Free-range use and intestinal parasites in organic/free-range laying hens

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SUMMARY

Intestinal parasites are commonly found in non-cage laying hens. Some of these parasites reduce welfare and performance. Anthelmintics are not always effective and may lead to residues in eggs and in the environment. The aim of this study was to evaluate the relationship between free-range use and infections with intestinal parasites in organic laying hens, in order to identify directions for preventive measures. The study included 40 farms in 3 countries. Per farm, 6 pooled soil and 14 pooled fecal samples were analyzed using the McMaster method. Range use on flock level was assessed in several ways. Of the fecal samples, 71% (median) contained ascarid eggs, with a median of 143 eggs/gram (EPG). *Capillaria* eggs were found in 7% (median) of the fecal samples (median EPG = 5). Of the soil samples, 0% (median) contained ascarids eggs. *Capillaria* eggs were only detected in Italian soil samples. No relationship was found between parasite eggs in feces and range use or flock performance (% of lay, mortality). The low number of ascarid eggs and regionally the absence of *Capillaria* eggs in free-range soil suggest to focus further investigations on the conditions inside the hen house rather than in the free-range.

Key words: *Ascaridia galli*, *Heterakis gallinarum*, *Capillaria* spp., organic, free-range

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DESCRIPTION OF PROBLEM

Intestinal worm infections in poultry are found in all housing systems, especially in systems where the hens come into contact with their feces (Permin et al., 1999; Jansson et al., 2010). Some of these parasites reduce welfare. *Heterakis* and *Capillaria* may cause inflammations in the gut (Permin and Hansen, 1998). Ascarids worsen health problems caused by other pathogens (Dahl et al., 2002; Eigaard et al., 2006; Permin et al., 2006) and *Heterakis* acts as a vector for Blackhead (Permin and Hansen, 1998). Another concern is that adult *A. galli* worms erratically migrate from the intestine to the oviduct and inside the hen's egg, ending up with a consumer. This rarely happens; publications about the phenomenon describe only single cases (Fioretti et al., 2005; Gamit et al., 2017; Yasur-Landau et al., 2022).

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In non-cage systems, a large amount of litter is available to the hens in order to meet their behavioral needs for foraging and dustbathing (EU Council Directive, 1999). In organic/free-range egg production, a free-range area is mandatory (EU Commission Regulation, 2008, 2021) to provide hens with even more possibilities for dustbathing, foraging and sunbathing. A study by Jansson et al. (2010) on 169 Swedish flocks in 2008, before anthelmintics became available there, found 4.3% of caged flocks to be infected, compared with 29% and 52% of flocks in single-tiered and multi-tiered indoor systems, respectively, and 77% of free-range/organic flocks. In a study including 55 organic flocks in eight European countries, Thapa et al. (2015) found *Ascaridia galli* in 70% of the flocks, *Heterakis* spp. in 29% and *Raillietina* spp. in 14%, which is in agreement with findings by Jansson et al. (2010). In German and Italian studies, it has been found that 99 to 100% of the hens were infected with at least 1 helminth species in 18 and 4 organic flocks investigated, respectively (Kaufmann et al., 2011; Wuthijaree et al., 2017).

The effect of the mandatory free-range area on helminth (parasitic worm) infections in laying hens is not always clear. Swedish studies on 169 flocks (Jansson et al., 2010) and six flocks (Hoglund and Jansson, 2011) found no significant differences in ascarid infections between barn and free-range systems. However, a Danish study of 16 farms found that free-range/organic hens had a higher prevalence of *A. galli* and *H. gallinarum,* infections than hens in indoor systems (Permin et al., 1999). An Austrian study on 79 flocks found a higher *H. gallinarum* infection rate in indoor systems than in organic/free-range systems, but a higher *A. galli* infection rate in organic/free-range hens than in indoor hens (Grafl et al., 2017). On the other hand, a study on 50 flocks in eight European countries found that flocks which were able to spend more time ranging had lower levels of *A. galli* (Thapa et al., 2015). A British survey of 19 flocks found lower fecal egg counts for *A. galli* and *H. gallinarum,* in flocks with a higher proportion of hens using a free-range area and lower fecal egg counts for *A. galli* when more outdoor space was available per hen (Sherwin et al., 2013). All studies cited above were based on measures at flock level but in recent studies hens have been tracked individually, making it possible to link individual ranging patterns to health and welfare aspects. An Australian study that classified 307 experimental hens (all with access to a free-range area) into 'indoor hens,' 'low outdoor hens,' or 'high outdoor hens,' depending on the frequency and duration of their individual use of the free-range area, found no differences in the number of *A. galli* nematodes in hens from the three different groups (Bari et al., 2020). Another Australian study found that 'rangers' were more often infected with *A. galli* and cestodes than 'stayers,' that is, hens that rarely or never went outside (Sibanda et al., 2020).

Suggested reasons for higher levels of parasite infection in free-range/organic hens include contact with feces of wild birds, earthworms as intermediate hosts (for *H. gallinarum* and some *Capillaria* species) or residual contamination from previous flocks (Permin et al., 1999). Embryonated ascarid eggs can survive and remain viable for at least two years in Danish pasture soil (Thapa et al., 2017). Transmission of parasites from wildlife to domestic species via soil cannot be ruled out, while transmission from domestic to wild species is also gaining attention (Walker and Morgan, 2014). The initial introduction of parasite (eggs) into the free-range soil might come from wild birds, but also from young hens. After the initial introduction, successive flocks of hens may infect each other. Permin et al. (1999) suggest that lack of disinfection of the henhouse could also be a risk factor. Reported risk factors for parasite infection, other than the free-range, are absence of a hygiene barrier at the farmers entrance of the henhouse or unit and age of the equipment used in the henhouse (Jansson et al., 2010). An explanation for lower levels of parasite infections in hens using a free-range area might be a lower risk of contact with parasite eggs, as feces containing parasite eggs may potentially be spread over a larger area than in an indoor system (Thapa et al., 2015). Another explanation might be that free-range use decreases the density of feces indoors, and therefore lowers the risk of infection indoors (Sherwin et al., 2013). Although several authors suggest that

soil in free ranges plays a role in the infection of free-range hens with parasites, only one study (Heckendorn et al., 2009) actually measured parasite eggs in soil from a (less than 3 years 'old') free range. Such measurements are necessary for the empirical support of considering soil as a risk factor.

