaplan and Vidyashankar, 2012).

Wide-spread multiple-drug resistance in GIN parasites has brought about a need for new GIN control options. The realization that parasite control cannot and should not be based solely on chemical anthelmintics, and knowing that novel anthelmintics are developed slowly, has led to an expansion of research into non-chemical antiparasitic agents (Jackson and Miller, 2006; Kaplan and Vidyashankar, 2012).

In addition to strategic use of available anthelmintics, general grazing management (Waller, 2006), condensed-tanning (CT) containing forages (Min et al, 2004; Terrill et al, 2007), nematophagous fungi (Larsen et al, 1994; Kahn et al, 2007), vaccination (Knox et al, 2003; LeJambre et al, 2008; Olcott, 2006) and copper oxide wire particles are all forms of non-chemical GIN parasite control that are being evaluated for parasite control strategies (Jackson and Miller, 2006).

Copper oxide wire particles (COWP) are small pieces of copper oxide that are marketed in the United States as a supplement for copper deficiency in cattle. They are administered in gelatin capsules or mixed in feed (Terrill, 2012). Efficacy is similar for the two methodologies (Burke, 2010). The particles pass to the rumen, mix with the ingesta and pass into the abomasum. Particles that remain in the rumen have no effect on GIN or systemic copper uptake. COWP adhere to the mucosa in the abomasum, and the acidity of the abomasum causes dissolution of copper ions from the particles (Patten, 2006).

The chemistry associated with the release of copper ions requires the acidity of the abomasum for the COWP to be effective as a copper supplement or a GIN control agent (Bang et

al, 1990), therefore COWP is maximally effective against abomasal nematodes, especially *Haemonchus contortus*. *Ostertagia ostertagia* and *Teladorsagia circumcincta* are also abomasal GIN, but COWP has been shown to have limited efficacy against these parasites, possibly due to the increase in abomasal pH associated with the pathology associated with these nematodes (Bang et al, 1990; Lawton et al, 1996; Dimander et al, 2003).

COWP have been shown to be effective in reducing fecal egg count (FEC) and abomasal nematode burdens in sheep and goats (Burke et al, 2004; Burke et al, 2010; Soli et al, 2010), reduction in worm fecundity and larval contamination of grazing areas (Knox, 2002), and reduction of FEC in some exotic hoofstock species (Fontenot, 2008). With the reduction of blood feeding *H. contortus* an increase in packed cell volume (PCV) also occurs (Burke et al, 2004; Soli et al, 2010).

A major concern with the use of COWP is copper toxicity. Copper toxicity is a result of accumulation of copper in the liver, and when the liver can accumulate no more, the excess copper remains in the blood, causing a hemolytic crisis, destroying red blood cells (RBC). The anemia associated with the destruction of RBC can lead to mortality (Orlik, 2010). If an animal is not copper deficient prior to treatment, toxicity can be an issue. Deficiencies in copper present clinically with ataxia due to degeneration of the spinal cord, spastic paralysis in newborns, anemia, color changes in pigmented wool or hair. Primary deficiencies, caused by dietary copper deficiencies, are less common in the United States in domestic ruminants than secondary deficiencies, caused by impaired absorption and utilization, frequently associated with confliction with other minerals, including molybdenum, sulfur, and iron (Leon et al, 2000).

For GIN parasite control and with copper sensitivity issues in mind, the goal is to find the least amount of COWP that still results in acceptable levels of control and no toxicity. Sheep are more susceptible to copper toxicity than goats, (Meschy, 2000; Solaiman et al, 2001), and copper sensitivity varies among sheep breeds and across geographical regions (Burke et al, 2007). Low doses of COWP (0.5 g or 1.0 g) given multiple times at 6 week intervals did not cause liver copper concentration to be above the normal range, but treated animals had higher liver copper concentrations than untreated (Burke and Miller, 2006). Monitoring liver enzymes such as sorbitol dehydrogenase (SDH), aspartate aminotransferase (AST; previously called serum

glutamic oxaloacetic transaminase [SGOT]), and  $\gamma$ -glutamyl transferase (GGT) is important if multiple treatments are to be administered (Solaiman et al, 2001; Burke and Miller, 2006).

Prior to the advent of COWP, copper sulfate had been associated with control of *H. contortus*, but it is more readily absorbed than COWP, therefore, more readily induces toxicity. Copper sulfate added to supplement feed or administered orally as a drench has been shown to be ineffective for controlling *H. contortus* (Burke and Miller, 2008).

Zoological facilities housing ruminant species can also have GIN parasite control problems. Anthelmintic dosages for exotic hoofstock are frequently extrapolated from information available for domestic species. Incorrect dosing can occur when herds are treated together using average weights or when animal compliance issues result in partial ingestion of treatments (Hunter and Isaza, 2002). Anthelmintic resistance in zoo hoofstock (Garretson et al, 2009; Fontenot et al, 2008; Young et al, 2000) has lead to their exploration of anthelmintic alternatives, including COWP (Fontenot et al, 2008), as part of their GIN parasite control programs.

The objectives of these studies were: 1) to evaluate the efficacy of COWP in giraffe (*Giraffa camelopardalis*) at Busch Gardens Tampa (BGT) in Tampa, Florida to control *Haemonchus* and 2) to investigate a potential mechanism of action of COWP for controlling *Haemonchus*.



## **CHAPTER TWO**

### **COPPER OXIDE WIRE PARTICLES FOR CONTROL OF *HAEMONCHUS* IN GIRAFFE (*GIRAFFA CAMELOPARDALIS*) AT BUSCH GARDENS TAMPA**

#### **2.1 Introduction**

Giraffe (*Giraffa camelopardalis*) are frequently a high priority for parasite control programs in zoos, particularly in the Southeastern United States. Giraffe commonly have very high FECs, and frequent administration of broad-spectrum anthelmintics has led to increased anthelmintic resistance (Garretson et al, 2009).

Anthelmintics are normally administered according to manufacturer directions, either orally, by injection, or by topical applications. In zoos, administration of medications of any kind presents a problem in that almost all are used extra-label in non-approved species. Extrapolation of dosages and methodologies of administrations is a necessity for zoo veterinarians (Hunter and Isaza, 2002). Orally-dosed medications, including anthelmintics such as levamisole, are often mixed in feed or feed supplements (Melbourne, 1978; Hunter and Isaza, 2002). Injectable medications usually require restraint or potentially hazardous immobilization, but cooperative species including giraffe can be trained to enter chute systems and tolerate physical examinations and injections (Hunter and Isaza, 2002). Topical application of anthelmintics (Garretson et al, 2009) is an option that also requires cooperation of the animals, but is easier in some instances than injectable or orally-administered anthelmintics. Topical anthelmintics may also require dosage adjustments to account for absorption differences (Hunter and Isaza, 2002).

In addition to broad-spectrum anthelmintics, alternative control methods have become increasingly popular as resistance to available anthelmintics increases. Anthelmintic resistance in zoological facilities is not unprecedented (Garretson et al, 2009; Young et al, 2000), and a need

for alternative control methods corresponds with the increasing prevalence of anthelmintic resistance.

Copper oxide wire particles (COWP) are available commercially for copper supplementation and recent antiparasitic activity has focused on small ruminants, particularly sheep and goats, as they are frequently infected with *H. contortus*. While sheep and goats differ in response to many medications, COWP are equally effective against *H. contortus* in both (Soli, 2010). Further work showed that COWP could be administered orally in a gelatin capsule directly or mixed with feed, with equivalent results (Burke, 2010).

The objective of this study was to evaluate the efficacy of COWP and determine the lowest effective dose to control *Haemonchus* species in giraffe at Busch Gardens Tampa (BGT).

## **2.2 Materials and Methods**

### **2.2.1 Location and Animals**

Seven adult female giraffe (*Giraffa camelopardalis*) were housed at BGT, and maintained by the standards of care dictated by the attending veterinarians.

### **2.2.2 Study Design**

Infection level in 7 giraffe was monitored by fecal egg count (FEC) at weekly to monthly intervals. A FEC of 600 eggs per gram (EPG) was established by the BGT veterinarians as the limit above which a COWP treatment was administered; therefore, treatments were not administered to all animals in the study at the same time. For each animal, dosage was decreased sequentially, from 25 grams to 12.5 grams to 6.3 grams, as the FEC increased above 600 EPG. The goal was to determine the lowest amount of COWP that could be given to each giraffe that could control infection, based on FEC data.

### **2.2.3 Fecal Sample Collection and Processing**

Giraffe fecal samples were collected from the ground immediately after deposition by BGT staff. Samples were immediately placed in Whirl-Pak® bags and sealed. The bags for BGT were either processed immediately or refrigerated to be processed later. Samples were processed

using a quantitative fecal analysis kit (Paracount-EPG™, Chalex Corporation; see Appendix B). The bags for LSU were divided into two groups. Groups marked for FEC were refrigerated until they were packed for overnight shipment to LSU in insulated boxes with ice packs; bags marked for culture were left at room temperature until packed for overnight shipment in insulated boxes with ice packs.

Upon arrival at LSU, the samples for FEC were refrigerated up to five days until they were processed using a Modified McMaster technique (Whitlock, 1948; see Appendix C). Culture samples were individually cultured using the technique described in Appendix D.

FEC reduction (FECR) was calculated by the formula (Pre-treatment FEC – Post-treatment FEC)/ Pre-treatment FEC x100%.

#### **2.2.4 Copper Oxide Wire Particles Administration**

The initial 25 g dose of COWP (Copasure®, Butler Schein™ Animal Health) was fed in the marketed capsule in a whole fruit (cored out apple or inside a banana), or as loose particles. Loose particles were administered mixed in sweet feed, mixed with Colife V Liquid® (PRN Pharmacal), or in a “fruit roll-up”: wet romaine lettuce with the particles sprinkled on it, then Gatorade® powder sprinkled on top to mask the odor, and the whole thing was rolled up to be fed as a single small roll so that the animal eats it entirely. The method of administration varied depending on the preference of each animal. For subsequent lower doses of 12.5 g and 6.3 g, the 25 g boluses were opened and the particles were weighed out into the appropriate amount.

#### **2.2.5 Statistical Analysis**

Data were analyzed using SAS® (version 9.3) mixed procedure in a repeated measures analysis of variance. FEC was log transformed to stabilize variance. Differences were considered significant when  $p < 0.05$ .

### **2.3 Results**

FEC conducted at LSU and BGT were not statistically different (data not shown,  $p \geq 0.05$ ). Therefore, data from each location was pooled to maximize the number of samples per week.

For the 25 g dose, pre-treatment mean FEC for the 7 giraffe was 2090 EPG and ranged from 675 EPG to 2775 EPG (Figure 2.1). FEC was significantly reduced for weeks two through five post-treatment ( $p = 0.0475$ ;  $p = 0.0144$ ;  $p = 0.0013$ ;  $p = 0.0215$  respectively). FECR was 55.9%, 70.6%, 52.9%, 64.7%, 76.5% and 64.7% for Weeks 1-6, respectively.

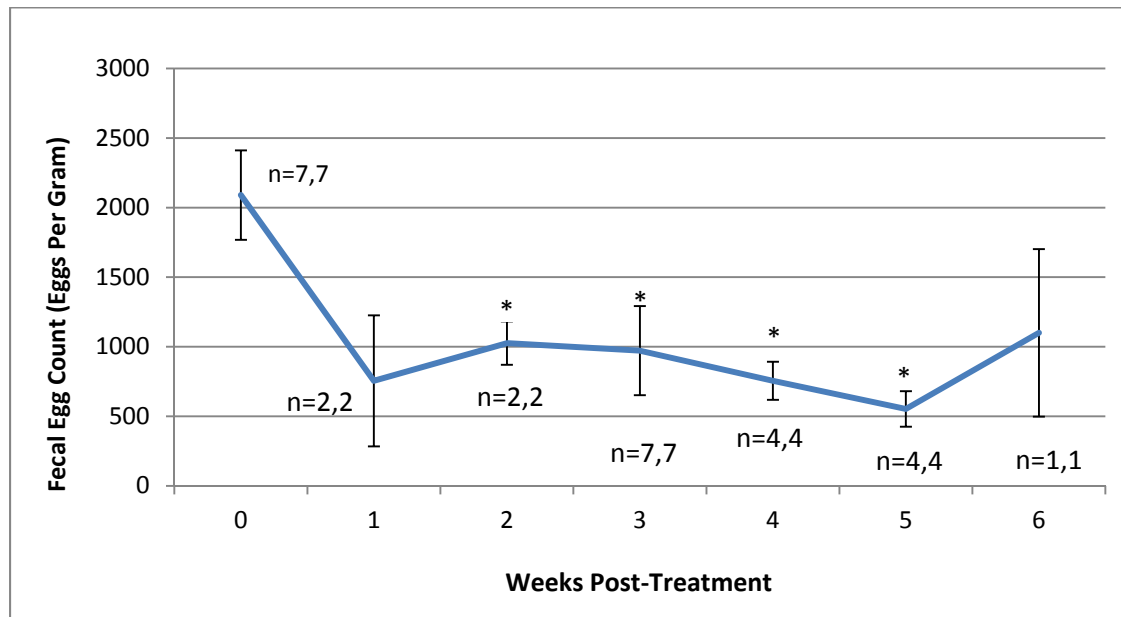


Figure 2.1. Mean (SEM) for fecal egg count of giraffe (*Giraffa camelopardalis*,  $n=7$ ) at Busch Gardens Tampa after treatment with 25 g of copper oxide wire particles. \*= significant ( $p < 0.05$ ) reduction compared to Week 0. n = total animals Busch Gardens Tampa, Louisiana State University

FECR was significant ( $p < 0.05$ ) for mean post-treatment (Weeks 1-6) FEC compared to pre-treatment (Week 0) FEC (Figure 2.2).

Fecal cultures showed that pre-treatment larval population distribution was 93% *Haemonchus* (range 85-100%) and 7% *Cooperia* (range 0-15%) and post-treatment distribution was 86% *Haemonchus* (range 73-100%) and 14% *Cooperia* (range 0-27%; Figure 2.3).

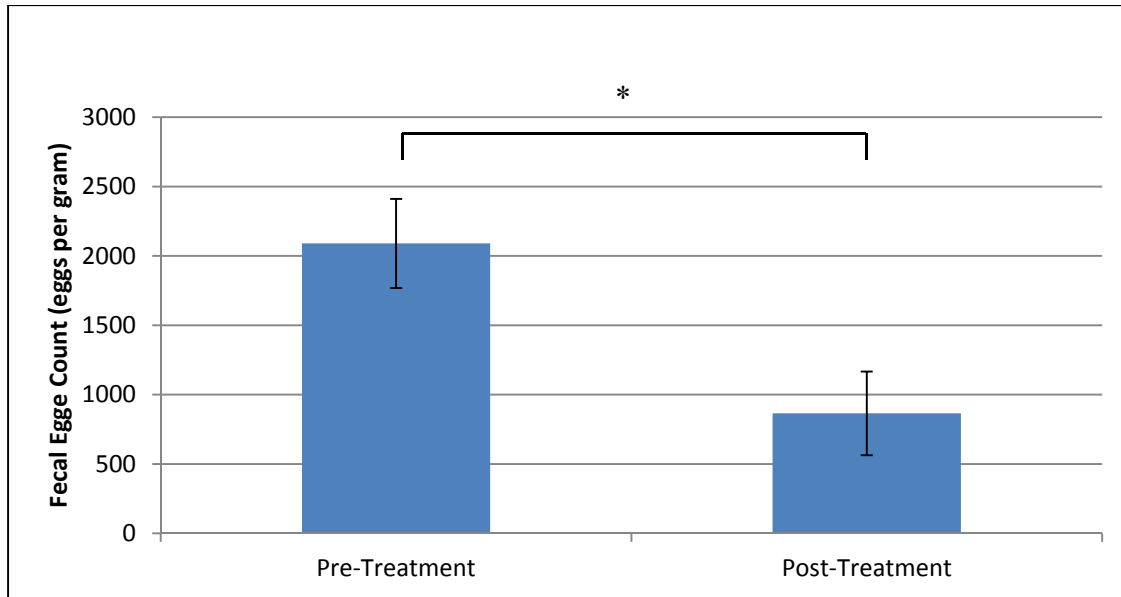


Figure 2.2. Mean (SEM) pre-treatment (Week 0) and post-treatment (Weeks 1-6 combined) fecal egg count of giraffe (*Giraffa camelopardalis*, n=7) at Busch Gardens Tampa after a treatment with 25 g copper oxide wire particles. \*= Significant ( $p < 0.05$ ) difference.

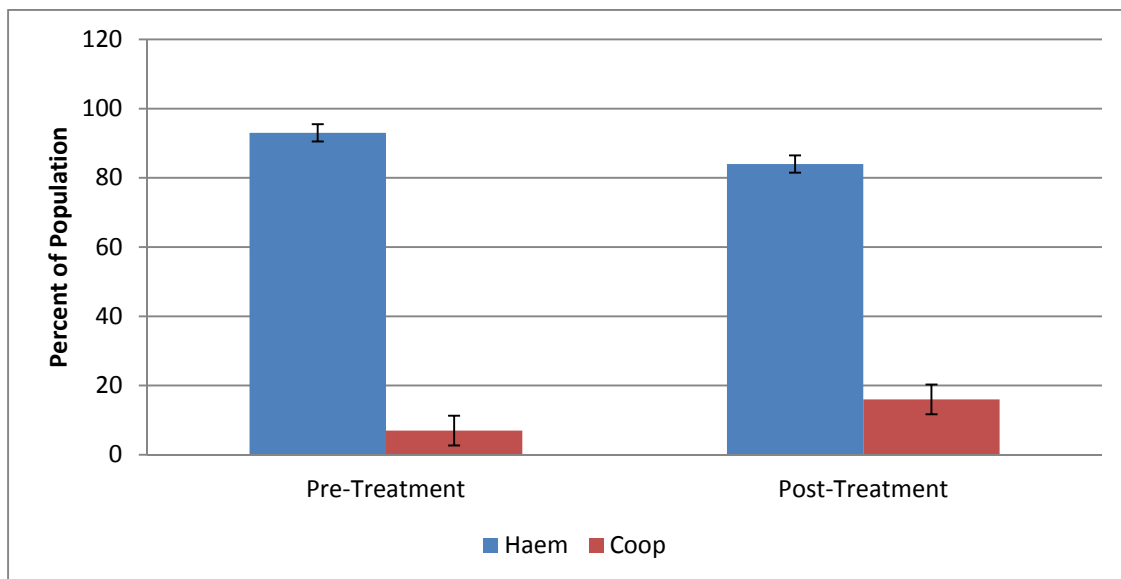


Figure 2.3. Mean (SEM) pre-treatment (Week 0) and post-treatment (Weeks 1-6 combined) fecal larval population distribution of giraffe (*Giraffa camelopardalis*, n=7) at Busch Gardens Tampa after a treatment with 25 g copper oxide wire particles. Difference was not significant ( $p \geq 0.05$ ). Haem= *Haemonchus*, Coop= *Cooperia*.

For the 12.5 g dose pre-treatment mean FEC for the 7 giraffe was 680 EPG and ranged from 375 EPG to 1075 EPG (Figure 2.4). FEC was significantly reduced for Week 2 ( $p = 0.0055$ ), but not ( $p \geq 0.05$ ) for Weeks 1 and 3-6 post-treatment. FECR was 43.9%, 53.0%, 9.1%, 39.4%, 0%, and 0% for Weeks 1-6, respectively. FECR was not significant ( $p \geq 0.05$ ) for mean post-treatment (Weeks 1-6) FEC compared to pre-treatment (Week 0) FEC (Figure 2.5).

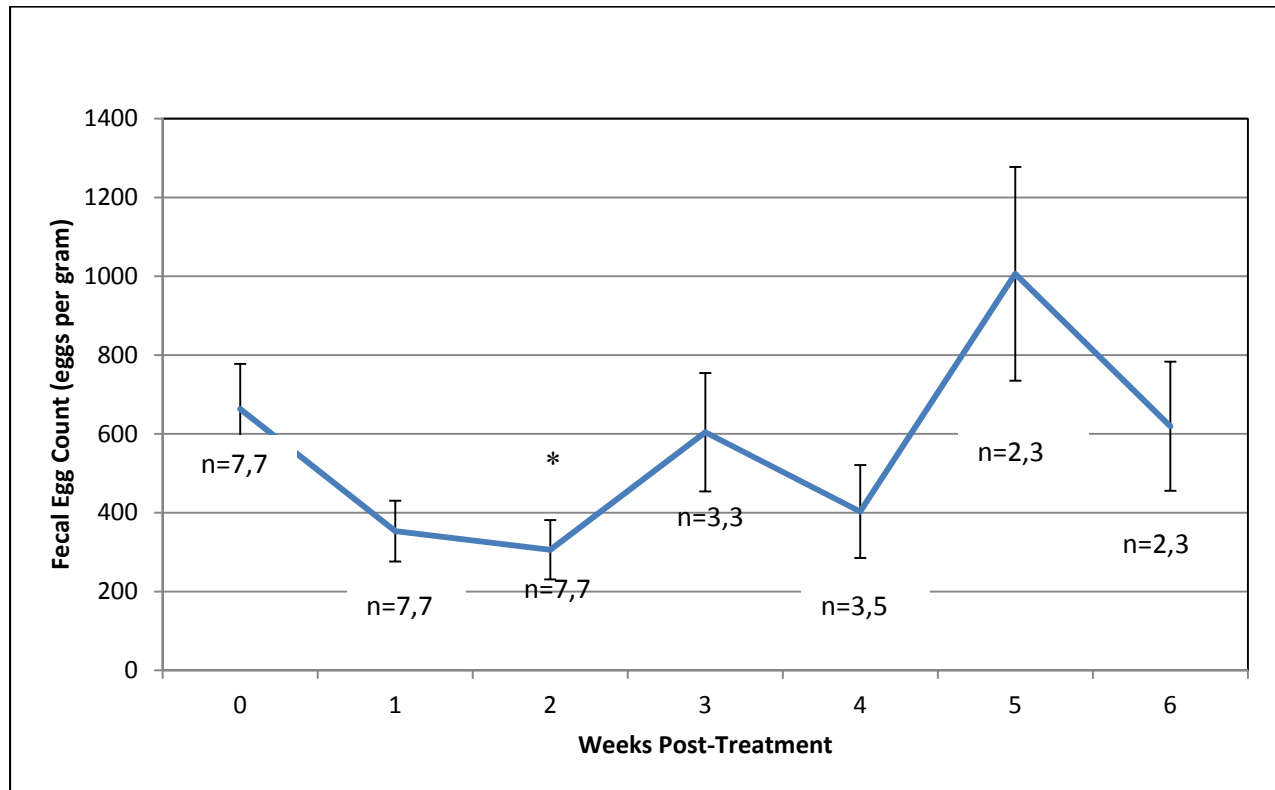


Figure 2.4. Mean (SEM) fecal egg count of giraffe (*Giraffa camelopardalis*,  $n=7$ ) at Busch Gardens Tampa after treatment with 12.5 g copper oxide wire particles. \*= significant ( $p < 0.05$ ) reduction compared to Week 0. n = total animals Busch Gardens Tampa, Louisiana State University

Fecal cultures showed that mean pre-treatment larval distribution was 86% *Haemonchus* (range 73-100%) and 14% *Cooperia* (range 0-27%) and post-treatment distribution was 89 % *Haemonchus* ( $n=7$ ; range 79-100) and 11% *Cooperia* ( $n=7$ ; range 0-21; Figure 2.6).

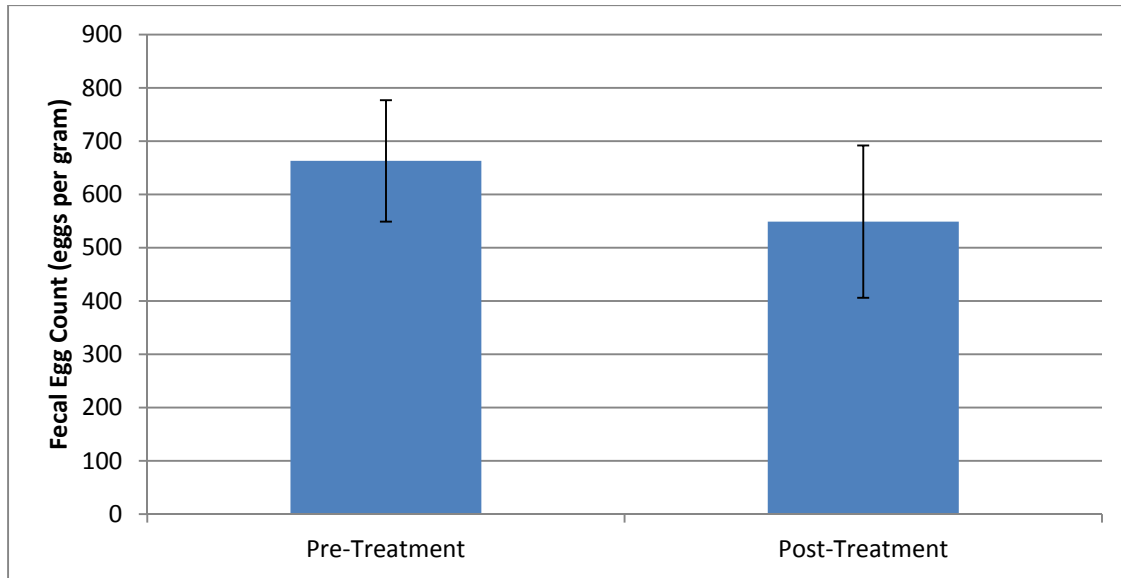


Figure 2.5. Mean (SEM) pre-treatment (Week 0) and post-treatment (Weeks 1-6 combined) fecal egg count of giraffe (*Giraffa camelopardalis*, n=7) at Busch Gardens Tampa after treatment with 12.5 g copper oxide wire particles. Difference was not significant ( $p \geq 0.05$ ).

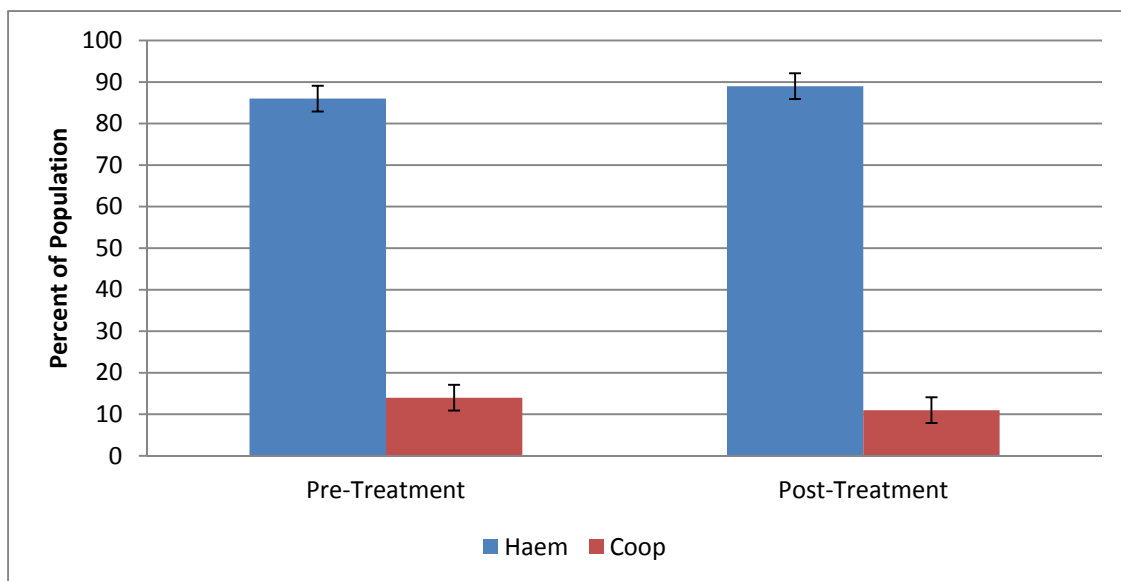


Figure 2.6. Mean (SEM) pre-treatment (Week 0) and post-treatment (Weeks 1-6 combined) fecal larval population distribution of giraffe (*Giraffa camelopardalis*, n=7) at Busch Gardens Tampa after a treatment with 12.5 g copper oxide wire particles. Difference was not significant ( $p \geq 0.05$ ). Haem= *Haemonchus*, Coop= *Cooperia*.

For the 6.3 g treatment there was no significant ( $p \geq 0.05$ ) reduction in FEC any time (Figure 2.7). FECR was not significant ( $p \geq 0.05$ ) for mean post-treatment (Weeks 1-4) FEC compared to pre-treatment (Week 0) FEC (Figure 2.8).

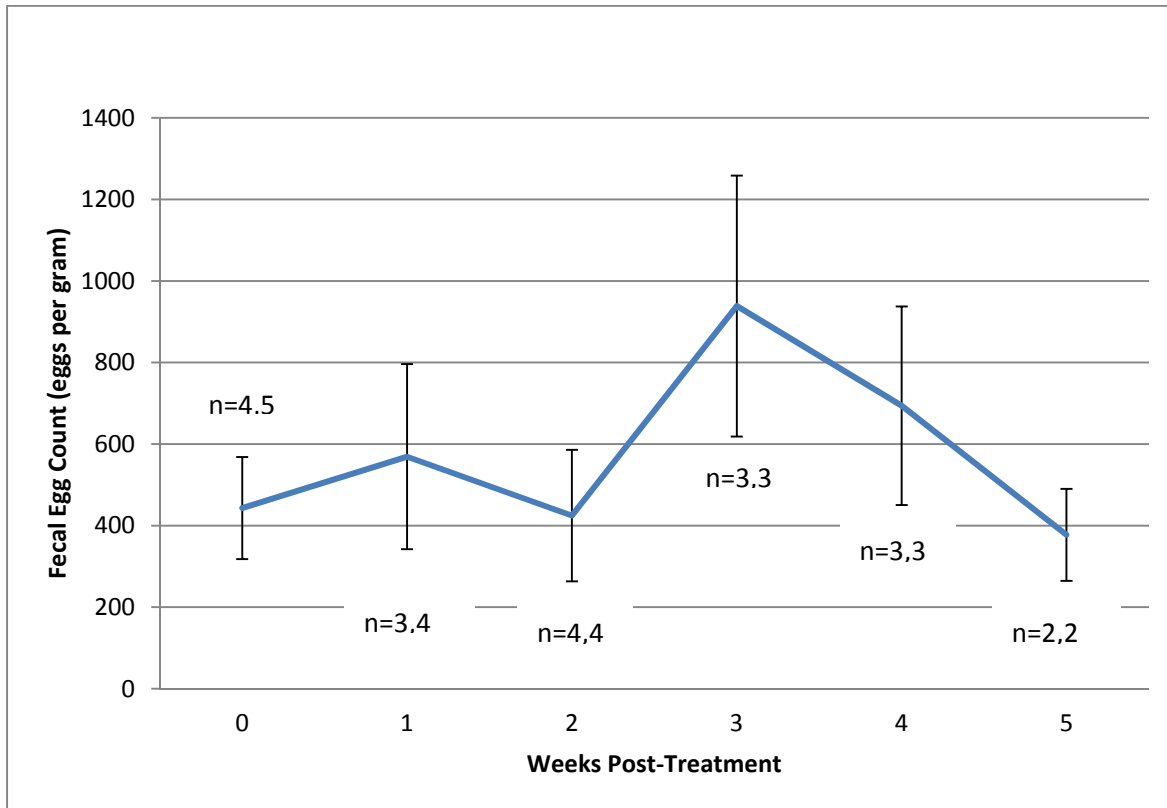


Figure 2.7. Mean (SEM) fecal egg count of giraffe (*Giraffa camelopardalis*,  $n=5$ ) at Busch Gardens Tampa after treatment with 6.3 g copper oxide wire particles. Difference was not significant ( $p \geq 0.05$ ) for any weeks post-treatment.  $n$  = total animals Busch Gardens Tampa, Louisiana State University

Fecal cultures showed that pre-treatment larval distribution was 97% *Haemonchus* (range 89-100%) and 3% *Cooperia* (range 0-11%); post-treatment distribution was 95% *Haemonchus* (range 84-100%) and 5% *Cooperia* (range 0-16%; Figure 2.9).



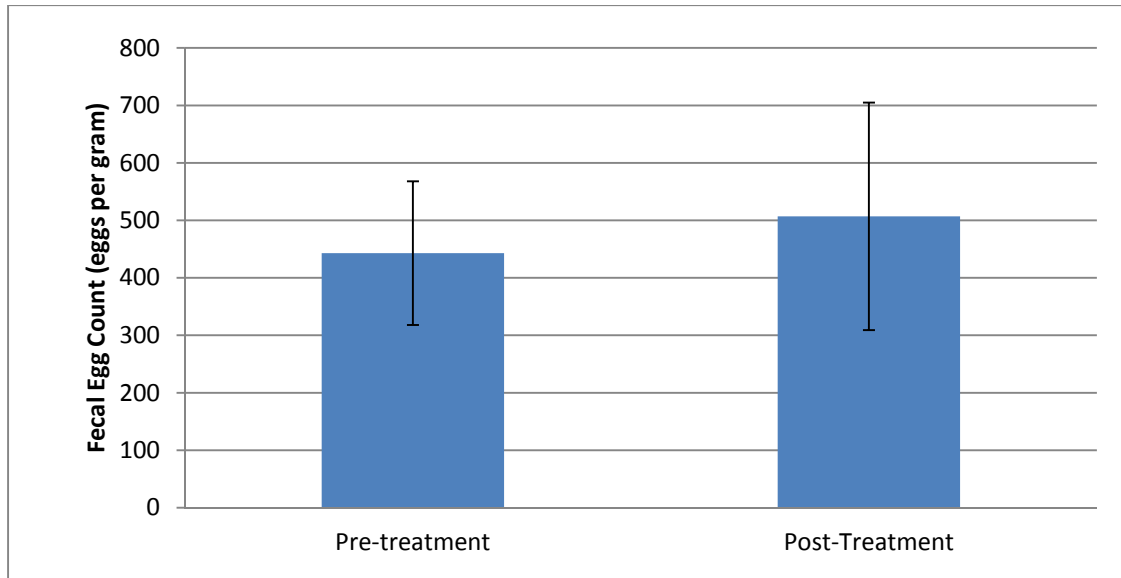


Figure 2.8. Mean (SEM) pre-treatment (Week 0) and post-treatment (Weeks 1-6 combined) fecal egg count of giraffe (*Giraffa camelopardalis*, n=5) at Busch Gardens Tampa after treatment with 6.3 g copper oxide wire particles. Difference was not significant ( $p \geq 0.05$ ).

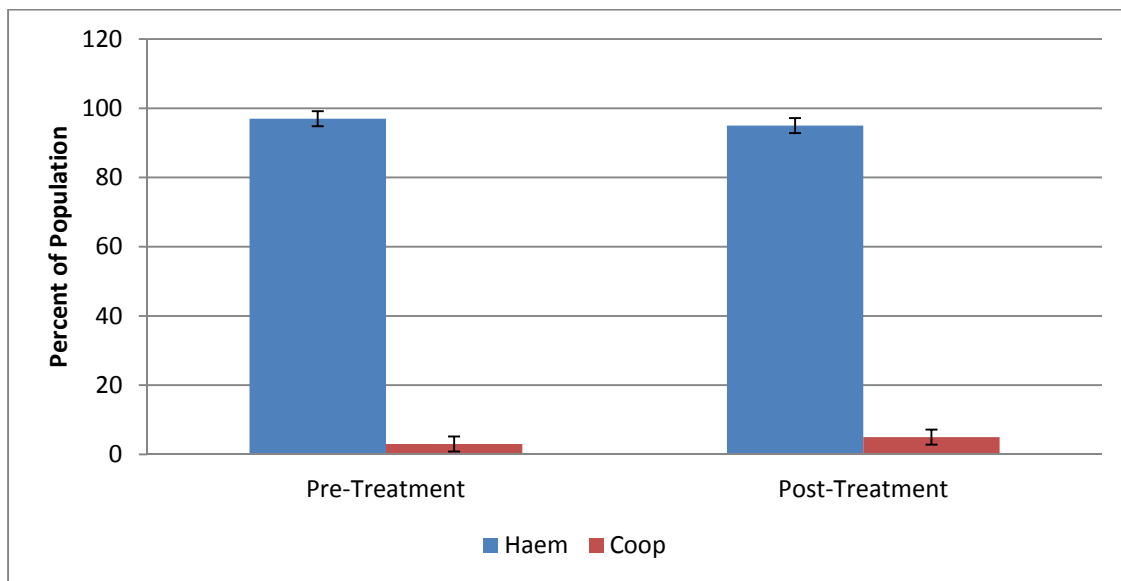


Figure 2.9. Mean (SEM) pre-treatment (Week 0) and post-treatment (Weeks 1-6 combined) fecal larval population distribution of giraffe (*Giraffa camelopardalis*, n=5) at Busch Gardens Tampa after a treatment with 6.3 g copper oxide wire particles. Difference was not significant ( $p \geq 0.05$ ). Haem= *Haemonchus*, Coop= *Cooperia*.

## 2.4 Discussion

Results from this study indicated that COWP can be used as a part of a control program for *Haemonchus* in giraffe. Overall, the FECs conducted at LSU and BGT were similar, but in some cases they were quite different. These differences could be related to storage and packaging conditions associated with shipping, improper sample handling, and/or technique. Samples were shipped with cold packs in insulated containers, but temperature differences during transport could have allowed some eggs to hatch, thus decreasing the number of eggs available. It is also possible that samples left at room temperature before being refrigerated and problems with refrigeration (temperature too high or too low) could affect egg counts as well (DeLisi et al, 2008). Samples being processed by either technique should be read quickly; failure to do this would leave eggs in a hypertonic solution, which can result in crenation and falsely low FEC (J.E. Miller, personal communication).

Fecal sample numbers varied with each week, as samples were not always collected on a weekly basis. These missing data points were accounted for in the statistical analysis. Regardless, based on the lack of significant difference between techniques, either technique would be acceptable; however, any decision to administer treatment (anthelmintics or COWP) should be based on a consistent technique.

The 25 g COWP treatment significantly reduced FEC from Weeks 2-5. The Week 1 and 6 reductions were not significant, but the FECR was similar to that of the other weeks. This was probably due to the large variation on Weeks 1 and 6 compared to the other weeks. One animal at Week 1 had a very high FEC, possibly due to decreased gut motility slowing the progression of COWP into the abomasum so that not enough of the COWP was present to have an effect by Week 1, but by Week 2 there was enough in the abomasums to see the effect. At Week 6, animals would be expected to have experienced reinfection and because individuals acquire infection at different rates, that could have accounted for the large variation. This is consistent with other studies that indicated the period of activity of COWP is probably within the first 2 weeks of administration (Burke et al, 2006). This, coupled with the prepatent period of about 3 weeks, would result in increasing FEC due to reinfection about 6 weeks after treatment.

The 12.5 g treatment significantly reduced FEC only at Week 2. Week 1 was approaching significance ( $p = 0.07$ ), and as with the 25 g treatment, one animal had a high FEC at Week 1 that decreased at Week 2, so reduced motility could have slowed the effect of COWP. Because FEC returned to pre-treatment levels from Weeks 3-6, the indication was that 12.5 g was sufficient to provide a short term effect that dissipated quickly. This could also be related to study design and seasonality of GIN parasites. The design of this study allowed for variation in administration times, according to FEC level (Table 2.1). This is similar to the manner in which treatment administration decisions are made in practice. Seasonal differences including temperature and moisture variations can account for some differences in FECR over time (O'Connor et al, 2006). The 6.3 g treatment did not affect infection at any time and should not be considered in control programs for giraffe.

Table 2.1. Calendar for copper oxide wire particle administration for giraffe (*Giraffa camelopardalis*) at Busch Gardens Tampa. D1 = first treatment dose, 25 g. D2 = second treatment dose, 12.5 g. D3 = third treatment dose, 6.3 g.

	October				November					December				January					Feb
Week	1	2	3	4	1	2	3	4	5	1	2	3	4	1	2	3	4	5	1
Anml																			
1			D1					D2								D3			
2						D1										D2			
3		D1					D2							D3					
4						D1								D2					D3
5										D1						D2			
6		D1						D2						D3					
7		D1					D2									D3			

FECR was acceptable for the 25 g dose, marginal (only the first 2 weeks post-treatment) for the 12.5 g dose and unacceptable for the 6.3 g dose. There were times when FECR for individual animals could have been influenced by timing of treatment. In one case (12.5 g dose), the pre-treatment FEC was 650 EPG, but when the treatment was administered it was 375 EPG. This was due to a delay in treatment following the attending veterinarian order to treat. The FEC continued to decrease over the time of the delayed treatment, but the reduction was not

significant. Because the fecal sample was processed at the time of treatment, the result was not yet known. If it had been known, that animal would not have been treated at that time.

Accounting for animal variation can be difficult in a study such as this. Animals used in this study varied in age, reproductive state, and health maintenance histories. Three had calves prior to administration of the 25 g COWP treatment (4 months, 3 months, and 2.5 months pre-treatment), and four were open. Three were treated with anthelmintics (albendazole and levamisole) 10 weeks, 13 weeks, and 14 weeks, respectively, prior to administration of the 25 g COWP treatment. All presented with clinical signs of GIN parasitism during the time of study, including edema around the jaw (bottle jaw), weakness, loose stool, weight loss and decreased appetite.

One giraffe died one month following administration of a 6.3 g COWP treatment. Liver samples collected during necropsy tested within normal range for copper content, indicating copper toxicity was not the cause of death.

These results indicated that only the 25 g dose of COWP effectively controlled *Haemonchus* in giraffe, and could be included in a GIN control program if *Haemonchus* is the GIN of concern. As with any treatment, animal compliance issues can complicate administration, and care should be taken to ensure that animals receive the treatment amounts at the correct time to prevent misuse.

## **CHAPTER THREE**

### **POTENTIAL MECHANISM OF ACTION OF COPPER OXIDE WIRE PARTICLES FOR CONTROL OF *HAEMONCHUS CONTORTUS***

#### **3.1 Introduction**

The primary use of copper oxide wire particles (COWP) is for supplementation of copper deficient animals, but COWP have been used to control *Haemonchus contortus* in sheep (Knox, 2002; Burke et al, 2004), and goats (Burke et al, 2008; Soli et al, 2010), and *Haemonchus* species in several exotic hoofstock species including giraffe (Garretson et al, 2009), scimitar-horned oryx (*Oryx dama*), blackbuck (*Antelope cervicapra*), roan antelope (*Hippotragus equines*), and blesbok (*Damaliscus pygargus phillipsi*; Fontenot, 2008).

Despite substantial evidence of the effectiveness of COWP in controlling *Haemonchus* spp., an extensive review of the literature revealed that the mechanism of action (MoA) is not known. If the MoA is a direct effect on the nematode, it could be associated with an interaction of COWP resulting in physical damage to the cuticle which can be visually assessed using electron microscopy, by quantification of copper content of nematodes, and/or the presence of elemental copper detected along the surface of the cuticle. If the MoA is an indirect effect on the nematode, it could be associated with an alteration in the abomasal environment such that it was no longer suitable for habitation.

The objective of this study was to evaluate a potential MoA of COWP by evaluating whether there may be direct or indirect effects on the nematode.

#### **3.2 Materials and Methods**

##### **3.2.1 Location and Animals**

Twelve Suffolk lambs were removed from pasture at the Louisiana State University (LSU) Ben Hur Research Farm Sheep Unit and transferred to the LSU School of Veterinary Medicine (LSU-SVM), in Baton Rouge, Louisiana. They were maintained at the LSU-SVM in concrete pens with wood shavings, in groups of two animals per pen (LSU IACUC protocol #06-094).

### **3.2.2 Study design**

The 12 lambs were initially dewormed with albendazole (Valbazen, 10 mg/kg) and levamisole (Levisole, 8 mg/kg). Residual infection was monitored weekly by fecal egg count (FEC) and blood packed cell volume (PCV). After a 3 week acclimation and stabilization period, abomasal fistulas were surgically created in each lamb. After a 3 week surgery recovery period, each lamb was inoculated with *H. contortus* infective larvae (L3). Infection was allowed to mature for 5 weeks. Initial baseline fecal (-12 and 0 hr) and blood (-12 hr) samples were collected and abomasal content with nematodes collection (through fistula) commenced at 0 hr. All lambs were then given 2 g of COWP orally in a gelatin capsule. Subsequently, abomasal, fecal and blood samples were collected at 12, 36, 60, and 84 hours post-treatment (HPT).

### **3.2.3 Fecal Samples**

Fecal samples were collected directly from the rectum of the lambs. FEC was determined using a Modified McMaster technique (Whitlock, 1948; see Appendix C). FEC was expressed as eggs per gram (EPG) of feces.

### **3.2.4 Blood Samples**

Blood was collected from the jugular vein and stored in purple top EDTA vacutainer tubes (BD Vacutainer® Glass Whole Blood Tubes, Becton, Dickinson, and Company, Franklin Lakes, NJ). After inverting the tubes several times to ensure thorough mixing of the whole blood, microhematocrit capillary tubes were filled, sealed with crit-o-seal, and centrifuged for five minutes in a microhematocrit centrifuge (Autocrit Ultra 3 Microhematocrit Centrifuge, Becton, Dickson and Company). PCV was determined by reading directly from the scale in the centrifuge.

### **3.2.5 Fistula Placement**

Fistulas (approximately 1.5 cm in diameter) were surgically created by suturing an incision through the right side exterior abdominal wall to an incision into the abomasum. The fistula was oriented slightly downward, covered with a bandage and the entire abdomen was wrapped to protect the open fistula. At the start of the study, one fistula was no longer patent and that lamb was removed from the study.

### **3.2.6 Inoculation with L3**

At time 0 hr, each lamb was inoculated with 15,000 *H. contortus* L3. The L3 were recovered from fecal cultures of other sheep at the LSU Sheep Farm and were verified as 100% *Haemonchus*.

### **3.2.7 Abomasal Contents and pH Measurement**

Abomasal contents were collected by insertion of a 25 ml sterile plastic pipette approximately 1.0 cm in diameter through the open fistula and sliding it along the mucosa several times. Abomasal contents were drained by gravity from the pipettes into sterile 50 ml centrifuge tubes and labeled for each animal. Paper pH strips (ColorpHast® plastic pH indicator strips, EMD Millipore, Merck KGaA, Darmstadt, Germany) were used to measure the pH of the contents.

### **3.2.8 Nematode Recovery and Processing**

Nematodes were recovered and counted by hand-picking them from water-diluted abomasal contents using tuberculin syringes with angled needles under a dissecting microscope. Nematodes were preserved for electron microscopy in a 1.25% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate (CAC) buffer solution, or frozen in a phosphate-buffered saline (PBS) solution for copper content analysis.

### **3.2.9 Electron Microscopy**

Two nematodes from each collection time (0, 12, 36, 60, and 84 hrs) were processed for scanning (SEM; FEI model Quanta 200; see Appendix E) and transmission (TEM; JEOL model JEM-1011; see Appendix F) electron microscopy at the Microscopy Center at the LSU-SVM. TEM samples were cut for cross-sectional (0, 12, 36, 60, and 84 hrs) or longitudinal (0, 12, and 36 hrs) viewing. SEM is used to view three dimensional features of samples up to 5 inches in size. SEM lenses generate a focused spot of electrons that is scanned over the surface of an electrically conductive specimen. The electrons strike the specimen, producing secondary electrons from the uppermost layer of the specimen. These secondary electrons are translated into a series of pixels, which blend to form what appears to be a continuous picture on the monitor. The brightness of the pixels is directly related to the number of secondary electrons

generated from the surface, and the image displayed in shades of gray gives the appearance of depth (Bozzola and Russell, 1992). TEM is used to view ultrastructures in thin slices of specimens. TEMs function similarly to light microscopes. A stream of electrons is focused through a specimen, and through a series of lenses produces a magnified image. The image is projected onto a viewing screen coated with phosphorescent zinc-activated cadmium sulfide powder, light is produced, and the image can be seen. Darker areas of the image indicate a denser area where fewer electrons have passed through the specimen; lighter areas of the image indicate a less dense area where more electrons have been transmitted through the specimen (Bozzola and Russell, 1992).

Elements present at 5-6 spots on the surface of the two nematodes at 0, 12 and 84 hrs were identified by Energy Dispersive X-ray Spectroscopy (EDS). EDS allows for chemical analysis of a specimen using a stream of electrons, based on the fact that each element is atomically unique. The electron beam interacts with outer shell electrons within an element in the sample, and the excitement causes a release of electrons from low-energy electron shells. This ejection of electrons temporarily causes an electron void that is filled with electrons from higher energy shells. The energy difference from the low-energy shell and high-energy shell electron exchange is measureable by energy-dispersive spectrometer. This energy difference is unique to its respective element, allowing for elemental identification (Bozzola and Russell, 1992; Goldstein et al, 2003).

### **3.2.10 Nematode Copper Content**

Nematodes were pooled to obtain at least 500 mg from each collection. Nematodes were processed according to established procedures and analyzed for copper content by the Louisiana Animal Disease Diagnostic Laboratory in Baton Rouge, Louisiana.

## **3.3 Results**

### **3.3.1 Fecal Egg Count and Packed Cell Volume**

Mean FEC for the 11 lambs at -12, 0, 12, 36, 60, and 84 HPT was 6096, 5155, 5327, 4318, 2782, and 2105 EPG, respectively (Figure 3.1). FEC decreased continually from 12 to 84 HPT.



Mean PCV for the 11 sheep at -12, 12, 36, 60, and 84 HPT was 22.2, 22.0, 23.0, 21.6, and 22.5, respectively (Figure 3.2). Blood was not taken for PCV at 0 hr. There was no obvious change in PCV over the period of the study.

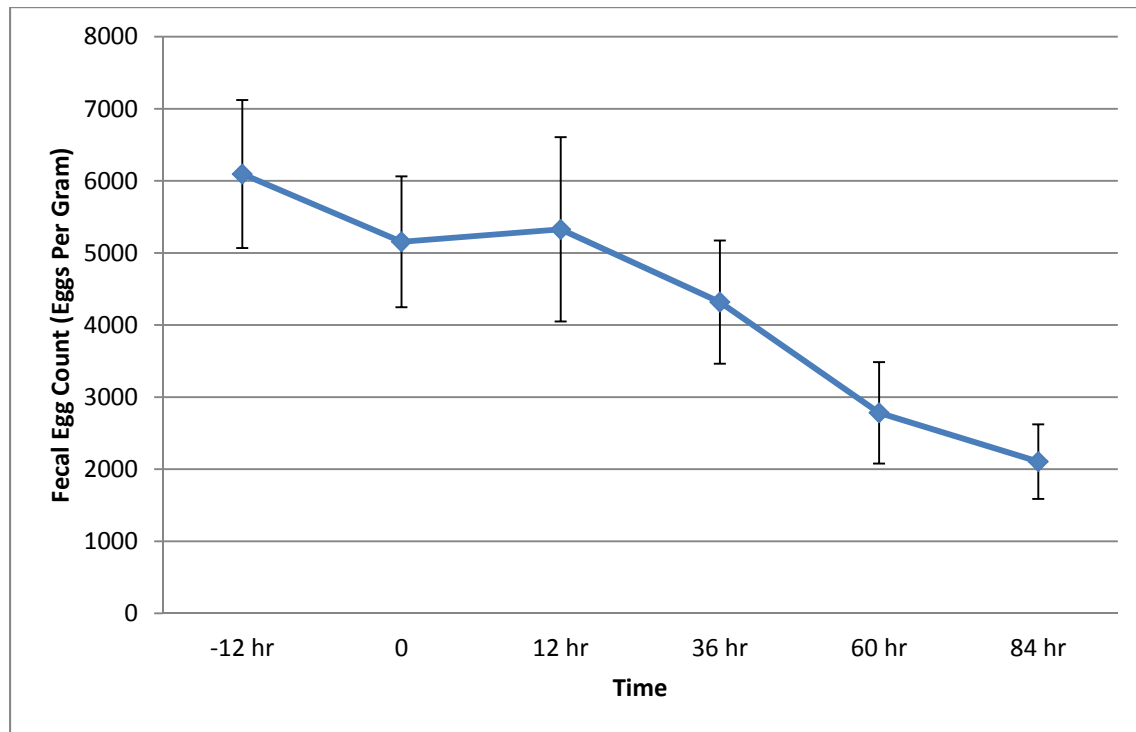


Figure 3.1. Mean (SEM) fecal egg count of lambs (n = 11) treated with 2 g copper oxide wire particles.

### 3.3.2. Abomasal Content pH

Mean abomasal content pH is shown in Figure 3.3. Content pH ranged from 2.5 to 5.0 for 0 hr samples; 2.5 to 6.0 for 12 HPT samples; 2.5 to 5.5 for 36 HPT samples; 3.0 to 6.0 for 60 HPT samples; and 3.0 to 6.0 for 84 HPT samples.

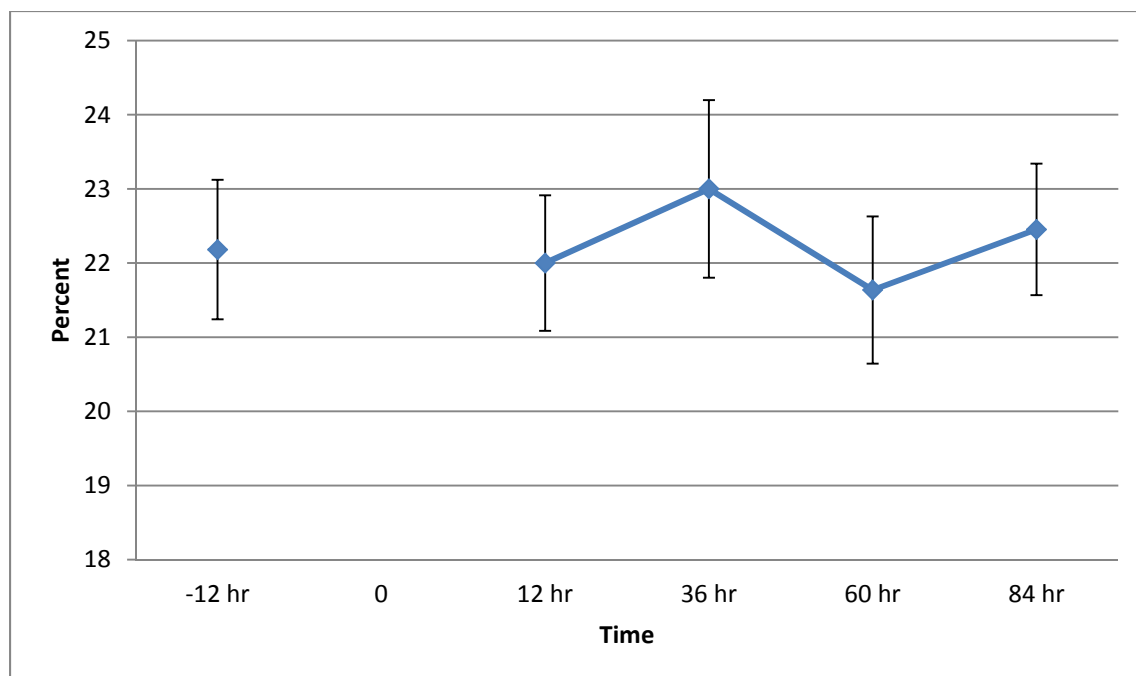


Figure 3.2. Mean (SEM) packed cell volume of lambs (n = 11) treated with 2 g copper oxide wire particles.

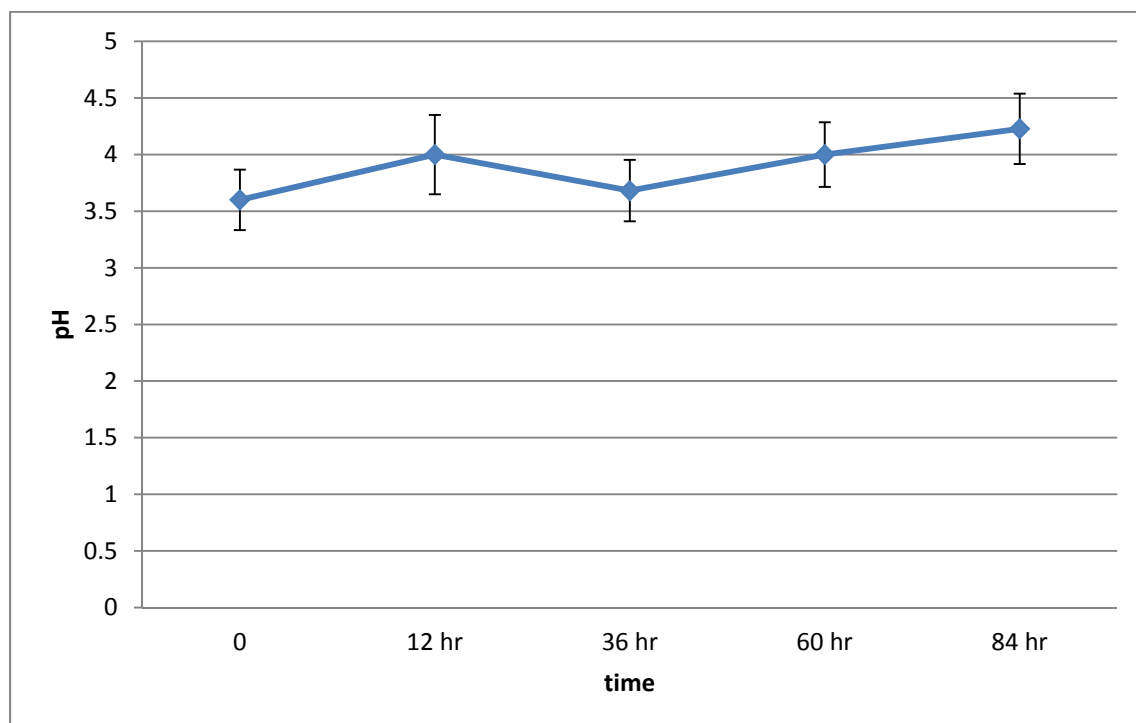


Figure 3.3. Mean (SEM) pH of abomasal contents of lambs (n = 11) treated with 2 g copper oxide wire particles.

### 3.3.3 Scanning Electron Microscopy

Adult *H. contortus* viewed with standard SEM showed no obvious cuticular differences between pre-treatment (0 hr) and PT samples. Anterior views of pre-treatment and 84 HPT samples are shown in Figure 3.4. Lateral views of pre-treatment and 36 HPT are shown in Figure 3.5.

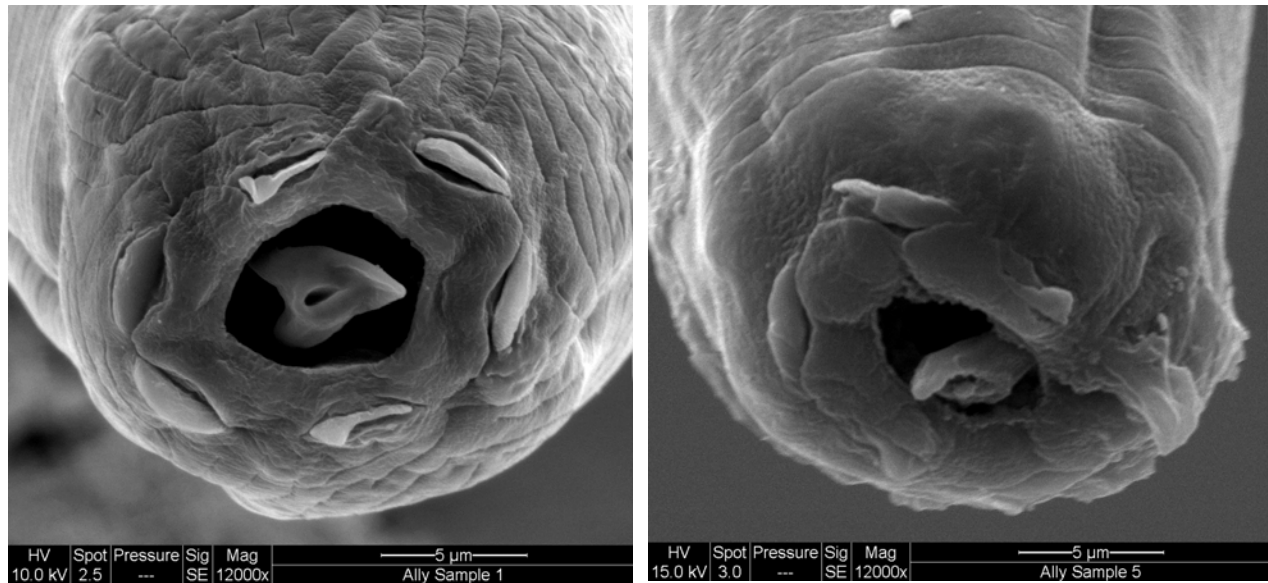


Figure 3.4. Scanning electron microscopy images of anterior end of *Haemonchus contortus*, collected from abomasal contents of lambs. Pre- (left) and 84 hrs post- (right) treatment with 2 g copper oxide wire particles.

### 3.3.4 Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy

EDS showed copper (Cu) present in 10 of 11 post-treatment and 0 of 5 pre-treatment spots tested. No other identified elements were present in post-treatment samples and absent in pre-treatment samples.

Pre-treatment *H. contortus* showed no copper registered in any of the 5 spots checked (Figure 3.6). *H. contortus* at 12 HPT showed copper in 4 of the 5 spots checked (Figure 3.7). *H. contortus* at 84 HPT showed copper in all 6 spots that were checked (Figure 3.8).

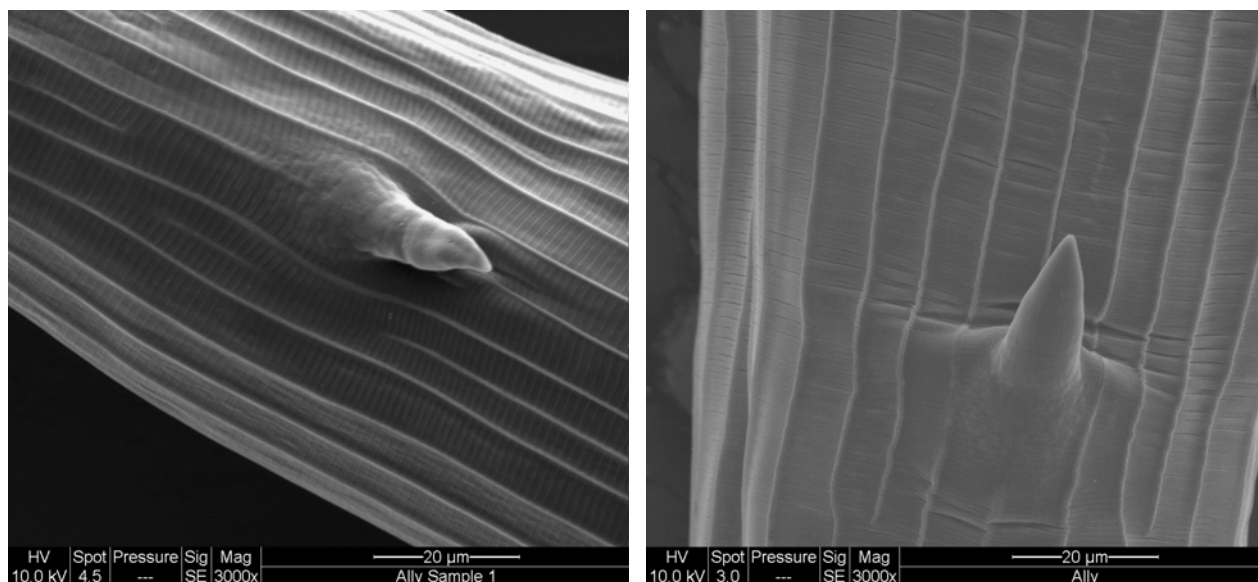


Figure 3.5. Scanning electron microscopy images of lateral view of *Haemonchus contortus*, collected from abomasal contents of lambs. Pre- (left) and 36 hrs post- (right) treatment with 2 g copper oxide wire particles.

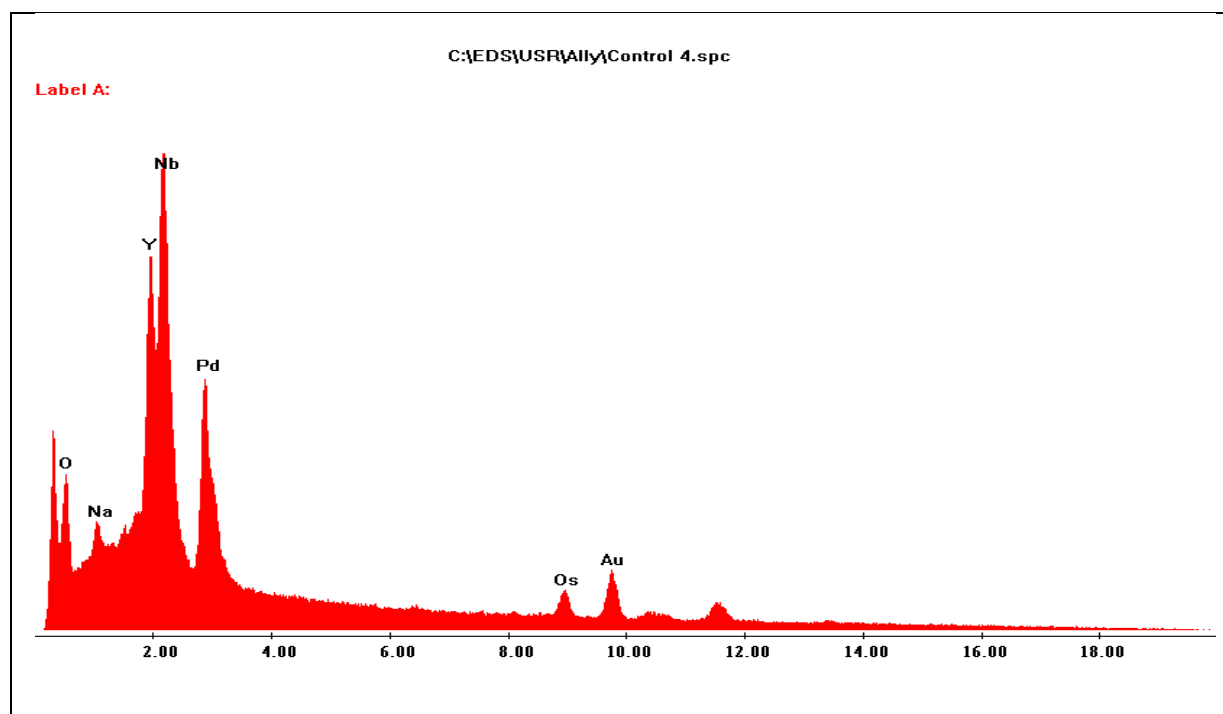


Figure 3.6. Elements identified by energy dispersive x-ray spectroscopy at a single spot on an adult *Haemonchus contortus* collected from abomasal contents of a lamb before treatment with 2 g copper oxide wire particles.

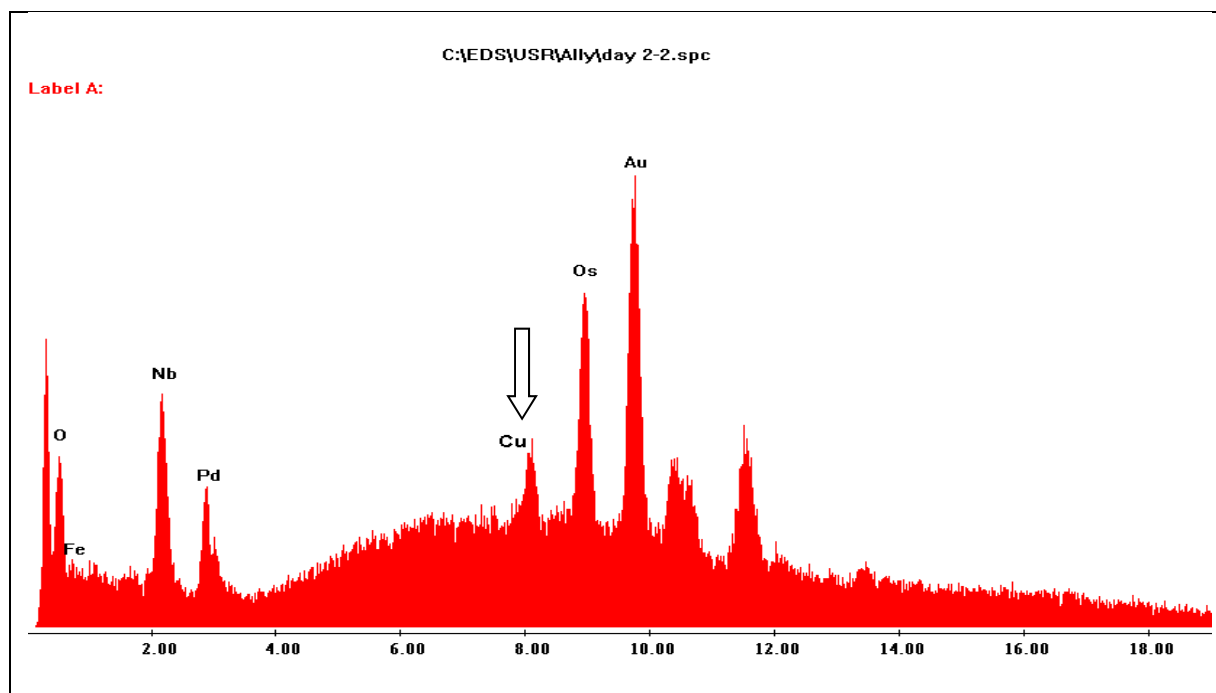


Figure 3.7. Elements identified by energy dispersive x-ray spectroscopy at a single spot on an adult *Haemonchus contortus* collected from abomasal contents of a lamb 12 hours after treatment with 2 g copper oxide wire particles. Arrow indicates copper.

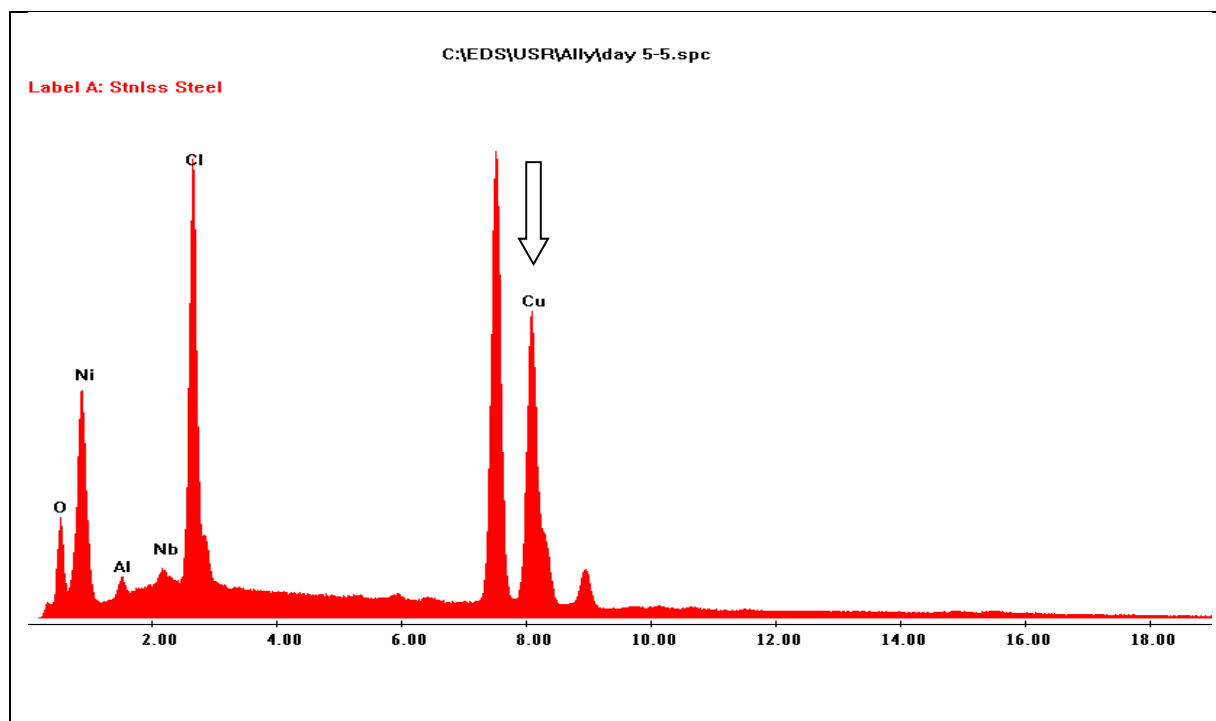


Figure 3.8. Elements identified by energy dispersive x-ray spectroscopy showing at a single spot on an adult *Haemonchus contortus* collected from abomasal contents of a lamb 84 hours after treatment with 2 g copper oxide wire particles. Arrow indicates copper.

### 3.3.5 Transmission Electron Microscopy

Cross-sectional aspects of pre-treatment adult *H. contortus* showed no cuticle damage (Figure 3.9). Cuticle lesions were present on all PT nematodes (Figures 3.10-3.13). Cuticle lesions on PT worms were visually consistent in depth, but varied in number. The frequency of cuticle lesions was maximal at 12 hours PT, and decreased as time progressed.

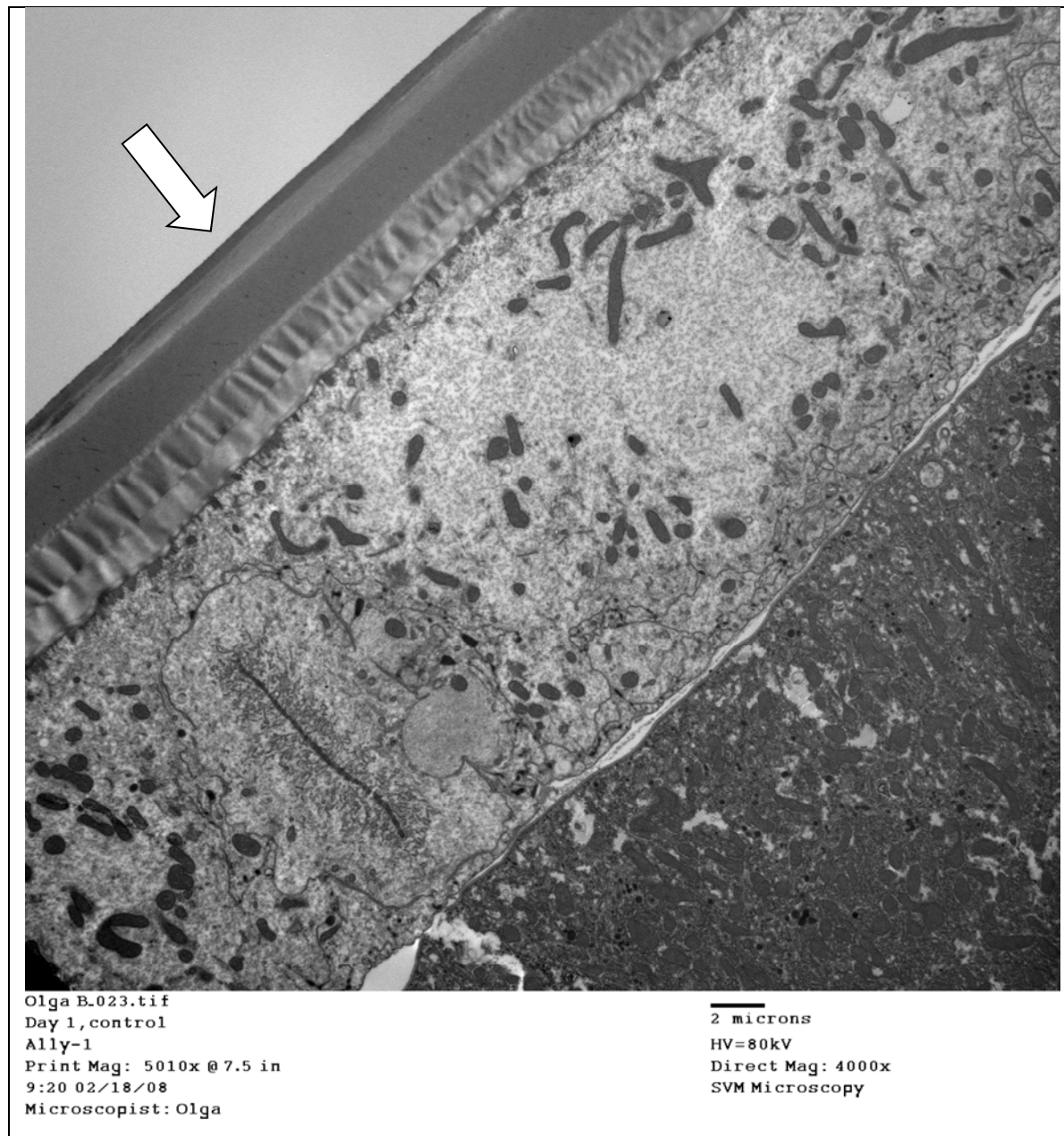


Figure 3.9. Cross-sectional transmission electron microscopy image of *Haemonchus contortus* adult collected from abomasal contents of a lamb before administration of a 2 g copper oxide wire particle treatment. 4000X Magnification. Cuticle appears unaffected (arrow).

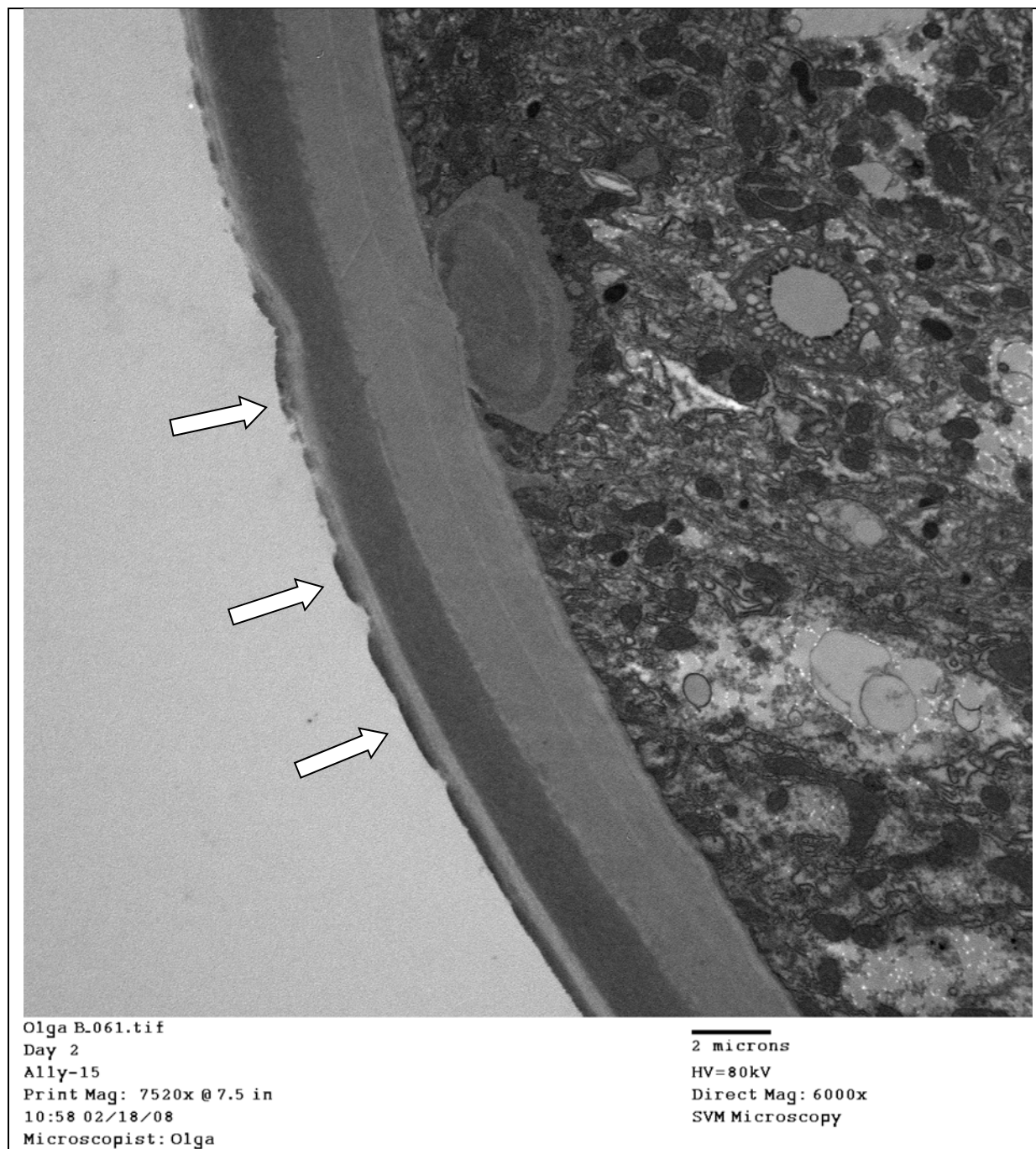
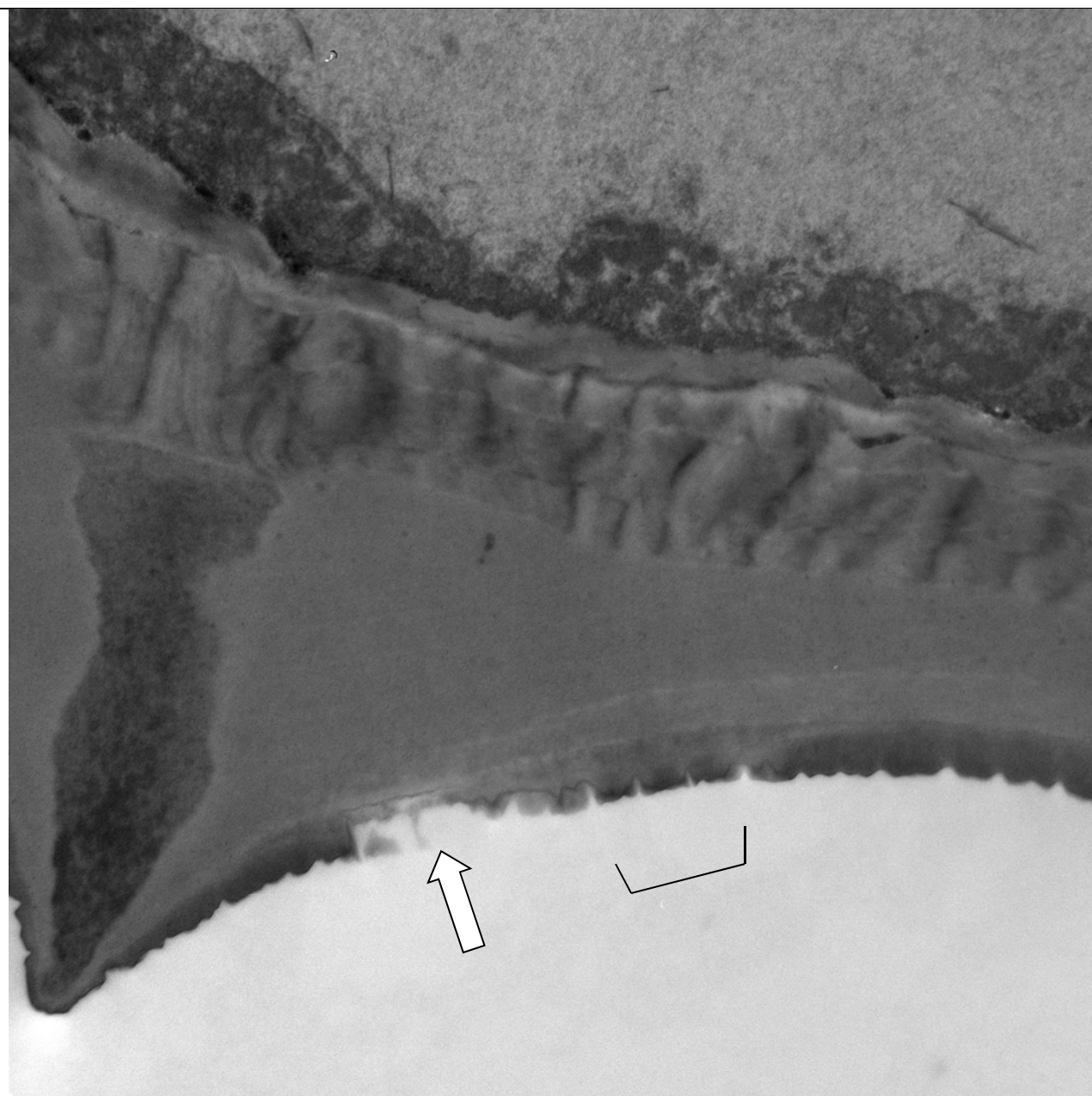


Figure 3.10. Cross-sectional transmission electron microscopy image of *Haemonchus contortus* adult collected from abomasal contents of a lamb 12 hours after administration of a 2 g copper oxide wire particle treatment. 6000X Magnification. Cuticle shows multiple affected areas (arrows).



Olga B.109.tif

Day 3

Ally-13

Print Mag: 25200x @ 7.5 in

10:47 02/21/08

Microscopist: Olga

500 nm

HV=80kV

Direct Mag: 15000x

SVM Microscopy

Figure 3.11. Cross-sectional transmission electron microscopy image of *Haemonchus contortus* adult collected from abomasal contents of a lamb 36 hours after administration of a 2 g copper oxide wire particle treatment. 15000X Magnification. Cuticle shows affected area (arrow). Cuticle differences due to angle from projected skeletal rod are also shown (bracket).



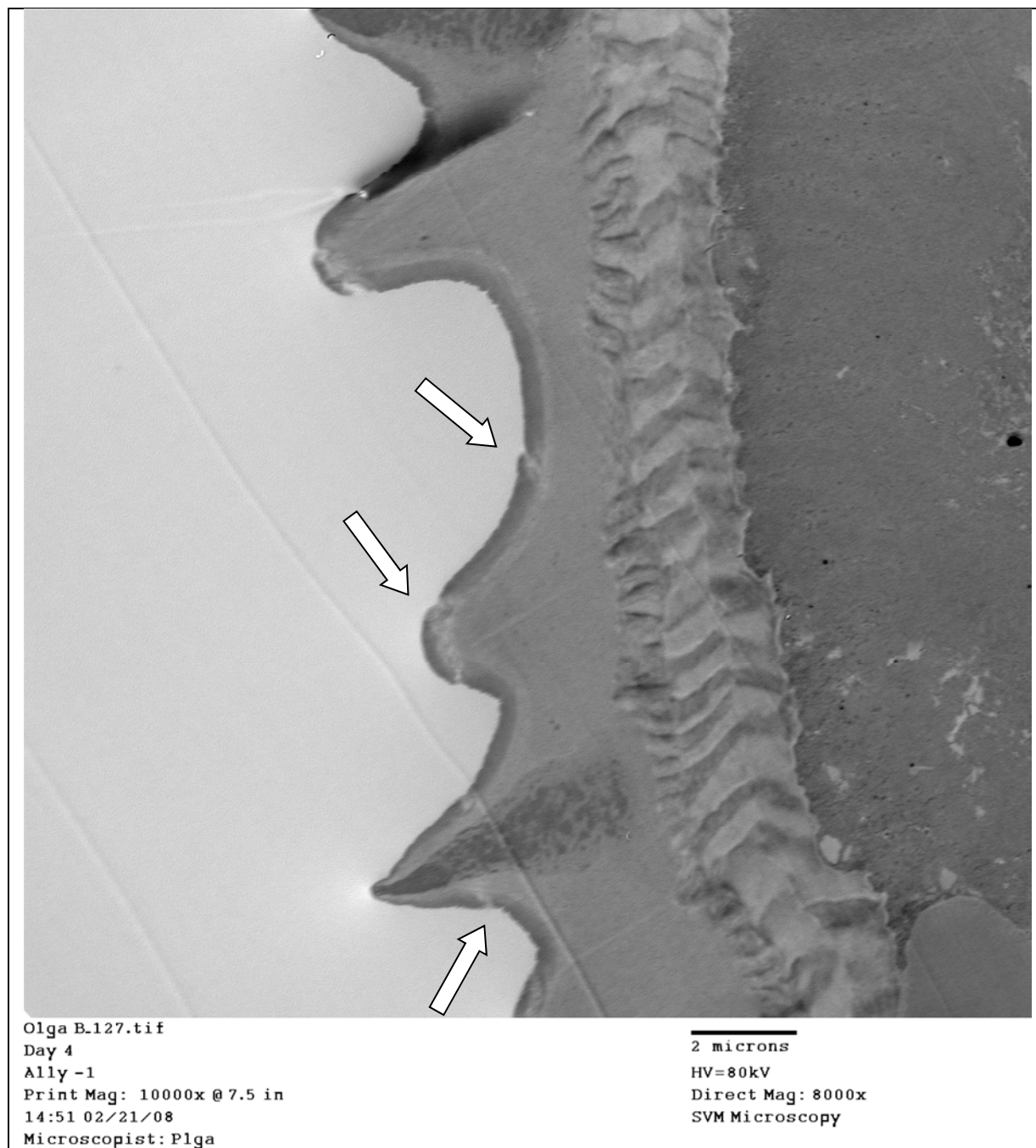


Figure 3.12. Cross-sectional transmission electron microscopy image of *Haemonchus contortus* adult collected from abomasal contents of a lamb 60 hours after administration of a 2 g copper oxide wire particle treatment. 8000X magnification. Cuticle shows affected areas (arrows).

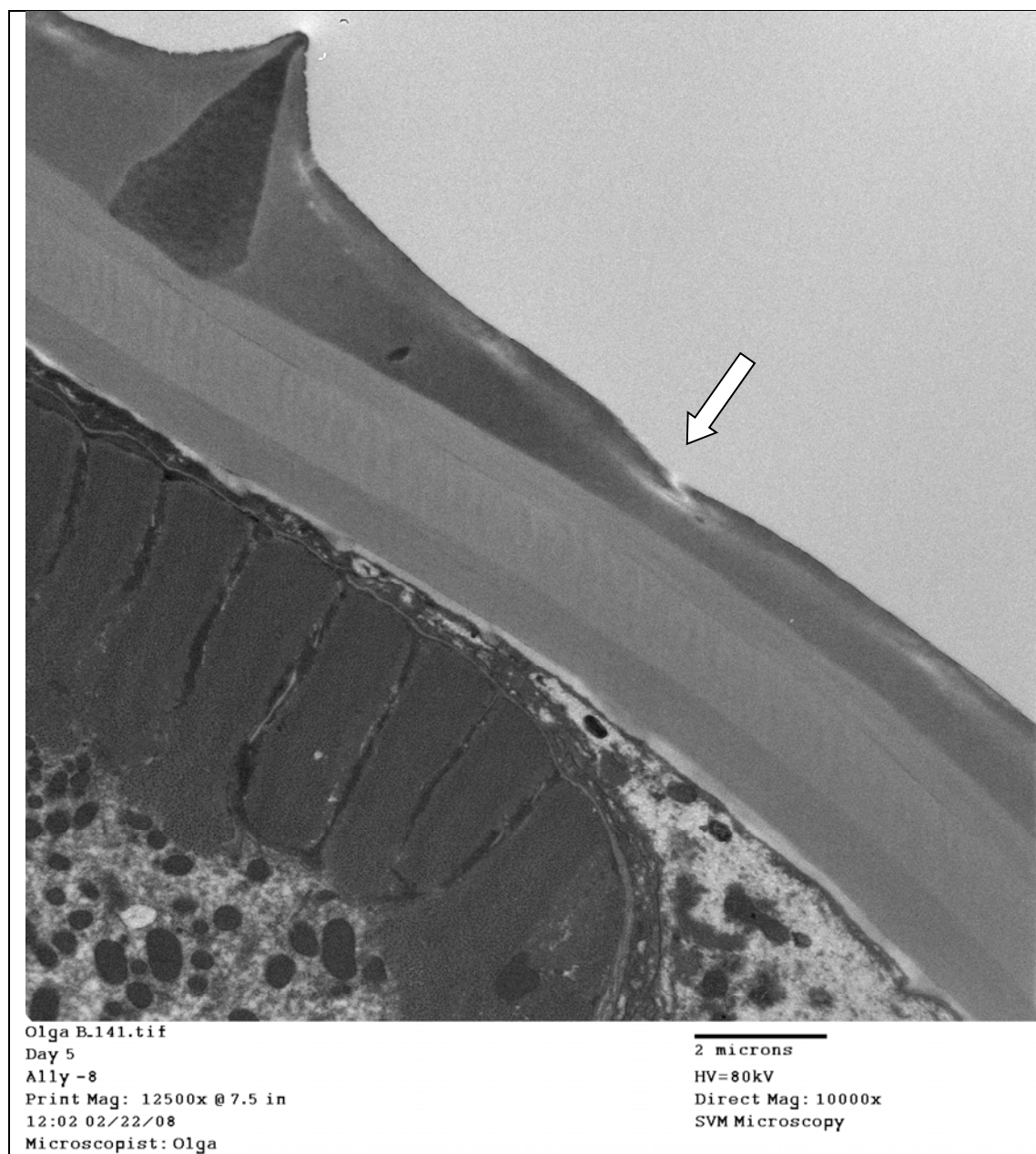


Figure 3.13. Cross-sectional transmission electron microscopy image of *Haemonchus contortus* adult collected from abomasal contents of a lamb 84 hours after administration of a 2 g copper oxide wire particle treatment. 10000X magnification. Cuticle shows affected area (arrow).

Longitudinal TEM images showed no distinct differences between pre-treatment and post-treatment (12 and 36 hours) samples (data not shown).

### 3.3.6 Copper Content of Nematodes

The pre-treatment nematode pool had a copper level of 19.4 ppm (Figure 3.14). The nematode pools for 12, 36, 60, and 84 HPT had copper levels of 30.5, 62.9, 95.9, and 78.0 ppm, respectively. The PBS solution in which the nematodes were stored was also tested and no copper was detected.

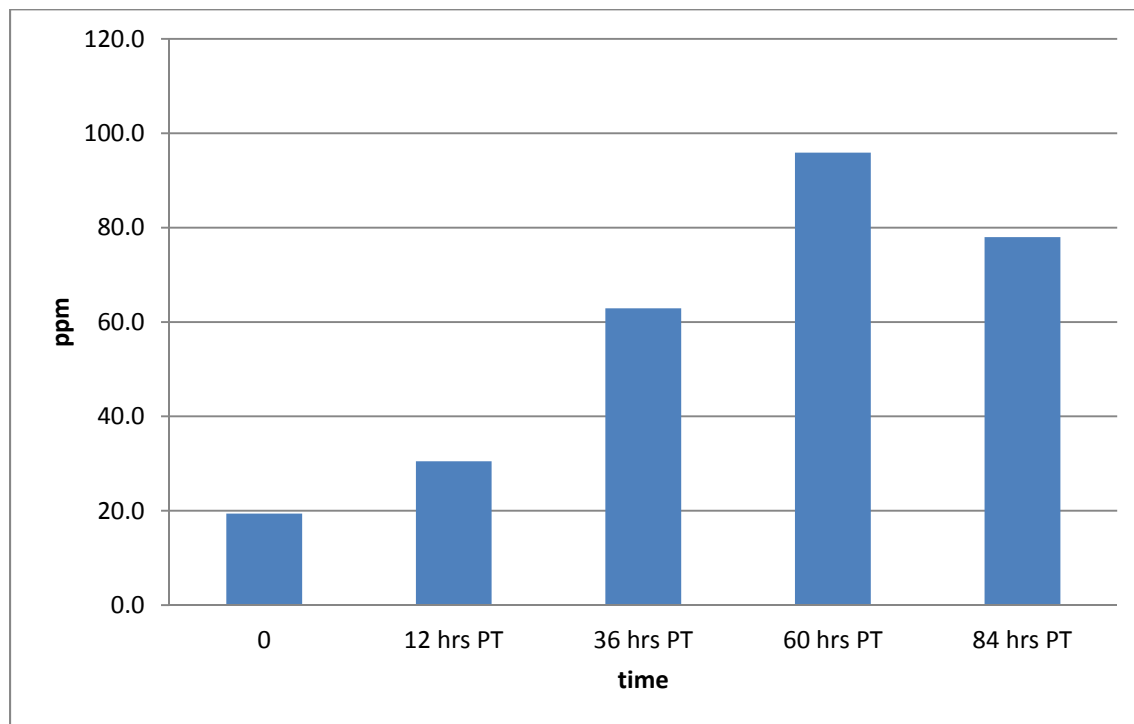


Figure 3.14. Copper content of *Haemonchus contortus* adults collected from abomasal contents of lambs before (0hr) and 12, 36, 60, and 84 hours after administration of a 2 g copper oxide wire particle treatment.

### 3.4 Discussion

This study was done to investigate a potential MoA of COWP on controlling *H. contortus*. The 2 g dose of COWP administered to the lambs resulted in a decrease in FEC over the 84 hr period of the study. This would indicate that infection dissipated most likely due to the effect of COWP. However, some nematodes were also physically removed at each time point which could have partially contributed to the observed decrease. Therefore, the decrease cannot be entirely attributed to COWP. At the start of the study, the level of anemia was moderately low

and as nematodes were eliminated due to COWP or physically removed, it might be expected that PCV would have increased due to decreased blood loss; however, the time frame of the study was not long enough to generate recovery from anemia (Gabuzda et al, 1968).

The SEM surface imagery of the nematodes appeared similar and did not reveal any obvious change between pre- and post-treatment. That indicated that any cuticle damage, if present, was not of the magnitude that could be seen with SEM.

The TEM imagery showed no cuticle lesions in pre-treatment samples and multiple cuticle lesions in all post-treatment samples. The 36 hour image included a skeletal rod which protrudes from the body, giving the cuticle there a different angle from the cuticle layers along the body wall (Roberts and Janovy, 2005). The lesions along this area resemble the lesions on other areas of the cuticle. The 60 hour image includes multiple diagonal lines through the image, a defect that occurred during the cutting portion of the processing. This defect could be responsible for some of the apparent cuticle lesions, but there are several cuticle lesions that do not appear within the cutting defect.

The EDS showed no copper present in any of the pre-treatment spots tested, and copper present in 10 of 11 post-treatment spots tested. No other elements were present in post-treatment samples and absent in pre-treatment samples. This indicated that Cu could be associated with the observed cuticle damage.

The frequency of cuticle lesions appeared to be higher at 12 hrs post-treatment when nematode copper concentration was lowest, and then decreased over time as nematode copper concentration increased. As the animals were not exposed to new infection during the study, the same population of nematodes was present throughout the study; therefore, the longer the nematodes survived in the abomasum, the greater potential opportunity for free Cu ions to adhere to the cuticle. COWP are present in the mucosa of the abomasum up to about 40 days or longer (Judson et al., 1984), but it has been observed that the efficacy may be limited to the first week or less (Burke et al., 2006). That is, the particles may be present, but the concentration of free Cu ions may peak quickly and then decrease which would result in nematodes that emerge into the lumen of the abomasum after the first week being unaffected by the lower concentration of free Cu ions. The fact that nematodes with lesions and associated copper concentration were still

present at each post-treatment time point suggests that the number of lesions may not be dependent on the concentration of copper. Also, it may be that nematodes that succumbed to treatment actually were expelled within the first 12 hrs and, therefore, surviving worms would have cuticle lesions, but not detrimental enough to cause elimination.

The lack of any obvious pH difference suggests that there was no environmental alteration by the COWP. However, it should be noted that the tissue at the opening of some of the fistulas became irritated during collection, causing blood to seep into the contents before the pH could be tested. Sheep blood ranges from pH 7.30 to 7.48 (Bartko et al, 1975), so the pH of the contents tinged with blood could be higher than the pH of the contents without blood. The range of pH indicted that blood probably affected the pH reading. Therefore, the presence of blood in many samples made the pH data inconclusive for this experiment

The results of this study suggested that a potential MoA for COWP may be by means of a direct effect on the adult nematode, and that an indirect effect via alteration of abomasal environment is less obvious. The SEM and longitudinal TEM showed no obvious evidence of COWP-related damage to the cuticle, but cross-sectional TEM showed definite evidence of treatment-inciting damage to the cuticle. Further, SEM with EDS showed points of copper accumulation coupled with increased copper concentration only in PT nematodes, which supported cuticle damage as a potential MoA.

This study provided evidence that the MoA of COWP, as an antiparasitic agent, may be cuticle damage that results in the inability of the nematode to maintain establishment. Further investigation using a fixed fistula device would make it easier to collect samples and more frequent collections, especially within the first 12 hrs of treatment could help to clarify the timeline of how COWP works. It would also help to have untreated control animals to coincide with PT collection times.

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**APPENDIX A**  
**LIST OF ACRONYMS USED**

BGT = Busch Gardens Tampa

COWP = Copper Oxide Wire Particles

EDS = Energy Dispersive X-ray Spectroscopy

EPG = Eggs Per Gram

FEC = Fecal Egg Count

FECR = Fecal Egg Count Reduction

GIN = Gastrointestinal Nematodes

HPT = Hours Post Treatment

LSU = Louisiana State University

LSU-SVM = Louisiana State University School of Veterinary Medicine

MoA = Mechanism of Action

PBS = Phosphate Buffered Solution

PCV = Packed Cell Volume

PT = Post Treatment

RBC= Red Blood Cells

SEM = Scanning Electron Microscopy

TEM = Transmission Electron Microscopy

## **APPENDIX B**

### **MCMASTER TECHNIQUE USED AT BUSCH GARDENS TAMPA**

1. Add flotation solution\*\* to line A (26 ml) of calibrated vial from Paracount-EPG™ Quantitative Fecal Analysis Kit (Chalex Corporation, Wallowa, OR).
2. Add feces until solution is even with fill line B of vial.
3. Stir mix thoroughly for 20- 30 seconds using wooden spatula.
4. Insert tuberculin syringe, stir briefly, and draw sample into syringe.
5. Charge one side of the McMaster slide with the sample, making sure the grid is completely covered.
6. Repeat steps 4 and 5 for the other side of the McMaster slide.
7. View slide using light microscope. Count all trichostrongyle-type eggs within the grids on each side of the slide.
8. Results are presented as eggs per gram (EPG) and are determined by multiplying the number of eggs counted by a factor of 25.

\*\*Flotation solution is mixed in the laboratory using Fecatect Zn 118 (Butler Schein™, Butler Schein Animal Health, Dublin, OH) zinc sulphate dry concentrate fecal flotation medium. Solution is made by mixing with slightly less water than manufacture recommendations, to a specific gravity of 1.200, slightly denser than the manufacturer recommendation. Each time a new container of solution is mixed, the specific gravity is measured via manual hydrometer.

**APPENDIX C**  
**MODIFIED MCMASTER TECHNIQUE FOR**  
**LOUISIANA STATE UNIVERSITY LABORATORY**

1. Weigh 2.0 g of feces into 100 ml plastic cup.
2. Crush pellets with wooden spatula (tongue depressor).
3. Add 15 ml of salt solution to cup; stir into a slurry with wooden spatula.\*
4. Add 15 ml of salt solution to cup.
5. Mix thoroughly with electric mixer.
6. Using plastic pipette with the end clipped to an approximately 45° angle, immediately after removing from mixer, pipette solution into one side of a wet McMaster slide (Chalex Corporation, Wallowa, OR).
7. Mix thoroughly again with electric mixer, and pipette sample immediately into the other side of the wet McMaster slide.
8. Repeat process up to five times before reading slides.
9. To read slides, count all trichostrongyle-type eggs within the grids on each side of the McMaster slide. Note other species that are seen, but do not count them.
10. Results are presented in eggs per gram (EPG) and determined by multiplying the number of ova counted within the McMaster slide grids by a factor of 50.

\* Salt solution is produced by pouring one box of salt (737 g) into a 3000 ml beaker, filling the beaker with tap water, and cooking with a stir stick for 2-3 hours, or until the salt is completely dissolved.

## **APPENDIX D**

### **INDIVIDUAL FECAL CULTURE TECHNIQUE**

1. Weigh sample into 100 ml plastic cup with three small holes drilled in the bottom. Weight should be between 4.0 g and 10.0 g.
2. Crush pellets thoroughly.
3. Mix vermiculite (approximately one-third of the amount by volume of feces) into sample and moisten with room-temperature tap water. Add more or less vermiculite and water to make a crumbly mixture that is moist enough to stick together when pressed, but not wet enough to pool water in the bottom of the cup.
4. Cover with cheesecloth secured by a rubber band around the top of the cup.
5. Fill the bottom of a 250 ml plastic tri-corner cup with hot water, approximately one-half inch in depth. Label tri-corner cup with sample information, including date.
6. Invert the cheesecloth-covered cup into the tri-corner cup, leaving space between the cheesecloth and the surface of the water.
7. Leave at room temperature for a minimum of 14 days, maximum of 21 days.
8. Flood the cheesecloth-covered cups with warm water, pressing the cup down into the tri-corner cup as much as possible. Leave alone overnight, at least 12 hours.
9. Pull the cheesecloth-covered cup up and carefully prop it on the side of the tri-corner cup to drain. At least an hour later, remove the top cup completely and discard its contents. Allow the tri-corner cup to sit at room temperature overnight, at least 12 hours.
10. Vacuum the liquid down to approximately 50 ml. Swirl cup gently and pour contents into 50 ml conical tubes. Transfer the label from the tri-corner cup to the 50 ml tube. Allow the tubes to sit at room temperature overnight, at least 12 hours.

11. Vacuum the 50 ml conical tubes to approximately 12 ml. Swirl the tube gently and pour contents into 15 ml conical tubes. Transfer the label from the 50 ml tube to the 15 ml tube. Add 1 ml of formalin, secure lid tightly, and invert twice.

12. Allow the tubes to settle at least overnight before counting larvae.

13. To count larvae, remove the liquid at the top of the tube with a clean pipette and discard. The amount of liquid removed varies inversely with the amount of solid material at the bottom of the tube, but should be removed to a measurable level for ease of calculations later. After removal of liquid, replace top and invert solution several times to mix thoroughly. Quickly remove 10  $\mu$ l of solution, place on a slide, mix with an iodine-soaked stir stick. Identify the first 100 larvae by genus to determine the population distribution. If fewer than 100 larvae were present in the first 10  $\mu$ l, invert several times again and repeat the process with another 10  $\mu$ l subsample. Continue to count all larvae after reaching 100 to determine the total larval count in 10  $\mu$ l. Extrapolate based on total amount of liquid, eggs per gram, and total weight of feces used for each culture sample to determine the total larval count and percent hatch.



## **APPENDIX E**

### **SCANNING ELECTRON MICROSCOPY PROCESSING**

1. Place sample in 1.25% Gluteraldehyde and 2% Formaldehyde in a 0.1 M Sodium Cacodylate (CAC) buffer at room temperature for at least 1 hour for fixation.
2. Wash: Place sample in a 0.1 M CAC buffer with 5% sucrose at room temperature for 10 minutes, three times, for a total of 30 minutes wash time to remove fixative.
3. Post-Fixation: Place sample in a 1% Osmium Tetroxide in a 0.1 M CAC buffer at room temperature for 1 hour.
4. Water Wash: Rinse sample in water three times for ten minutes each.
5. Dehydration: Ascending concentrations of ethyl alcohol (ETOH) at room temperature as follows:

% ETOH	Number of changes	Time (minutes) per change
30	1	5
50	1	5
70	2	5
80	2	10
90	2	10
100	3	10

6. Critical point drying in CPD “bomb” device.
7. Mount to aluminium electron microscopy stubs using double stick tape.
8. Sputter coat with gold/palladium 60/40 for 6 minutes.
9. Store in EM dessicator when not viewing through electron microscope.

**APPENDIX F**  
**TRANSMISSION ELECTRON MICROSCOPY PROCESSING**

1. Place sample in 1.25% Gluteraldehyde and 2% Formaldehyde in a 0.1 M Sodium Cacodylate (CAC) buffer at room temperature for at least 1 hour for fixation.
2. Wash: Place sample in a 0.1 M CAC buffer with 5% sucrose at room temperature for 10 minutes, three times, for a total of 30 minutes wash time to remove fixative.
3. Post-Fixation: Place sample in a 1% Osmium Tetroxide in a 0.1 M CAC buffer at room temperature for 1 hour.
4. Water Wash: Rinse sample in water three times for ten minutes each.
5. Stain: Place sample in 0.5% uranyl acetate in water in refrigerator overnight. Repeat water wash (step 4).
6. Dehydration: Ascending concentrations of ethyl alcohol (ETOH) at room temperature as follows:

% ETOH	Number of changes	Time (minutes) per change
30	1	5
50	1	5
70	2	5
80	2	10
90	2	10
100	3	10

7. Infiltration: Place sample in epoxy resin (ER) and 100% ETOH at room temperature as follows:

ER: 100% ETOH	Time
1:3	at least 1 hour
1:1	at least overnight
3:1	1-4 hours, or overnight
ER Only	1-4 hours
ER and DMP-30	4 hours at room temp; overnight in refrigerator

8. Polymerization for 12-48 hours at 60° F.

## **VITA**

Allyson Kinney Moscona, a native of Millbrook, Alabama, received her B.S. in Animal Science from Louisiana State University in 2003. Since then, she has gotten married and had two children. A love of zoos and a natural curiosity has led her to graduate school, where she will receive her master's degree in August 2013.