

1 **FENBENDAZOLE RESISTANCE IN *HETERAKIS GALLINARUM***

2 **Fenbendazole resistance in *Heterakis gallinarum*, the vector of *Histomonas meleagridis*, the**
3 **causative agent of Blackhead Disease in poultry.**

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17 **ABSTRACT**

18 Due to their ubiquity, management of parasites is a common and important factor for
19 profitable production of poultry. *Heterakis gallinarum*, the cecal nematode, is the most common
20 nematode parasite of poultry. While typically causing no pathology on its own, *H. gallinarum* is
21 the vector of *Histomonas meleagridis*, a protozoan parasite that causes blackhead disease.
22 *Histomonas meleagridis* is highly pathogenic in turkeys, potentially causing high mortality. In

23 contrast, disease caused by *H. meleagridis* is much less severe in chickens, where it primarily
24 reduces productivity without manifestations of clinical disease. There are no approved treatments
25 for *H. meleagridis*, making control reliant on control of the helminth vector through the use of
26 fenbendazole (FBZ) the only drug labeled for treatment of *H. gallinarum* in the United States We
27 were contacted by an industry veterinarian regarding health-related concerns in a broiler-breeder
28 house due to histomoniasis, despite frequent anthelmintic treatments. Since we had recently
29 diagnosed resistance to FBZ in *Ascaridia dissimilis*, a closely related nematode of turkeys, we
30 were interested to determine if *H. gallinarum* had also evolved resistance to FBZ. *Heterakis*
31 *gallinarum* eggs were isolated from litter collected from the house and used to infect 108
32 chickens. Treatment groups included a non-treated control, a label-dose and a 2X-label dose of
33 FBZ, with 36 birds per group divided into two replicate pens of 18 birds each. Birds were placed
34 at 1-day post hatch, and at 3 weeks of age were infected with 150 embryonated eggs via oral
35 gavage. Two weeks post infection, treated birds were administered a minimum of either a label-
36 or 2X label-dose of FBZ in water for 5 days (SafeGuard® Aquasol, 1mg/kg BW). To ensure that
37 all birds consumed the full intended dose at a minimum, the dosage was calculated using 1.25
38 times the average body weight. One-week post treatment, birds were euthanized, ceca removed,
39 and parasites enumerated. Efficacy was calculated by comparing the total numbers of worms
40 recovered from each treatment group to the numbers recovered in the non-treated control group.
41 There were no significant differences in worm numbers recovered from any of the three groups
42 (p-value=0.81). There also was no efficacy benefit to treatment with a 2X dose; *H. gallinarum*
43 worm counts were reduced by 42.7% and 41.4%, for the label and 2X dosages, respectively.
44 These data provide strong evidence that *H. gallinarum* has developed resistance to FBZ.
45 Consequently, in houses infected with FBZ-resistant *H. gallinarum*, *H. meleagridis* will be able

46 to cycle through the birds in an unrestricted manner. Further investigation is needed to determine
47 the prevalence of resistance in *H. gallinarum* on chicken farms, but it is clear this has the
48 potential to have a large-scale economic impact on the poultry industry. These data when viewed
49 together with our recent findings of FBZ resistance in *A. dissimilis*, suggest that drug resistance
50 in ascarid nematodes may be an important emerging problem on poultry operations.

51 **KEYWORDS**

52 *Heterakis*, *Histomonas*, fenbendazole, resistance

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1. INTRODUCTION

70 The near ubiquity of parasites on poultry farms makes the management of parasites a
71 common and important factor effecting profitable production of poultry. A study of birds from
72 10 different production companies Southeastern United Stated reported that 98.6% of birds are
73 infected by parasitic helminths, with 96% being infected with the cecal worm, *Heterakis*
74 *gallinarum* (Yazwinski, et al., 2013). *Heterakis gallinarum* belongs to the Family Ascarididae,
75 which also contains the closely related *Ascaridia galli* and *Ascaridia dissimilis*, important small
76 intestinal nematodes of chickens and turkeys, respectively. Helminth eggs from this family are
77 resistant to environmental pressures such as temperature, dehydration, and pH extremes, causing
78 a cycle of continuous infection and transmission within the house environment (Cauthen, 1931;
79 Tarbiat, et al., 2015). *Heterakis gallinarum* is a small nematode that rarely causes significant
80 direct pathology, but it serves as the vector for *Histomonas meleagridis*, a highly pathogenic
81 protozoal parasite that is the causative agent of Blackhead disease in poultry.

82 *Histomonas meleagridis* currently ranks as the highest research priority in broilers of any
83 parasite of poultry (Armour, et al., 2020). *Histomonas meleagridis* is carried within the eggs of
84 *H. gallinarum*, and Histomonads are released into the gut when the larvae hatch from the
85 nematode egg in the intestine. *Histomonas meleagridis* causes the disease histomoniasis, which
86 is characterized by necrosis in the mucosal tissues of the ceca and liver, and may cause dark
87 discoloration of the head, hence the name Blackhead. Historically, infections in turkeys often
88 produced high levels of mortality, whereas in chickens, infection was largely asymptomatic.
89 Recently, this view has shifted as studies show that both chickens and turkeys demonstrate
90 clinical signs such as apathy, depression, and ruffled feathers, together with decreased feed and
91 water uptake (Liebhart, et al., 2017). *Histomonas meleagridis* has now been shown to impact

92 chickens in multiple different production systems, including reduced feed conversion in broilers,
93 and decreased egg quality and production in layers and breeders, both caged and cage-free (Clark
94 and Kimminau, 2017; Grafl, et al., 2011; Liebhart, et al., 2013) Despite these significant effects
95 on health and production, there are currently no FDA approved treatments for histomoniasis,
96 making control of this disease dependent on control of the *H. gallinarum* vector.

97 Currently, fenbendazole (FBZ) is the only anthelmintic approved for use against ascarids of
98 poultry in the United States. Fenbendazole belongs to the benzimidazole class of anthelmintics, a
99 drug class used widely across multiple livestock species. In registration studies for SafeGuard®
100 Aquasol®, a formulation of FBZ that is suspended in water for delivery, average efficacy against
101 *H. gallinarum* was 96.2%, similar to that of *Ascaridia galli*, at 97.6% (United States Food and
102 Drug Administration, 2018). Likewise, in registration studies for the feed additive formulation of
103 SafeGuard®, average efficacy against *H. gallinarum* was 97.85% in growing turkeys (United
104 States Food and Drug Administration, 2000). Both formulations are delivered from a central
105 ration, or medication tank, and then distributed throughout the house. These methods of
106 administration, along with human error, may result in poor delivery of treatment, leading to
107 underdosing. Underdosing is recognized as one of the major contributors to the development of
108 anthelmintic resistance (Jackson and Coop, 2000; Silvestre, et al., 2001), and combined with the
109 high frequency of treatment, in as little as every four weeks, development of resistance is a major
110 concern (Smith, et al., 1999).

111 Resistance to benzimidazoles in many of the most economically important strongylid
112 nematodes of livestock is highly prevalent (Howell, et al., 2008; Kaplan, 2004; Kaplan and
113 Vidyashankar, 2012), however resistance in ascarid nematodes appears to be rare. In poultry,
114 reduced efficacy was first reported in the turkey nematode *Ascaridia dissimilis*, leading to

115 speculation that drug resistance may have developed (Yazwinski, Tucker et al. 2013). This
116 suspicion was recently confirmed in a controlled efficacy study, where FBZ resistance was
117 clearly demonstrated in *A. dissimilis* (Collins, et al., 2019). This confirmation of resistance
118 highlights the potential of ascarid nematodes of poultry to develop resistance to FBZ, and since
119 birds treated with FBZ may be infected with both *A. dissimilis* and *H. gallinarum*, FBZ
120 resistance in *H. gallinarum* may already exist.

121 Given the recent concern of increased infection and disease from *H. meleagridis* in breeder
122 chickens and having demonstrated FBZ resistance in one ascarid species of poultry, we wanted
123 to determine if *H. gallinarum*, had also developed resistance. Through collaboration with
124 industry veterinarians, we identified a farm with suspected-resistant *H. gallinarum* and
125 conducted a controlled efficacy trial to determine if the worms on that farm were in fact resistant
126 to FBZ.

127 2. MATERIALS AND METHODS

128 2.1 Chickens

129 One hundred eighteen, Cobb 500, chicks were hatched and placed the following day in
130 housing at the Poultry Science Farm at the University of Georgia. Nipple drinkers and hanging
131 feeders were used to provide water and feed *ad libitum*. Birds were fed a diet of non-medicated
132 Nutrena® NatureWise® Chick Starter Grower feed.

133 2.2 Parasite Isolates

134 A potentially resistant isolate of *Heterakis gallinarum*, AmFa 1.0, was identified through
135 collaboration with an industry veterinarian. Prior to May of 2017, the farm of origin for this
136 isolate, treated birds with a variety of treatments including FBZ, but after May of 2017,

137 exclusively treated six flocks, four treatments per flock, with FBZ. Litter was obtained from the
138 suspect farm, and eggs were isolated using previously established protocols (Collins et al., 2019).
139 Briefly, litter was washed through a series of sieves to remove debris, and then the remaining
140 sediment was added to a solution with specific gravity of 1.15 and centrifuged at 433g for 7
141 mins. Eggs within the fluid phase were collected on a 32uM sieve, rinsed with deionized water,
142 and stored in tissue culture flasks in deionized water containing 0.5% formalin at 10°C. Prior to
143 infecting the birds, flasks were incubated at 30°C for four weeks, until eggs were fully
144 developed, and then stored at 10 °C until infection.

145 ***2.3 Infection & Treatment***

146 Birds were divided into two replicates of 18 birds each for the following three treatments:
147 non-treated control, label dosage of FBZ, and 2x label dosage of FBZ. An additional 10 birds
148 remained uninfected as environmental controls to confirm that there was no prior contamination
149 of the study environment with *H. gallinarum* eggs. Birds were allowed to grow to three weeks of
150 age before being infected with approximately 150 embryonated eggs via oral gavage. Mesh
151 curtains were placed between pens to prevent any cross-over of birds between treatment groups.

152 Two weeks post infection, birds were treated with either the label or a 2x dose of
153 SafeGuard® Aquasol® (Label Dosage: 1mg/kg BW), a FBZ formulation designed for delivery in
154 water. To increase the likelihood that every bird received the target dosage at a minimum, the
155 average bird weight of the groups on the day before treatment plus 25% was used to calculate the
156 dose administered. Calculated volumes of FBZ were mixed into 90% of the volume of water
157 estimated to be consumed, as per production guidelines. For delivery of the FBZ, water lines
158 were connected to carboys with both replicates of each treated group receiving water from the

159 same carboy. As per label directions, treatment was administered over the course of five days,
160 resuspending the drug daily.

161 **2.4 Worm Recovery**

162 Seven days post-treatment, all birds were humanely euthanized for worm recovery. Ceca
163 were removed, opened, and placed in physiological saline. Samples were incubated overnight at
164 37C to aid in the recovery of tissue associated nematodes. Cecal contents were then washed over
165 a 50uM mesh sieve to remove small debris, and cecal cores if present were manually disrupted.
166 Contents were then examined under a dissecting microscope, and all nematodes were recovered
167 and enumerated.

168 **2.5 Statistical Analysis**

169 Differences between the three groups were evaluated using Kruskal–Wallis test by ranks
170 with Dunn’s correction, a non-parametric analysis of variance with multi-comparisons (Graph
171 Pad Prism 8, San Diego, CA). Analysis of control vs. label dosage and control vs. 2x dosage
172 were done with the following parameter: Two-samples unpaired with zero-inflation, and a
173 correction factor of 1. Efficacy was calculated using eggCounts, an R package using a Bayesian
174 hierarchical model to determine anthelmintic efficacy (Wang, et al., 2018).

175 **2. RESULTS**

176 No significant differences were observed between the three groups ($p>0.81$). Model adjusted
177 efficacies for the label dose and 2x label dose groups were 42.7% and 41.4%, with upper 95% CI
178 of 74.2 and 74.1%, respectively (Table 1). Both of these upper 95% CIs are well below reported
179 efficacies of 96.2% and 97.85%, for the water and feed formulations, respectively (United States

180 Food and Drug Administration, 2000; United States Food and Drug Administration, 2018).

181 These data provide strong evidence that these *H. gallinarum* are resistant to FBZ.

182 **4. DISCUSSION**

183 We confirm here for the first time, resistance to FBZ in the poultry nematode, *Heterakis*
184 *gallinarum*. Efficacies of the label dosage and 2X the label dosage were 42.7% and 41.4%
185 respectively, with no significant difference between the two dose levels, nor between these two
186 treated groups and the non-treated controls ($p>.9999$). The lack of differentiation in efficacy for
187 the two dose levels indicate not only that resistance has developed, but there appears to be a
188 virtual total lack of efficacy. The observed efficacies are likely a due to random variation
189 between birds and groups, as the worm counts were highly over-dispersed with many zeros in all
190 three groups (Supplementary table 1). Similar to the resistant isolate of *A. dissimilis* we
191 previously identified, this farm of origin has a history of FBZ use, further highlighting the risks
192 for resistance associated with having only one approved compound for use against helminths of
193 poultry. As compared to *A. dissimilis*, *H. gallinarum*, by itself, poses less disease risk to its host.
194 However, due to its role as a vector for *H. meleagridis*, FBZ resistance in *H. gallinarum* poses
195 important health challenges in poultry operations.

196 *Histomonas meleagridis* is one of the most concerning disease pathogens of poultry
197 production today, due to its severe impact on animal productivity and welfare. Since there are no
198 approved drugs for the treatment and control of *H. meleagridis*, prevention of histomoniasis
199 relies heavily on the control of *H. meleagridis* using anthelmintics. Consequently, failure to
200 control FBZ-resistant *H. gallinarum* would lead to a continuous cycle of infection and disease
201 with *H. meleagridis*. This then presents a scenario of production loss and animal welfare
202 concerns that cannot be readily prevented.

203 In conclusion, we now have identified resistance to FBZ in two separate species of
204 poultry nematodes in two successive trials. This highlights the possibility that FBZ resistance is
205 much more common on poultry farms than is currently appreciated. Drug resistance in poultry
206 ascarids may have important impacts both directly and, in the case of the *H. meleagridis* life
207 cycle, indirectly on animal welfare and production loss. Given the ease with which we have
208 found farms with drug-resistant ascarids, there is an important need to determine the scope and
209 magnitude of this problem by investigating the prevalence of FBZ resistance in nematodes of
210 poultry.

211 If the prevalence of resistance is as high as we believe it could be, it is possible that
212 anthelminthic resistance is playing a role in the recent resurgence of *H. meleagridis* as a concern
213 in chickens. While historically not seen as a significant problem, recent evidence shows
214 significant production impacts in broilers in layers due to histomoniasis. There are likely many
215 complex factors contributing to this resurgence, but a lack of vector control due to anthelminthic
216 resistance may play an important role, further highlighting the need for methods for surveillance.

217 Currently, we are investigating the genetic mechanisms of FBZ resistance in poultry
218 ascarids, which appear to differ from that of strongylid nematodes. Identifying the genetic
219 mechanisms of resistance is important, as this would facilitate the development of a diagnostic
220 test, which would facilitate the measurement of resistance prevalence on a wide geographic
221 scale. In addition, new alternative treatments for both for *H. gallinarum* and *H. meleagridis* are
222 greatly needed.

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227

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229

230 **ETHICAL STATEMENT**

231 All birds were handled under protocols approved by the University of Georgia Institutional
232 Animal Care and Use Committee (IACUC) under animal use policy A2019 01-005-Y2-A3.

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285 **Table 1.** Zero-inflated adjusted average worm count and efficacy.

Treatment	Mean Number of Worms	% Reduction	95% CI
Control	12.2	-	-
Label Dosage	6.8	42.7%	(4.5%,74.2%)
2x Dosage	6.8	41.4%	(3.4%,74.1%)

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