In 2006, 50% of organic flocks in the Netherlands were treated with the anthelmintic flubendazole (Iepema et al., 2006). Discussions about the use of anthelmintics highlight the adverse side-effects caused by residues ending up in products intended for human consumption (Kan et al., 1998; De Ruyck et al., 2004) or in the environment (Wagil et al., 2015; Lahr et al., 2018). Moreover, use of anthelmintic products does not prevent reinfection (Tarbiat et al., 2016a). So to keep infections low, deworming is done at regular intervals. Anthelmintics are also known to have an adverse effect on poultry. For example, Levkut et al. (2019) found a potential inflammatory effect of flubendazole on broiler chicken intestines. Another risk of widespread use of anthelmintics is that nematodes can develop resistance, as seen for nematode parasites in cattle (Sutherland and Leathwick, 2011), but not yet for benzimidazole and *A. galli* in laying hens (Feyera et al., 2022; Tarbiat et al., 2017). One option for reducing the use of anthelmintics may be to only treat the hens when fecal egg counts surpass an assigned threshold, for example 200 eggs/g feces. In a Swedish study (Tarbiat et al., 2016b) this led to three treatments instead of one (one treatment being the conventional situation in Sweden). In the Netherlands, three treatments per flock would be a reduction, compared to the 'deworming by calendar'-strategy, which results in treatments every 4-6 weeks (Bestman and Wagenaar, 2014; Iepema et al., 2006).

The aim of this study was to investigate the relationships between free-range use and parasite infection in organic laying hens. New in this study, compared to earlier studies, was that besides fecal samples, also soil was sampled from free ranges, which were in use at least for 8-10 years, and was analysed for the presence of parasite eggs. Also features as cover of soil in the free range with vegetation, litter, stones and management of the soil were included.

MATERIALS AND METHODS

Ethical Treatment of Animals

The study was conducted on commercial farms. The animals involved were not handled or restricted in their movement or daily routine otherwise. Therefore, approval of an ethical commission was not needed.

Recruitment of Flocks

A total of 40 organic flocks of laying hens were recruited in Sweden, the Netherlands and Italy. The housing in these countries, all belonging to the European Union, is very well comparable, i.e. indoor stocking density of 6 hens/m^2 and outdoor stocking density 4 m^2/hen, because of the EU-regulation (EU Commission Regulation, 2021). The flocks in these countries were expected to differ mainly in climate. In Sweden, a set of 16 farms was provided by an advisor on organic poultry production. Of these, farmers who were successfully contacted and willing to participate, did so. In the Netherlands, organic poultry farmers were invited to join the study by a letter (103 Dutch farmers) and a call in a Dutch poultry farmers' journal. Italian farmers were invited to join the study through veterinarians and organic producer associations. The following criteria were set for participation: free-range already in use for poultry for at least 8 to 10 years and no other animals (e.g., sheep or horses) on the free-range area in the past five years. Farm visits were planned when the hens were at least 45 weeks of age and had had outdoor access continuously for the past two months (e.g., no interruption because of avian influenza). If the farmer was planning to treat the hens for parasites, the visit was planned as soon before the treatment as possible. This was done in order to maximize the period between the last treatment and the sampling. All flocks sampled from October to March were regarded as winter flocks, while all flocks sampled from April to September were regarded as summer flocks (Kaufmann et al., 2011).

Data Collection

In accordance with the EU legislation on organic production, the farms kept their hens in

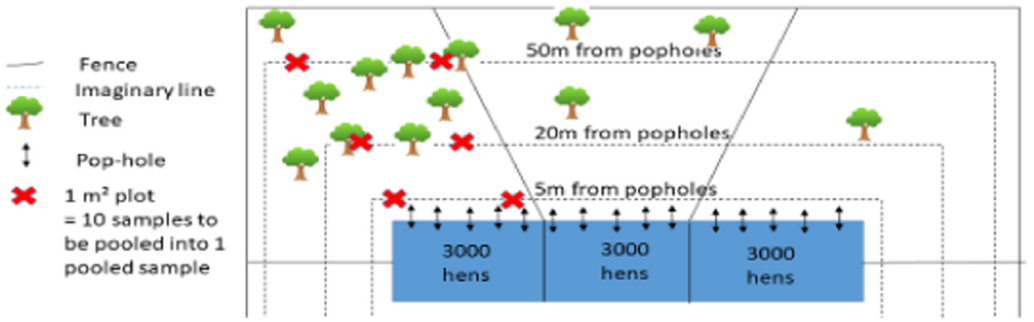

Figure 1. Schematic representation of a fictitious free-range area with soil sampling locations.

groups of no more than 3,000 birds. If a farm had multiple groups, only one of these was sampled. Free-range use was measured as number of years that the free-range area was in use for poultry, the number of weeks the sampled flock had uninterrupted access to the free-range area, the percentage of hens seen outside by the farmer until then under optimum conditions (before sunset, in calm, dry weather), the mean number of signs of hen presence in the 6 soil sampling locations and the mean proportion of soil covered with grass/herbs (Heckendorn et al., 2009) in the six soil sampling locations. The variation among farmers for the estimate of range use was accounted for by the observations by the researchers on signs of hen presence and of proportion of soil covered with vegetation, whereas the latter was a proxy for range use. See 'soil samples' section for further details on the signs of hen presence and the description of the sample locations.

Questionnaire

A questionnaire was prepared in order to collect data about the flocks participating in the study (date of hatch, breed name), farm (age of the free-range area, number of hens on the farm, number of hens per compartment), hen performance (laying percentage at 60 wk of age, mortality percentage to 60 wk of age, health (see below), free-range use (see below), treatments for parasites (name of drug/product, age of the hens when treated), and management of the soil (new layer of soil or litter added, ploughing or other inversion of the upper soil layer, rotational use) in the zones sampled. The farmers were asked to rate the overall health of their hens until then by giving a score on a scale of 1 (very bad) to 10 (perfect). The farmers were also asked to estimate the proportion of hens seen outside under optimum conditions until then. All questions were asked orally and all responses were written down by the researcher or technical assistant prior to the sampling of soil and feces. If the hens were younger than 60 wk at the time of the visit, the farmer was contacted again when the birds had reached 60 wk of age, for information on production and mortality percentages.

Soil Samples

Figure 1 shows a schematic representation of the free-range area and sampling locations. A total of 6 soil samples were taken from the free-range soil at 3 different distances from the popholes (or as close to this distance as possible): 2 samples at 5 m, 2 at 20 m, and 2 at 50 m. At each distance from the popholes, 2 different plots of 1 m^2 each were chosen. This was done in order to sample a variety of locations. These plots differed in distance from the fence and from trees. All 1 m^2 plots were described on a recording sheet in terms of proportion of soil visible, proportion of cover by grass/herbs, proportion of cover by shade (tree/bushes canopy, artificial cover), soil cultivation since removal of last flock, depth of soil cultivation, cover with litter or stones (yes/no, type, proportion of surface covered). Furthermore, the presence (yes/no) of 6 different signs of hens was recorded: hen(s), dropping(s), dust bath or scratching pit, scratch marks, feathers and footprints. In every 1 m^2 plot, 10 samples to 0 to 10 cm depth were taken using a soil sampling

device (Eijkelkamp 'grass plot sampler' diameter 23 mm). All 10 samples from each 1 m^2 plot were pooled to one sample and stored in a refrigerator at the end of the day, until further processing for McMaster worm egg counts (see 'McMaster counts' section).

Fecal Samples

Fecal samples and soil samples were collected from the same group of hens. In total, seven mixed samples were collected outside and seven were collected inside. Each mixed sample contained 10 fresh droppings pooled together. Outdoor feces were collected at a minimum of 50 m distance from the popholes. Collecting feces both outside and inside was done to include feces from hens with different ranging patterns. Inside the henhouse, as far away from the popholes as possible, another seven mixed samples were collected. Droppings were considered to be fresh if they had a shiny (moist) appearance and were soft. They were collected with gloves and a spoon, with a new set for each mixed sample, scooping without touching the ground or litter. Only intestinal droppings were collected, as cecal droppings seemed not to be sufficiently abundant to compile all mixed samples with the same ratio of cecal and intestinal droppings. The fecal samples were stored in a refrigerator at the end of the day, until further processing for worm egg counts.

McMaster Counts

Counts of eggs of *A. galli, H. gallinarum,* and *Capillaria* were made on all samples, using the simple McMaster method (Permin and Hansen, 1998). Eggs of *A. galli* and *H. gallinarum,* were all counted as coming from the group 'ascarids.' Hereafter, 'ascarids' refers to both *A. galli* and *H. gallinarum*. The *Capillaria* genus contains different species, but eggs from the genus *Capillaria* were all counted as *Capillaria* eggs. In Italy, 3 g of both feces and soil were used. In Sweden and the Netherlands, after repeatedly not finding eggs in soil samples, larger quantities of soil were analysed. In the Netherlands, 6 g of soil was used and ascarid and *Capillaria* eggs were counted in the two McMaster chambers. In Sweden, 10 g of soil was used and all ascarid and *Capillaria* eggs were counted, not only those in the two McMaster chambers. The Dutch and Swedish soil results were then back calculated to EPG.

Statistical Analyses

All data were entered into a MS Excel spreadsheet and all descriptive and analytical statistics were produced using SPSS 26 (IBM, 2019).

Mean ascarid EPG and mean *Capillaria* EPG at flock level were calculated as a mean of ascarid or *Capillaria* EPGs for all samples within a flock. These means were calculated separately for fecal and soil samples. Per flock, the proportions of fecal and soil samples testing positive for either ascarids or *Capillaria* were also calculated. Thus, parasite infection was expressed in 2 sets of 4 different variables, one set for fecal samples and one set for soil samples. These variables were: percentage of samples testing positive for ascarids, mean number of ascarid eggs/g sample material, percentage of samples testing positive for *Capillaria* and mean number of *Capillaria* eggs/g sample material. Parasitological and descriptive flock and farm variables were checked for normality (Kolmogorov-Smirnov, Shapiro-Wilk and histogram tests in SPSS). The means of variables that were normally distributed were compared between countries with one-way independent ANOVA, using post-hoc Bonferroni tests. The means of variables that were not normally distributed were compared with the Kruskal-Wallis test with pairwise comparisons and Bonferroni corrected. Correlations between parasitological parameters in soil and feces were calculated with Spearman's rho. Correlations between feces collected outdoors and indoors were calculated with the Wilcoxon signed ranks test.

Three variables were used to reflect the degree of free-range use by a flock: 'Percentage of hens outside,' 'Mean soil vegetation percentage,' and 'Mean number of hen signs.' Mutual Pearson correlations were calculated to assess whether use of just one of these three variables was justified, which would be the case if they were highly correlated. The relationship between parasitological parameters for feces

Table 1. Mean value (standard deviation) of variables characterizing the flocks studied.

Variable	All	Sweden	Netherlands	Italy
No. of flocks	40	10	20	10
Hens/farm	11,714 (10,433)	19,435 (17,062)[a]	11,496 (3,971)[ab]	4,430 (4,687)[b]
Hens/flock	3,384 (3,322)	6,540[1] (5,918)[a]	2,771 (519)[b]	1,770 (1,098)[b]
Years free-range in use	16 (5.6)	18 (7.3)	15 (4.1)	14 (6.2)
Genotype: Brown		1	17	6
White		9	3	1
Mixed/other		0	0	3
Age of hens in weeks	62 (12)	66 (11)	62 (11)	60 (16)
Access to free-range in weeks	31 (9)	28 (10)	32 (5)	34 (13)
% Hens out	51 (25)	31 (20)[a]	48 (22)[a]	76 (10)[b]
% Soil covered by grass/weeds	30 (33)	61 (26)[a]	5.5 (11)[b]	47 (33)[a]
Mean number of signs of hen presence	4.2 (1.5)	3.4 (1.8)[a]	5.2 (0.6)[b]	2.9 (1.0)[a]
Health at 60 wks[2]	8.1 (1.3)	8.0 (1.5)	7.9 (1.1)	8.5 (1.4)
Laying % 60 wks	86 (7.5)	88 (3.1)[a]	89 (3.3)[a]	76 (8.1)[b]
Mortality % by 60 wks	5.1 (4.6)	4.3 (2.4)	4.5 (1.9)	7.2 (8.5)
Number of flocks treated with anthelmintics	19/40	0/10	19/20	0/10
Number of anthelminitic treatments by 60 wks	2.5 (2.8)	0 (0)[a]	4.8 (1.9)[b]	0 (0)[a]
Days since last anthelmintic treatment		Not applicable	41 (14)	Not applicable

[1]Because the wintergardens/free-ranges on the Swedish farms did not contain physical structures to separate hens, the hens from different compartments could thus come into contact with each others feces. This was a relevant aspect in this study and therefore these hens were considered as one flock. Indoors, the groups were separated and included no more than 3,000 hens.
[2]Health status of the flock, estimated by the farmer and expressed on a scale of 1 (=extremely bad) to 10 (=extremely good).
[a-b]Means within the same row with no common superscript differ significantly (one-way ANOVA; post-hoc Bonferroni $P < 0.01$).

and free-range use, farm and flock characteristics were analyzed using linear mixed models. The selection of variables that were expected to influence the parasitological variables, was based on literature and other expert knowledge. Variables indicating parasitological infection that were not normally distributed were $\log_{10}$-transformed to obtain a normal distribution. Variables that also after $\log_{10}$-transformation still were not normal distributed, were rank-transformed. Rank transformations are a bridge between parametric and nonparametric statistics (Conover and Iman, 1981). Dependent variables were: ranked proportion of fecal samples containing ascarid eggs, $\log_{10}$-transformed ascarid EPG, ranked proportion of fecal samples containing *Capillaria* eggs and ranked *Capillaria* EPG. Fixed effects were country, season, number of hens per flock, number of hens per farm, 'age' of the free-range area, number of weeks in which the hens had had uninterrupted access to the free-range area at the time of sampling, age of the hens, proportion of hens using the free-range area, mean number of signs of hen presence, flock health estimated by the farmer, laying percentage at 60 wk of age and mortality percentage by 60 wk of age. These variables/effects went into an univariable selection. Thereafter, the variables with $P < 0.1$ were entered together in linear mixed modelling. The final model was selected based on Akaike information criterion and p-values ≤ 0.05.

RESULTS AND DISCUSSION

In total, 40 flocks (10 in Sweden, 20 in the Netherlands and 10 in Italy) were visited from October 2018 to October 2020, when the hens were aged between 45 and 94 (mean 62) wk. If the flock had been treated with anthelmintics, the visit was made as long as possible after the last treatment (i.e., shortly before the next treatment), resulting in 21 to 68 (mean 41) d after treatment. The flocks had uninterrupted free-range access for at least 17 to 64 (mean 31) wk.

Flock Information and Parasite Occurrence in Soil and Feces

Table 1 shows the variables investigated for the 40 flocks. In all 3 countries, the free-range area had been in use for a similar period (on

Table 2. Descriptive statistics (median value and minimum-maximum) of parasitological parameters in fecal and soil samples.

Variable	All	Sweden	Netherlands	Italy
No. of flocks	40	10	20	10
No. of pooled* faecal samples	524	132	280	112
Median % of samples containing ascarid eggs	71 (0−100)	96 (21−100)	71 (21−100)	50 (0−100)
Median number of ascarid eggs/g faeces	143 (0−1936)	379 (22−1471)	141 (14−1936)	136 (0−300)
Median % of samples containing *Capillaria* eggs	7 (0−71)	0 (0−7)[a]	14 (0−71)[b]	18 (0−71)[b]
Median number of *Capillaria* eggs/g feces	5 (0−150)	0 (0−0)[a]	14 (0−150)[b]	12 (0−79)[b]
No. of pooled* soil samples	240	60	120	60
Median % of samples containing ascarid eggs	0 (0−67)	0 (0−0)[a]	17 (0−50)[b]	17 (0−67)[b]
Median number of ascarid eggs/g soil	0 (0−100)	0 (0−0)[a]	8 (0−100)[b]	8 (0−75)[b]
Median % of samples containing *Capillaria* eggs	0 (0−100)	0 (0−0)[a]	0 (0−0)[a]	83 (0−100)[b]
Median number of *Capillaria* eggs/g soil	0 (0−283)	0 (0−0)[a]	0 (0−0)[a]	83 (0−283)[b]

[a-b]Medians within the same row with no common superscript differ significantly (independent-samples Kruskal-Wallis test and Bonferroni corrected pairwise comparison $P < 0.05$).

*'Pooled' samples means that each sample consisted of 10 individual droppings (in case of fecal samples) or 10 samples taken from the same $1m^2$ plot (in case of soil samples) that were pooled (=mixed) together.

average 16 y), there were high scores for hen health (on average 8.1) and there was relatively low mortality (on average 5.1% to 60 wk of age). However, flocks and farms differed in size and genotype between the countries. One-way ANOVA revealed that Italian farmers estimated range use of their flocks highest ($F(2, 35) = 12.9$; $P < 0.001$). Based on the observers estimates of proportion of soil covered with grass/weeds ($F(2, 37) = 25.1$; $P < 0.001$) and mean number of signs of hen presence ($F(2, 37) = 17.5$; $P < 0.001$), range use was highest in the Netherlands. Italian flocks were rated as healthy as Dutch and Swedish flocks by farmers, but they showed a lower actual production level than Dutch and Swedish flocks ($F(2, 36) = 26.9$; $P < 0.001$) (Table 1). Only Dutch flocks (19 out of 20 studied) were treated with anthelmintics (mostly flubendazole, sometimes fenbendazole), on average 4.8 times by 60 wk of age.

The parasite levels found in fecal and soil samples are shown in Table 2. In general, the range of values (minimum to maximum) in fecal samples was rather high. A similarly high percentage (median 71%) of fecal samples was found to contain ascarid eggs (median 143 eggs/g feces) in all 3 countries. *Capillaria* eggs were rarely found in Swedish fecal samples; according to the results of the McMaster method, none of the Swedish fecal samples contained *Capillaria* eggs, but with additional effort (checking the whole 3 g sample instead of only the 2 McMaster chambers from the Permin and Hansen (1998) protocol) 1 *Capillaria* egg was found in 1 fecal sample. Kruskal-Wallis testing revealed that the results concerning *Capillaria* were similar for Dutch and Italian fecal samples: percentage of samples with *Capillaria* ($H(2) = 11.9$; $P = 0.003$) and mean number of *Capillaria* eggs/g ($H(2) = 11.3$; $P = 0.003$). Ascarid eggs were only found in Dutch and Italian soil (proportion of samples with ascarid eggs ($H(2) = 10.3$; $P = 0.006$) and mean number of ascarid eggs/g ($H(2) = 9.7$; $P = 0.008$). *Capillaria* eggs were extremely rare in Swedish soil samples. According to the McMaster method, none of the samples contained *Capillaria* eggs, but with additional effort (checking the whole 10 g sample instead of only the 2 McMaster chambers from the Permin and Hansen (1998) protocol) 1 and 2 *Capillaria* eggs were found in 2 soil samples. Thus, when sticking to the McMaster counts, *Capillaria* eggs were only found in Italian soil (proportion of soil samples with *Capillaria* ($H(2) = 33.4$; $P < 0.001$) and mean number of *Capillaria* eggs/g ($H(2) = 33.3$; $P \leq 0.001$) (Table 2).

Concerning the presence of ascarid eggs, no significant correlation was found between proportion of positive fecal samples and proportion of positive soil samples (Spearman's rho = −0.239; $P = 0.138$; n = 40) or between

ascarid EPG in fecal and soil samples (Spearman's rho = −0.035; P= 0.828; n = 40). This is probably due to the fact that hardly any ascarid eggs were found in soil. Concerning the presence of *Capillaria* eggs, the proportion of positive fecal samples was correlated with the proportion of positive soil samples (Spearman's rho = 0.410; P= 0.009; n = 40). The number of *Capillaria* eggs in feces was also related to the mean number of *Capillaria* eggs in soil (Spearman's rho = 0.336; P= 0.034; n = 40) (Table 2). These correlations are probably due to the fact that *Capillaria* eggs were found in substantial quantities in both fecal and soil samples from Italy. The latter finding also showed that helminth eggs, if present, could be found with the McMaster method. Thus, finding hardly or no ascarid eggs in soil probably means that there were hardly any such eggs present.

Regarding the four variables for parasite eggs in feces, no differences were found between samples collected outside at distances more than 50 m from the popholes and samples collected inside the henhouse (Wilcoxon signed rank test; n = 38; $-1.441 < Z < -1.034$; $0.301 < P < 0.150$). The same absence of difference was found by others too (Sherwin et al., 2013). If indoor and outdoor collected feces were produced by hens with different ranging patterns (low and high rangers), then ranging pattern did not result in a different EPG.

Choice of Variables Reflecting Free-Range Use

No correlation was found between the proportion of hens seen outside by the farmer and that estimated by the observer based on cover of grass/weeds (r = −0.02; P= 0.91) and signs of hen presence at the six sampling locations (r = 0.09; P= 0.59), when corrected for country and season. However, a strong negative correlation was found between the two types of estimates made (vegetation cover, hen presence) by the observer (r = −0.83; P< 0.001). Such a negative correlation is logic, when realizing that hens eat the grass completely and prevent regrowth by scratching the soil. Of these, the mean number of signs of hen presence was chosen for further calculations, since this estimate directly represented use of the free-range area, while absence of vegetation cover was an indirect indicator for outdoor stocking density (Heckendorn et al., 2009).

No model could be fitted for ascarid or *Capillaria* EPG in feces or for proportion of fecal samples containing ascarid or *Capillaria* eggs. A minority of soil samples contained ascarid eggs (median 0%) and these were found only in the Netherlands and Italy. *Capillaria* eggs were only found in Italian soil samples. Therefore, no model could be fitted for either EPG in soil or for percentage of soil samples containing parasite eggs.

Prevalence of Parasite Eggs in Free-Range Soil and Faeces

This study confirmed that ascarid infections are widespread in organic laying hen flocks. A median of 71% of fecal samples analyzed contained ascarid eggs. Other studies have also found high prevalence of ascarid infections in organic and/or free-range laying hens (Permin et al., 1999; Sherwin et al., 2013; Thapa et al., 2015; Grafl et al., 2017). The prevalence of *Capillaria* infections showed lower variation than the prevalence of ascarid infections. A median of 7% of the fecal samples contained *Capillaria* eggs. This is within the range found in other studies (Permin et al., 1999; Jansson et al., 2010; Grafl et al., 2017; Wuthijaree et al., 2017).

Capillaria eggs were present in more samples and in higher amounts in fecal samples from the Netherlands and Italy, compared to Sweden.

The median proportion of soil samples per flock containing ascarid eggs was 0%, but it ranged from 0% in Sweden to 17% in the Netherlands and Italy. Only very few studies looked at the presence of eggs of poultry gut parasites in soil. Heckendorn et al. (2009) found ascarid eggs in 100% of the soil samples they analyzed, but with fewer EPG (at most 2.5, compared with 18 in our study). However, the results of these studies are not directly comparable, since we expressed EPG in multiplies of 50 (original McMaster) instead of an exact number of eggs (e.g., a sample containing exact 2.5 EPG would probably be reported as EPG = 0 as the chance of catching these few eggs in the 2 McMaster

chambres would be very small). A median of 0% of soil samples per flock contained *Capillaria* eggs, but the value ranged from 0% in Swedish and Dutch soil up to 83% in Italian soil. The common denominator in regional aspects is that from north to south an increasing amount of parasite eggs is found in feces (*Capillaria*) and in soil (ascarids and *Capillaria*). This may be caused by climatic conditions, temperatures in soil and henhouses generally being lower in Sweden than in Italy with the Netherlands in between, assuming a lower suvival or embryonation of parasite eggs at lower temperatures (Permin and Hansen, 1998; Tarbiat et al., 2015). Furthermore, in Sweden it is allowed to keep hens inside during winter, which may reduce the build-up of parasite eggs in the soil.

This is the first study to detect *Capillaria* eggs in soil from free-range areas. A previous analysis of litter samples from henhouses revealed that 91% of the samples contained ascarid eggs and 13% contained *Capillaria* eggs. The mean number of ascarid eggs per gram of litter material was 400 and the number of *Capillaria* eggs ranged between 0 and 28 per gram (Maurer et al., 2009). This indicates that, for ascarids and depending on the region also for *Capillaria*, litter inside the henhouse may carry a higher risk of parasite infections than soil in the free-range area. In the present study, we found a positive correlation between *Capillaria* eggs in soil and in feces, but we could not determine causality. Maurer et al. (2009) did not find a correlation between parasitological parameters in litter and feces.

Use of Free-Range Area

We assessed use of the free-range area in several ways: as farmers' estimates of free-range use by the current flock, as observers' estimates of signs of hen presence, as observers' estimates of cover with grass/weeds, and 'age' of the free-range. However, none of these indicators for use of the free-range area was found to be associated with parasitological parameters. Other studies investigating the relationship between parasite infections and use of the free-range area have found a positive relationship, i.e. more parasites with more free-range use (Permin et al., 1999; Sibanda et al., 2020; Shifaw et al., 2021), a negative relationship (Sherwin et al., 2013; Thapa et al., 2015) or no relationship (Bari et al., 2020). It is possible that our sample size of 40 flocks was not large enough, when taking into account the ratio between median number of eggs/g and the range between minimum and maximum values. When finding a positive correlation between range use and gut parasite infections, the next necessary step would be to count parasite eggs in the soil, but the studies cited above lack such an empirical confirmation.

Anthelminitic Treatments

Ascarid infections did not differ between Dutch and Italian flocks, even though 19 out 20 Dutch flocks studied were treated on average five times up to 60 wk of age with flubendazole or fenbendazole, whereas the Italian flocks were not treated at all. Because all treated flocks were Dutch and all untreated flocks except one were Swedish or Italian, we could not assess the effect of anthelmintic treatment on parasite eggs in soil or fecal samples or determine whether this difference was caused by other differences between the three countries. However, the parasitological parameters for Dutch flocks raise questions about the effectiveness of anthelmintic treatment. This confirms findings in experimental studies in which laying hens were treated with flubendazole and found to be parasite-free only one for week (Tarbiat et al., 2016a) or 2 to 4 wk (Höglund and Jansson, 2011) post-treatment. An experiment with a 'targeted treatment', consisting of measuring fecal egg count every two weeks and treatment in cases of >200 eggs/g, found that the number of eggs/g was lower in the targeted treatment flocks than in untreated or standard-treated (i.e., treated once) flocks (Tarbiat et al., 2016b). Together, these findings suggest to reconsider the use of anthelmintic treatments 'by calendar', which is a standard approach in the Netherlands.

Health and Productivity

Generally, an impaired health may lead to measurable changes in several aspects, such as feed- or water intake, reduced production, shell

abnormalities, et cetera.. Health assessment by the farmers as done in our study, was a crude estimate, which was justified because it was a secondary objective of this study. We wanted to collect all data, including the health, in a one-time visit. If one wants to do it correctly, overall health, even by a veterinarian, cannot be expressed in one score. Furthermore, indicators as shell quality, feed and water intake would be a snapshot. Within these restrictions, we did not find a relationship between any of the parasitological parameters studied and hen health or productivity. Other studies also generally found no relationship between parasitological parameters and mortality (Gauly et al., 2008; Sherwin et al., 2013; Wongrak et al., 2015). However, Stehr et al. (2019) found a lower laying rate and lower egg weight in experimentally infected hens. Mortality has sometimes been found to be higher in hens with an *A. galli* infection, but with other factors also playing a part, for example, too low protein content in the feed (Ikeme, 1971) or a bacterial infection (Dahl et al., 2002; Eigaard et al., 2006; Permin et al., 2006). However, Hinrichsen et al. (2016) found higher mortality in peak-of-lay hens on highly infected organic farms in summer. The lack of relationship we found between parasitological parameters and egg production is in line with other studies (Gauly et al., 2007; Sherwin et al., 2013). Decreased egg production was reported by Stehr et al. (2019), who found a lower laying rate and lower egg weight in hens experimentally infected with *A. galli* and *H. gallinarum* and in case of bacterial co-infection (Dahl et al., 2002). Generally, ascarid infection alone does not seem to be associated with higher mortality or lower egg production, but under commercial conditions bacterial co-infections can be expected (Sharma et al., 2019).

Management of the Free-Range Area

Because of the regionally low prevalence of parasite eggs in soil samples analyzed in this study, it was not possible to test relationships between soil treatment, presence of shade provided by tree canopies or artificial structures, and parasite eggs in soil samples. Heckendorn et al. (2009) investigated naturally 'infected' soil and counted absolute numbers of parasite eggs, whereas we expressed EPG in multiples of 50. They found very few ascarid eggs (≤2.5 eggs/g) in all soil samples, but observed no effect of mowing the free-range area. An experimental study by Maurer et al. (2020) found that ascarid eggs disappeared faster from gravel and wood chips than from soil. However, they mixed poultry feces with pea gravel, wood chips and soil, resulting in ≥350 ascarid eggs/g of chips/gravel/soil, that is, much more than the EPG found by Heckendorn et al. (2009) and in the present study. In order to investigate the effect of management on parasite eggs in soil samples, an experiment would be more controlled than studies on commercial flocks.

Limitations of the Study

Including flocks from at least 45 weeks of age in this study posed a risk of substantial age differences between the sampled flocks. However, this risk was justified because the alternative, choosing a shorter age period, posed the risk of us being unable to sample numerous flocks because of statutory confinement due to avian influenza, which was imposed regularly (almost yearly) by national authorities during the study period. Another of our criteria was that flocks should have had access to the free-range area for at least two months. Introduction of a confinement period would have seriously delayed sampling (by months of confinement + 2 months) and flocks might have been at slaughter age before this delay period was over. Furthermore, in retrospect, the application of the McMaster method, especially for soil samples should have been standardized between the different laboratories.

CONCLUSIONS AND APPLICATIONS

1. Ascarid and *Capillaria* eggs were widely present in fecal samples from laying hens in

respectively all three and the two southern countries, including samples from flocks treated repeatedly with anthelmintics.

2. In Sweden and the Netherlands (almost) no parasite eggs were found in soil, while the majority of Italian soil samples contained *Capillaria* eggs.

3. No associations were found between indicators of use of the free-range area and ascarid or *Capillaria* eggs in fecal samples.

4. No associations were found between parasite eggs in fecal samples and hen health and productivity.

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DISCLOSURES

The authors have no conflicts of interest.

REFERENCES

Bari, M. S., Y. C. S. M. Laurenson, A. M. Cohen-Barnhouse, S. W. Walkden-Brown, and D. L. M. Campbell. 2020. Effects of outdoor ranging on external and internal health parameters for hens from different rearing enrichments. PeerJ 8:e8720.

Bestman, M., and J.-P. Wagenaar. 2014. Health and welfare in Dutch organic laying hens. Animals 4:374–390.

Conover, W. J., and R. L. Iman. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. Am. Stat. 35:124–129.

Dahl, C., A. Permin, J. P. Christensen, M. Bisgaard, A. P. Muhairwa, K. M. D. Petersen, J. S. D. Poulsen, and A. L. Jensen. 2002. The effect of concurrent infections with Pasteurella multocida and *Ascaridia galli* on free range chickens. Vet. Microbiol. 86:313–324.

De Ruyck, H., E. Daeseleire, K. Grijspeerdt, H. De Ridder, R. Van Renterghem, and G. Huyghebaert. 2004. Distribution and depletion of flubendazole and its metabolites in edible tissues of guinea fowl. Br. Poul. Sci. 45:540–549.

Eigaard, N. M., T. W. Schou, A. Permin, J. P. Christensen, C. T. Ekstrøm, F. Ambrosini, D. Cianci, and M. Bisgaard. 2006. Infection and excretion of Salmonella Enteritidis in two different chicken lines with concurrent *Ascaridia galli* infection. Avian Pathol. 35:487–493.

EU Commission Regulation. 2008. Commission Regulation (EC) 589/2008 of 23 June 2008 laying down detailed rules for implementing Council Regulation (EC) No 1234/2007 as regards marketing standards for eggs. Official Journal of the European Union 24th June 2008.

EU Commission Regulation. 2021. Commission Implementing Regulation (EU) 2020/464 of 26 March 2020 laying down certain rules for the application of Regulation (EU) 2018/848 of the European Parliament and of the Council as regards the documents needed for the retroactive recognition of periods for the purpose of conversion, the production of organic products and information to be provided by Member States. Official Journal of the European Communities 25th November 2021.

EU Council Directive. 1999. Council directive 1999/74/EC of 19 July 1999 laying down the minimum standards for protection of laying hens. Official Journal of the European Communities 3rd August 1999.

Feyera, T., A. Shifaw, B. Sharpe, T. Elliott, I. Ruhnke, and S. W. Walkden-Brown. 2022. Worm control practices on free-range egg farms in Australia and anthelmintic efficacy against nematodes in naturally infected layer chickens. Vet. Parasitol. Reg. Stud. Reports. 30:100723.

Fioretti, D. P., F. Veronesi, M. Diaferia, M. P. Franciosini, and P. Casagrande Proietti. 2005. *Ascaridia galli*: a report of erratic migration. J. Anim. Sc. 4:310–312.

Gamit, A. B., P. K. Nanda, S. Bandyopadhyay, and R. Bhar. 2017. A report of *Ascaridia galli* in commercial poultry egg from India. J. World Poult. Res. 7:23–26.

Gauly, M., C. Duss, and G. Erhardt. 2007. Influence of *Ascaridia galli* infections and anthelmintic treatments on the behaviour and social ranks of laying hens (*Gallus gallus domesticus*). Vet. Parasitol. 146:271–280.

Gauly, M., A. Kanan, H. Brandt, S. Weigend, E. Moors, and G. Erhardt. 2008. Genetic resistance to *Heterakis gallinarum* in two chicken layer lines following a single dose infection. Vet. Parasitol. 155:74–79.

Grafl, B., S. Polster, T. Sulejmanovic, B. Pürrer, B. Guggenberger, and M. Hess. 2017. Assessment of health and welfare of Austrian laying hens at slaughter demonstrates influence of husbandry system and season. Br. Poult. Sci. 58:209–215.

Heckendorn, F., D. A. Haring, Z. Amsler, and V. Maurer. 2009. Do stocking rate and a simple run management practice influence the infection of laying hens with gastrointestinal helminths? Vet. Parasitol. 159:60–68.

Hinrichsen, L. K., R. Labouriau, R. M. Engberg, U. Knierim, and J. T. Sørensen. 2016. Helminth infection is

associated with hen mortality in Danish organic egg production. Vet. Rec. 179:196–196.

Höglund, J., and D. S. Jansson. 2011. Infection dynamics of *Ascaridia galli* in non-caged laying hens. Vet. Parasitol. 180:267–273.

IBM (2019). SPSS statistics for windows (version 26.0).

Iepema, G., J-P. Wagenaar, and M. Bestman. 2006. Parasitic worms in organic laying hens. Proceedings of Joint European Organic Congress May 30-31.

Ikeme, M. M. 1971. Observations on the pathogenicity and pathology of *Ascaridia galli*. Parasitology 63:169–179.

Jansson, D. S., A. Nyman, I. Vågsholm, D. Christensson, M. Göransson, O. Fossum, and J. Höglund. 2010. Ascarid infections in laying hens kept in different housing systems. Avian Pathol. 39:525–532.

Kan, C. A., H. J. Keukens, and M. J. H. Tomassen. 1998. Flubendazole residues in eggs after oral administration to laying hens: determination with reversed phase liquid chromatography. Analyst 123:2525–2527.

Kaufmann, F., G. Daş, B. Sohnrey, and M. Gauly. 2011. Helminth infections in laying hens kept in organic free range systems in Germany. Livest. Sci. 141:182–187.

Lahr, J., A. Derksen, M. Wipfler, M. Schans, B. van de Berendsen, M. Blokland, W. Dimmers, P. Bolhuis, and R. Smidt. 2018. Veterinary medicines and hormones in the environment through slurry application; an exploratory study in the province of Gelderland into antibiotics, antiparasitic agents, coccidiostats and natural hormones in manure, (aquatic) soil, groundwater and surface water Report No. 2898 Wageningen Environmental Research (in Dutch.

Levkut, M., V. Revajová, M. Levkutová, E. Selecká, Z. Ševčíková, and V. Karaffová. 2019. The influence of chestnut wood and flubendazole on morphology of small intestine and lymphocytes of peripheral blood, spleen and jejunum in broiler chickens. Helminthologia 56:273.

Maurer, V., Z. Amsler, E. Perler, and F. Heckendorn. 2009. Poultry litter as a source of gastrointestinal helminth infections. Vet. Parasitol. 161:255–260.

Maurer, V., E. Perler, Z. Amsler-Kepalaite, and A. Bieber. 2020. Ascarid eggs disappear faster from gravel and wood chips than from soil. Br. Poult. Sci. 62:1–7.

Permin, A., M. Bisgaard, F. Frandsen, M. Pearman, J. Kold, and P. Nansen. 1999. Prevalence of gastrointestinal helminths in different poultry production systems. Br. Poultr. Sci. 40:439–443.

Permin, A., J. P. Christensen, and M. Bisgaard. 2006. Consequences of concurrent *Ascaridia galli* and *Escherichia coli* infections in chickens. Acta Vet. Scand. 47:43–54.

Permin, A., and J. W. Hansen. 1998. The Epidemiology, Diagnosis and Control of Poultry Parasites. FAO Animal Health Manual. http://www.fao.org/docrep/018/x0583e/x0583e.pdf.

Sharma, N., P. W. Hunt, B. C. Hine, and I. Ruhnke. 2019. The impacts of *Ascaridia galli* on performance, health, and immune responses of laying hens: new insights into an old problem. Poult. Sci. 98:6517–6526.

Sherwin, C. M., M. A. F. Nasr, E. Gale, M. Petek, K. Stafford, M. Turp, and G. C. Coles. 2013. Prevalence of nematode infection and faecal egg counts in free-range laying hens: relations to housing and husbandry. Br. Poult. Sci. 54:12–23.

Shifaw, A., T. Feyera, S. W. Walkden-Brown, B. Sharpe, T. Elliott, and I. Ruhnke. 2021. Global and regional prevalence of helminth infection in chickens over time: a systematic review and meta-analysis. Poult. Sci. 100:101082.

Sibanda, T. Z., C. J. O'Shea, J. de Souza Vilela, M. Kolakshyapati, M. Welch, D. Schneider, J. Courtice, and I. Ruhnke. 2020. Managing free-range laying hens – part B: early range users have more pathology findings at the end of lay but have a significantly higher chance of survival – an indicative study. Animals 10:1911.

Stehr, M., M. Grashorn, D. Dannenberger, A. Tuchscherer, M. Gauly, C. C. Metges, and G. Daş. 2019. Resistance and tolerance to mixed nematode infections in relation to performance level in laying hens. Vet. Parasitol. 275:108925.

Sutherland, I. A., and D. M. Leathwick. 2011. Anthelmintic resistance in nematode parasites of cattle: a global issue? Trends Parasitol. 27:176–181.

Tarbiat, B., D. S. Jansson, and J. Höglund. 2015. Environmental tolerance of free-living stages of the poultry roundworm *Ascaridia galli*. Vet. Parasitol. 209:101–107.

Tarbiat, B., D. S. Jansson, L. Moreno, C. Lanusse, M. Nylund, E. Tydén, and J. Höglund. 2016a. The efficacy of flubendazole against different developmental stages of the poultry roundworm *Ascaridia galli* in laying hens. Vet. Parasitol. 218:66–72.

Tarbiat, B., D. S. Jansson, E. Tydén, and J. Höglund. 2016b. Comparison between anthelmintic treatment strategies against *Ascaridia galli* in commercial laying hens. Vet. Parasitol. 226:109–115.

Tarbiat, B., D. S. Jansson, E. Tydén, and J. Höglund. 2017. Evaluation of benzimidazole resistance status in *Ascaridia galli*. Parasitol 144:1338–1345.

Thapa, S., L. K. Hinrichsen, C. Brenninkmeyer, S. Gunnarsson, J. L. T. Heerkens, C. Verwer, K. Niebuhr, A. Willett, G. Grilli, S. M. Thamsborg, J. T. Sørensen, and H. Mejer. 2015. Prevalence and magnitude of helminth infections in organic laying hens (*Gallus gallus domesticus*) across Europe. Vet. Parasitol. 214:118–124.

Thapa, S., S. M. Thamsborg, N. V. Meyling, S. Dhakal, and H. Mejer. 2017. Survival and development of chicken ascarid eggs in temperate pastures. Parasitology 144:1243–1252.

Wagil, M., A. Białk-Bielińska, A. Puckowski, K. Wychodnik, J. Maszkowska, E. Mulkiewicz, J. Kumirska, P. Stepnowski, and S. Stolte. 2015. Toxicity of anthelmintic drugs (fenbendazole and flubendazole) to aquatic organisms. Environ. Sci. Pollut. Res. 22:2566–2573.

Walker, J. G., and E. R. Morgan. 2014. Generalist at the interface: nematode transmission between wild and domestic ungulates. Int. J. Parasitol. Parasites Wildl. 3:242–250.

Wongrak, K., M. Gauly, and G. Daş. 2015. Diurnal fluctuations in nematode egg excretion in naturally and in experimentally infected chickens. Vet. Parasitol. 208:195–203.

Wuthijaree, K., C. Lambertz, and M. Gauly. 2017. Prevalence of gastrointestinal helminth infections in free-range laying hens under mountain farming production conditions. Br. Poultr. Sci. 58:649–655.

Yasur-Landau, D., M. Hanovice-Ziyoni, E. Almogi, L. Fleiderovitz, and M. L. