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The efficacy of flubendazole against different developmental stages of the poultry roundworm *Ascaridia galli* in laying hens

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ABSTRACT

Infection with the poultry roundworm *Ascaridia galli* has increased in European countries due to the ban on battery cages. This study was conducted in two commercial laying hen flocks (F1 & F2) on different farms in central Sweden. The aims were to (1) investigate the efficacy of flubendazole (FLBZ, 1.43 mg/kg administered in drinking water for 7 days) against adult and larval stages including histotrophic larvae of *A. galli*, and (2) determine how long it took before the flocks were reinfected after deworming. Accordingly, 180 randomly selected hens were sacrificed before drug administration (bd), on day 3 and 7 during drug administration (dd), and on a weekly basis for up to five weeks post drug administration (pd). Intestinal contents and cloacal materials of each hen plus pooled faecal samples from manure belts were investigated to assess the worm burden and the parasite egg per gram faeces (epg). Additionally, drinking water, and serum and gastrointestinal digesta content samples obtained from ten treated animals were analyzed by HPLC to measure FLBZ and its reduced (R-FLBZ) and hydrolyzed (H-FLBZ) metabolites. No parasite eggs were observed in cloacal samples on day 21 and 28 pd on F1 and on day 21 pd on F2. The epg in manure decreased by 65% and 88% on day 3 dd and by 99% and 97% on day 35 pd on F1 and F2 respectively. Mean FLBZ concentrations quantified in duodenal contents ranged between 0.50 and 0.79 μg/g. Although, no histotrophic larvae were found dd, they reappeared one week pd (7 ± 7 F1, 0.5 ± 0.5 F2). Adult worms were found in both flocks before drug administration (44 ± 20 F1, 35 ± 25 F2), on day 3 dd (4 ± 3 F1, 2 ± 2 F2), and then not until day 35 (0.2 ± 0.6) on F1 and day 28 (0.4 ± 0.9) pd on F2. Thus, the only period in which no *A. galli* were found was on day 7 dd. Although FLBZ was highly efficient our results indicate that the birds were reinfected already within one week pd.

1. Introduction

In the past decades, the proportion of Swedish laying hens affected by the poultry roundworm *Ascaridia galli* has increased from 23% in 2004 to 38% in 2008 regardless of their housing system (Jansson et al., 2010) and further to 73% in organic production in 2014 (Thapa et al., 2015). A similar situation is present in some other European countries (Kaufmann et al., 2011; Sherwin et al., 2013). The life-cycle of *A. galli* is direct, but it undergoes a histotrophic phase in the mucosal layer of the proximal small intestine before the larvae reenter the intestinal lumen where they develop into adults (Luna-Olivares et al., 2012). The prepatency period in chickens is around 6–8 weeks (Anderson, 2000; Taylor et al., 2007). Adverse effect on the host increases with worm burden. Ascaridiosis in poultry may cause reduced animal health and welfare as well as production losses (Kilpinen et al., 2005; Katoch et al., 2012). Anthelmintic treatment in combination with other preventive measures e.g., biosecurity, disinfection and segregation of birds by age groups are the fundamental principles of the effective control of roundworms in poultry. Swedish egg producers often deworm their flocks once or twice during the egg production period. Deworming should lead to reduced parasite egg contamination of the barn, which would, at least theoretically, postpone the infection after placement and reduce worm burdens during the early lay period. However, it has been shown that the effect of deworming seems to be more transient than expected (Höglund and Jansson, 2011).

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The benzimidazoles flubendazole (FLBZ) and fenbendazole (FBZ) have the same mode of action, and are currently the only active substances certified for use in laying hens against ascarids in the European Union countries. Attempts to evaluate the efficacy of FLBZ or FBZ against *A. galli* have been made. However, there are however limitations with these studies e.g., the emphasis has been solely put on the elimination of the adult worms whereas the effect on lumenal and histotrophic larvae were overlooked (Ssenyonga, 1982; Sander and Schwartz, 1994). Others had evaluated the drug efficacies in research facilities without challenge infection (Squires et al., 2012) or in broiler parents treated via feed additive (Yazwinski et al., 1986). Little is known about the efficacy of the available anthelmintics and optimal design of deworming programs for laying hens under field conditions. It is also unclear whether deworming with benzimidazoles is effective against all internal life stages of *A. galli*.

The aims of this study were to investigate the efficacy of FLBZ against both larval and adult stages of *A. galli* in laying hens, and to determine how long it took before reinfection was established post drug administration (pd). Additionally, FLBZ concentrations in serum and gastrointestinal fluid were measured to assess the drug uptake in the investigated hens.

2. Material and methods

The study was conducted in two flocks (F1 and F2) during the autumn of 2012 and the summer of 2013 on two different commercial laying hen farms in Sweden. In the selection of infected flocks, the shortest possible distance to the laboratory was taken into consideration so that the larvae would remain viable in the mucosa during transport. Both flocks (each held ≈7000 hens) were housed indoors in NATURA-Nova aviaries (Big Dutchman) and wood shavings covered the floor. Both flocks were confirmed to be mono-infected with *A. galli* before the beginning of the study. Accordingly, an in-house species-specific PCR was run on faecal samples collected from manure belts from each flock. For further confirmation, post mortem examination of some hens (data not shown) from within each flock was carried out and the intestines were examined for parasite species presented.

The experiments started when the hens were 67 (F1) and 70 (F2) weeks old, and were then continued until adult parasites were observed in the intestines after deworming. Both flocks were treated daily for seven days with flubendazole (FLBZ, Verminator®, Elanco Animal Health/Eli Lilly Danmark A/S,1.43 mg/kg body weight) according to the manufacturer's recommendation. Both flocks were deprived of water for 2 h before FLBZ was administered orally by injecting medicated water into the water lines by electronic pumps.

2.1. Sampling

Types of samples and timing of sampling is summarized in Fig. 1. From F1, four water samples (50 ml each) one from the middle and one from the end of two separate water lines, were obtained from the nipples on day 1, 3 and 7 during drug administration period. The water samples were obtained at two and four hours after the start of the drug administration on each sampling day. From F2 the samples were collected as described above but in replicates.

Four manure samples (≈800–1000 g each) were collected from evenly distributed locations on two manure belts in each flock. The manure belts had been operated the day before to ensure that the samples represented the egg expulsion of the previous 24 h.

Ten randomly selected hens from each flock were bled from the wing vein (*v. cutanea ulnaris*), and then sacrificed under current Swedish legislation (stunning and cervical dislocation) on each sampling occasion. The hens were necropsied and the intestines and individual cloacal faecal material were collected. Each intestine was numbered and placed in a separate plastic container and transported to the laboratory at a temperature of 15–20 °C to minimize cooling of any histotrophic larvae. Blood and water samples were transported on ice. Handling of the birds and euthanasia were approved by the Swedish Ethical Committee for Scientific Experiments (C24/10).

2.2. Laboratory analyses

2.2.1. Parasitological analyses

Ascaridia galli eggs in faeces (3 g) collected from manure belts were analyzed with the McMaster method (Foreyt, 2001) and cloacal faecal contents (0.5 g) were analyzed with MiniFlotac (MiniFLOTAC®, University of Naples Federico II) (Cringoli et al., 2012) to determine the number of parasite eggs per gram faeces (epg). The reason for using MiniFlotac technique over McMaster for cloacal samples was the limitation of collecting sufficient faecal materials from the cloaca. The minimum detection level in both tests was adjusted to 50 epg.

Approximately 2.5–4 h after euthanasia, the intestines were cut open and intestinal contents from the small intestine were sieved (mesh size ~300 μm) and then rinsed several times with 1 L tap water. The numbers of *A. galli* (both larvae and adult worms) were observed by microscope (Olympus SZX9 equipped with Olympus DP50 camera), counted and sexed. Sex differentiation was based on the morphological characteristics of the worms, e.g., presence of bursa and caudal spicules in males and straight and pointed tip tail or presence of parasite eggs in the uterus in females. Worms were categorized into adult if they had a distinct sexual characteristics and the length of the worms were 40–70 mm for males and 80–120 mm for females (Taylor et al., 2007).

The histotrophic larvae in the intestinal mucosa were detected with an agar gel-incubation method (Ferdushy et al., 2012) with some modifications. Accordingly, the mucosal layer was removed from the intestinal wall using a glass slide. The collected material was mixed with 100 ml of 39 °C physiological (0.9%) NaCl, which in turn was mixed with 100 ml agar solution (3%) at the same temperature. The mixture was poured as a thin layer over non-woven cloths (Johnson universal) and positioned in a glass tank containing approximately 1.5 L 39 °C physiological NaCl and incubated at 39 °C overnight. After removal of the cloths, the tank was left for at least one hour to let the larvae settle at the bottom. Approximately 2/3 of the supernatant was removed by a water aspirator. The remaining liquid (50–70 ml) was examined with a stereomicroscope.

2.2.2. Pharmacological analyses

At the laboratory the blood samples were centrifuged to obtain serum. Sera and water samples were then frozen at −20 °C, and analyzed at the Laboratory of Farmacology, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Argentina.

Experimental and fortified samples of water, serum and intestinal content were analyzed for the parent FLBZ, its reduced (R-FLBZ) and hydrolyzed (H-FLBZ) metabolites, and the internal standard oxibendazole (OBZ) by high performance liquid chromatography (HPLC). Fifty microliters of each sample were injected in a Shimadzu 10A HPLC System (Kyoto, Japan). The compounds were identified with the retention times of 97–99% pure reference standards. A complete validation of the analytical procedures for extraction and quantification of FLBZ, H-FLBZ and R-FLBZ in each matrix was performed before starting the analysis of experimental samples. The calibration curves for each analyte constructed by least squares linear regression analysis, showed good linearity with correlation coefficients greater than 0.992. Recovery of the three molecules under study was estimated by comparison of the peak areas from

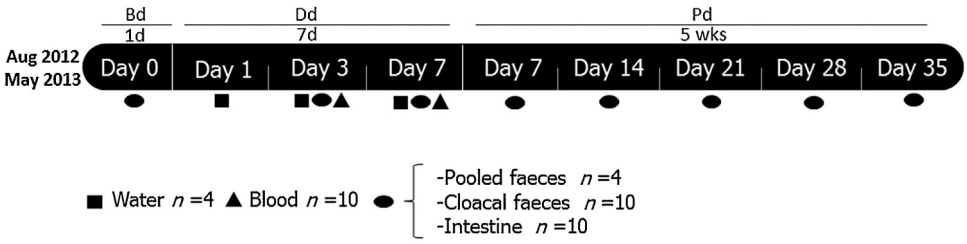

Fig. 1. Types of samples and timing of sampling during the study. Bd: before drug administration, Dd: during drug administration (flubendazole, 1.43 mg/kg body weight), Pd: post drug administration. *n* indicates the number of collected samples.

spiked water, hen serum and intestinal content samples with the areas resulting from direct injections of standards. The absolute recoveries in the different matrices for FLBZ, H-FLBZ and R-FLBZ ranged between 60–98% with coefficients of variation (CV) ≤10%. Precision (intra- and inter-assay) was determined by analyzing replicates of each matrix sample fortified ($n = 5$) with each compound at three different concentrations of the calibration curves with CV lower than 11%. The limit of quantification (LOQ) for FLBZ and its metabolites ranged between 0.02–0.10 μg/ml. These were defined as the lowest drug concentration on each standard curve that could be quantified with a precision not exceeding 20% and accuracy within 20% of nominal.

2.3. Statistical analyses

Data were summarized in Excel® (Microsoft® 2010), and exported to JMP™ version 11.0 (SAS Institute Inc., Cary, NC, USA) and/or GraphPad Prism® version 5.00 (GraphPad Software, La Jolla, California USA), where statistical analyses and graphical illustrations were carried out. Differences in the number of worms and parasite eggs recovered at the different time intervals post treatment were tested with T-test and/or two-way ANOVA. Data from FLBZ concentrations in water, blood and intestinal contents were tested with generalized linear regression (GLM). In analysing the FLBZ concentration in water samples, time of sampling (2 and 4 h) and place of sampling on the water lines (middle and end) were independent variables and FLBZ concentration was dependent variable.

3. Results

3.1. Parasitological findings

3.1.1. Parasite eggs from the manure belt samples

The mean number of parasite eggs in the manure belt samples differed significantly between the two flocks ($P < 0.0001$) on day 0. Mean ± SD of the epg values from F1 and F2 were 2350 ± 859 and 937 ± 306, respectively.

Despite deworming, parasite eggs were detected on all sampling occasions in both flocks (Fig. 2). Both flocks showed an overall reduction in epg up until 28 days pd (7.5 ± 5 F1, 27 ± 26 F2), thereafter epg levels were observed to increase again. This coincided with identification of adult worms in the intestinal samples (see below).

3.1.2. Parasite eggs in cloacal contents

Before treatment (day 0), the epg ± SD were 5435 ± 4730 and 1855 ± 2199 in F1 and F2, respectively. Following deworming, the mean epg levels decreased in both flocks (Fig. 3).

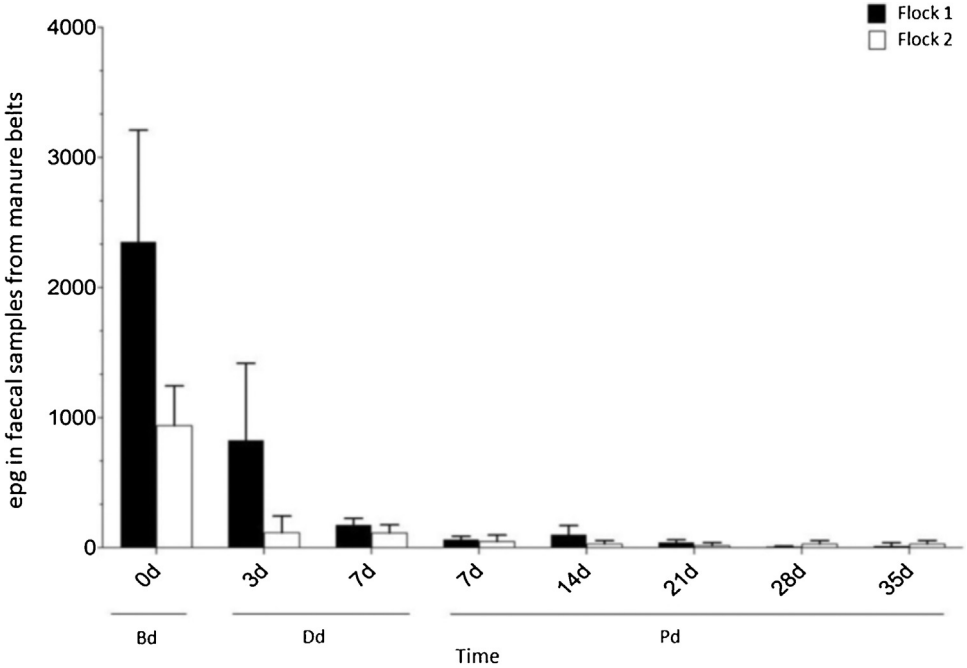

Fig. 2. Egg per gram faeces (epg) in faecal materials obtained from manure belts on different sampling occasions. Bd: before drug administration, Dd: during drug administration, Pd: post drug administration.

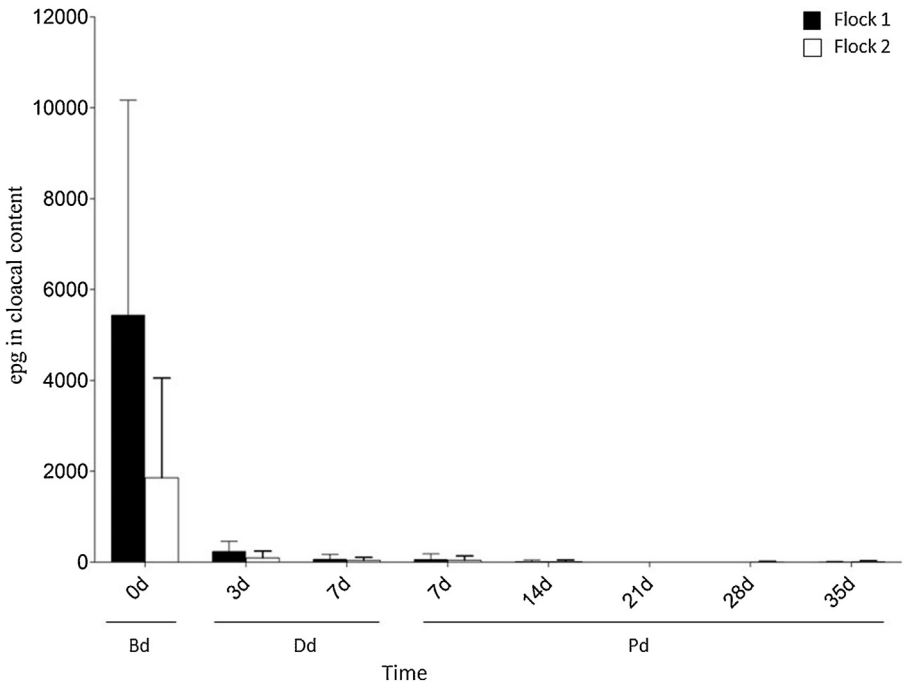

Fig. 3. Egg per gram faeces (epg) in samples obtained from cloacal contents of 10 hens on each sampling occasion. Bd: before drug administration, Dd: during drug administration, Pd: post drug administration.

At 21 and 28 days pd, no eggs were detected in samples from F1 (Fig. 3). Parasite eggs reappeared in one sample from F1 from one hen, which harbored adult worms of both sexes on the last sampling point 35 days pd. In F2, parasite eggs were detected during the entire sampling period except for day 21 pd (Fig. 3). Reappearance of parasite eggs on 28 and 35 days pd in F2 coincided with recovery of adult worms on these two occasions.

3.1.3. Adult A. galli

Before deworming, the mean ± SD worm burdens were 44 ± 20 in F1 and 36 ± 25 in F2. The female to male ratio at this point was 1:0.9 (F1) and 1:0.8 (F2). On the third day dd, the mean number ± SD of adults were 5 ± 4 in F1 and 2 ± 2 in F2, and the sex ratio showed an increased female to male ratio, e.g., 1:0.04 in F1 and 1:0.4 in F2. Thereafter, no adult *A. galli* were found between day 7 during deworming (dd) up to 28 days pd. Reappearance of adult worms

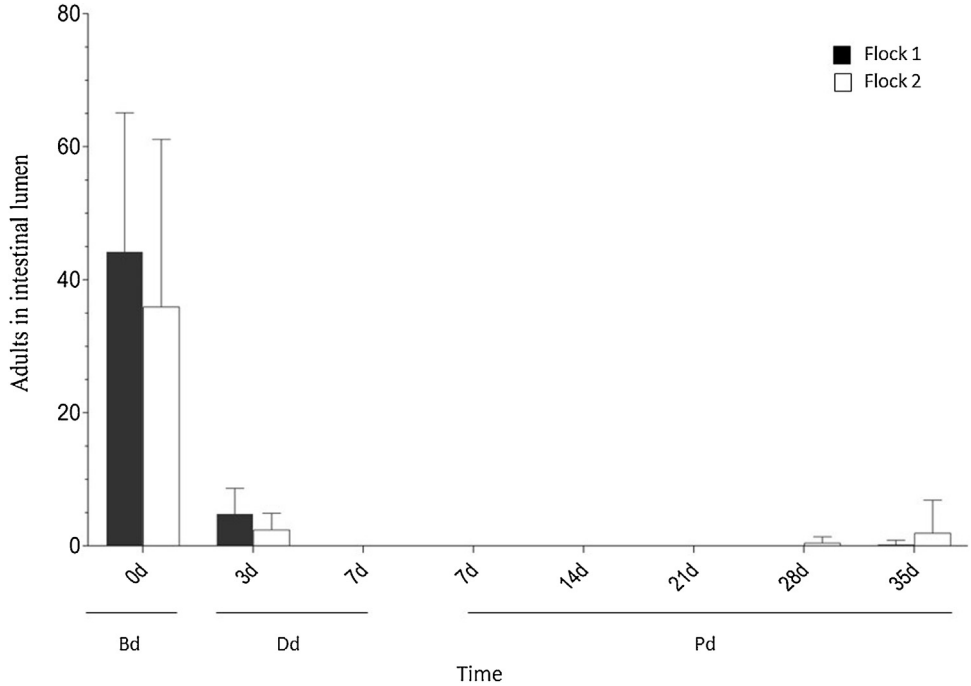

Fig. 4. Number of adult *A. galli* recovered from the intestinal lumens of 10 hens on each sampling occasion. Bd: before drug administration, Dd: during drug administration, Pd: post drug administration.

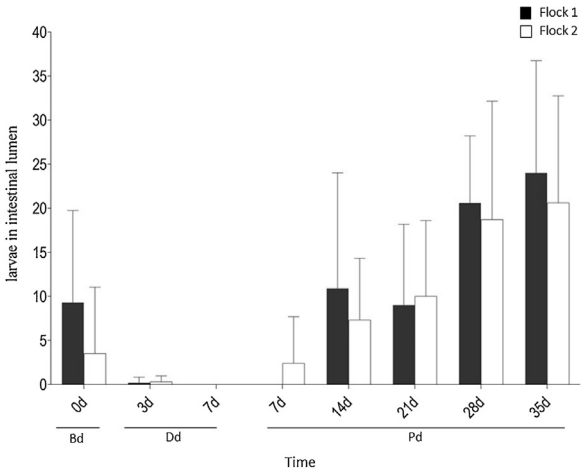

Fig. 5. Number of *A. galli* larvae recovered from the intestinal lumen of 10 hens on each sampling occasion. Bd: before drug administration, Dd: during drug administration, Pd: post drug administration.

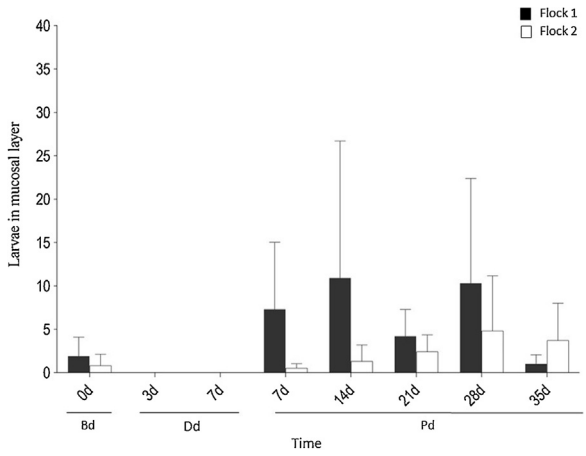

Fig. 6. Number of histotrophic larvae recovered from the mucosal layer of the intestinal wall of 10 hens on each sampling occasion. Bd: before drug administration, Dd: during drug administration, Pd: post drug administration.

was observed on day 28 and 35 pd in F2 and in F1, respectively (Fig. 4).

3.1.4. Lumenal larvae

Mean larval numbers ± SD decreased significantly from 9.3 ± 10 (F1) and 3.5 ± 7.5 (F2) prior to deworming on day 0, to 0.2 ± 0.6 and 0.4 ± 0.6, respectively, on day 3 dd. No larvae were detected in intestinal samples from any of the two flocks on day 7 dd. Lumenal larvae reappeared on day 7 pd in F2 (2 ± 5) and on day 14 pd in F1 (10 ± 13) (Fig. 5). The mean number ± SD of recovered larvae then gradually increased to 24 ± 12 in F1 and 20 ± 12 in F2 on the last sampling points.

3.1.5. Histotrophic larvae

Before deworming an average ± SD of 1.9 ± 2 (F1) and 0.8 ± 1.3 (F2) larvae were recovered from the mucosa. In contrast, no larvae were detected on day 3 and 7 dd. Histotrophic larvae (on average ± SD 7.3 ± 7.7 in F1 and 0.5 ± 0.5 in F2) reappeared in the mucosal layer on day 7 pd. In the samples from the last five sampling occasions (after the end of treatment period) 6.7 ± 4 (F1) and 2.5 ± 1.7 (F2) histotrophic larvae were detected (Fig. 6).

3.2. Pharmacological findings

There was a variation in FLBZ concentration between water samples in both flocks (1.04–4.72 μg/ml in F1 and 0.00–3.66 μg/ml in F2) (Fig. 7). The mean FLBZ concentration in water was 3.2 ± 1.2 μg/ml in F1 and 2.2 ± 0.7 μg/ml in F2.

FLBZ and R-FLBZ concentrations found in serum samples in both flocks were undetectable or close to the limit of quantification (0.02 ± 0.01 in F1, 0.03 ± 0.01 in F2) at both 3 and 7 days of treatment.

FLBZ concentrations were quantified in duodenal content, which represents the surrounding medium of the target parasite (*A. galli*). Mean concentrations (±SD) at times checked during treatment in both F1 (1, 3 and 7 days) and F2 (3 days) are shown in Fig 8. The mean FLBZ concentrations ranged between 0.50 and 0.79 μg/g.

4. Discussion

In this study, we have for the first time showed that FLBZ was effective against all internal developmental stages of *A. galli* in laying hens in connection with routine deworming of two laying hen flocks on different commercial farms. Both adult worms, and lumenal and histotrophic larvae were killed by oral FLBZ treatment for seven days. The significant FLBZ concentrations measured in duodenal content during treatment helped to explain the excellent anthelmintic effect observed for FLBZ after its administration in the drinking water. However, histotrophic larvae reappeared within seven days pd in both flocks. Adult worms were observed after 28 and 35 days pd in F2 and F1, respectively. Moreover, the numbers of both lumenal and histotrophic larvae after deworming exceeded those observed before deworming. This observation reflects the high level of exposure to infection following anthelmintic treatment. Contrary to our finding, a previous study has suggested that resistance to *A. galli* acquired during continuous infection followed by anthelmintic treatment, would lead to a lower establishment rate of subsequent *A. galli* infection (Ferdushy et al., 2014). It should however be noted that later study was conducted on a research facility under a strict control e.g., enumerated inoculation doses and certain inoculation intervals, whereas this study was conducted under field conditions within contaminated farm where the hens were constantly exposed to an unknown number of parasite eggs.

Our results show that the prepatency period of *A. galli* was 4–5 weeks. This is somewhat shorter than in previous studies e.g., 6 weeks or more in adult chickens (Taylor et al., 2007), 7 weeks in 12-weeks old chickens (Pankavich et al., 1974) and 8 weeks in chickens older than 12-weeks old (Anderson, 2000). Despite the finding that adult worms were killed after day 3 (Fig. 4), low numbers of parasite eggs were in general observed in cloacal contents throughout the entire sampling period. Parasite eggs up to 21 days pd were likely to represent intestinal passants as no adult worms were observed. In contrast, the cloacal egg counts on days 28 and 35 pd coincided with the identification of adult worms. Thus, our study shows that the life-cycle of *A. galli* can be completed within as little as four to five weeks in adult hens. This is in agreement with Idi et al. (2004) who recorded adult *A. galli* 4–5 weeks post inoculation of one-week old chickens. Furthermore, contrary to previous suggestion (Idi et al., 2004), the prepatancy of *A. galli* seems to be independent of host age. This will have implications for the design of the deworming strategies for laying hens.

Unlike in the cloacal faecal samples, small numbers of parasite eggs were detected throughout the experiment in the faecal samples collected from the manure belts. This was probably due to environmental parasite egg contamination, despite our attempt to exclude these by operating the manure belts prior to sampling. As previously suggested (Höglund and Jansson, 2011), our findings confirm that analysis of faecal samples from manure belts is not a reliable indicator of the anthelmintic efficacy in chicken flocks.

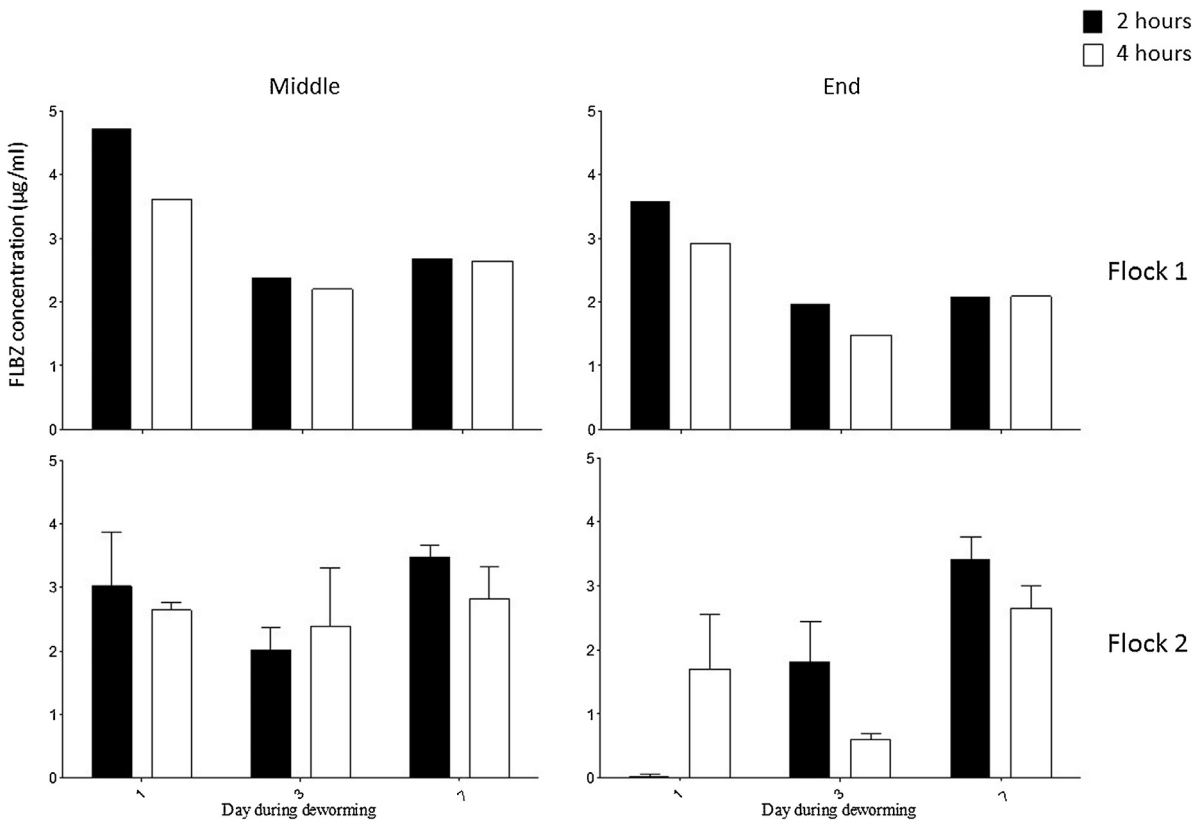

Fig. 7. Flubendazole concentration (µg/ml) in water samples obtained during the drug administration period from the middle and the end of the water lines, 2 and 4 h after the start of the treatment.

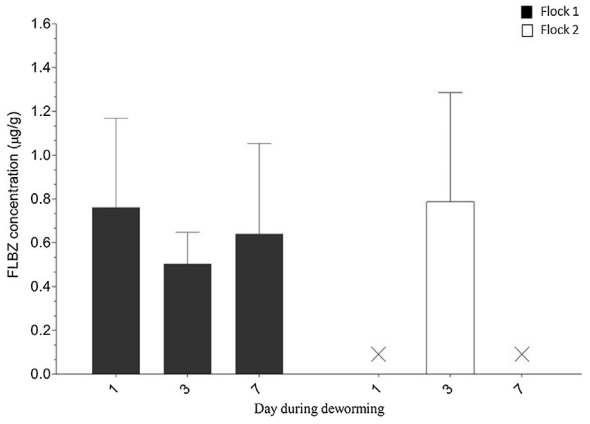

Fig. 8. Flubendazole concentration (µg/g) in intestinal samples obtained from 10 hens on each sampling occasion during flubendazole administration period. The cross indicates that no samples were obtained on day 1 and 7 in flock 2.

Flubendazole has poor solubility in water, but has been made available as a suspension for administration in the drinking water. This formulation makes it easy and inexpensive to administer the drug in commercial laying hen flocks (Boersema, 1985; Karimi Torshizi et al., 2010). There may however, be complications with administration of anthelmintics via drinking water. For example, water quality, drug solubility, medication equipment and varying water intake in birds may influence the success of drug administration (Vermeulen, 2002). Our study was conducted in conjunction with routine deworming in both flocks. A significant ($P < 0.05$) difference was observed in FLBZ concentrations between water samples taken from the middle or the end of water lines. Interestingly, the drug was not consumed within the stipulated time as recommended by the manufacturer as considerable amount of FLBZ was detected in water samples taken 4 h after the start of the treatment (Fig. 7). Sedimentation of the drug suspension in the stock solution and along the water lines may be responsible for the differences observed in the drug concentrations in our water samples (Fig. 7). Similar problems have been highlighted before (Sander and Schwartz, 1994). Since high FLBZ concentrations presented in drinking water, the low serum concentrations (µg/ml) for both FLBZ and R-FLBZ (0.02 ± 0.01 in F1, 0.03 ± 0.01 in F2, at both 3 and 7 days) could be explained by poor intestinal absorption of the parent drug after its administration in water. Variation in FLBZ blood concentrations between individual birds (Vermeulen, 2002) and differences in drug concentrations in the blood and in the intestinal content samples has been observed before for FLBZ (Michiels et al., 1982). Undetectable levels of FLBZ and R-FLBZ in some of the blood samples may be due to faster intestinal (first-pass effect) and liver metabolism of FLBZ in these hens (Baggot and McKellar, 1994) than that in the rest of the investigated hens. Nevertheless, the overall very low serum concentrations were not reflected by reduced drug efficacy. However, desired duodenal FLBZ concentrations were found in all the investigated hens, which is coherent with the parasitological findings in this study. Although the efficacy was 100% on all developmental stages during drug administration period, it was at the same time evident that female worms survived deworming longer than males. This is likely associated with the smaller surface area:volume ratio in males. To our knowledge, this has not been previously reported in *A. galli* and clearly needs more detailed investigation as it plays an important role in infection dynamic of *A. galli*.

5. Conclusion

Taken together, our results show that deworming with FLBZ had an adequate but ultimately short-lived effect in the studied flocks. It is also clear that laying hens were susceptible to reinfection very soon after the end of the treatment. Deworming laying hens once or twice during the production period (mainly during peak production) seems to be a temporary solution to reduce the worm burden in order to maintain the productivity. Clearly, the way anthelmintics are currently being used in commercial laying farms is of a less value with regard to efficient, long-lasting parasite control and promotion of animal health. Therefore, there is a need for a better deworming strategy against *A. galli*. To our knowledge, there are no reports on anthelmintic resistance to FLBZ in laying hens. However, as reported in this study, variation in drug consumption between birds should not be neglected as underdosing the birds may result in development of resistance in the future.

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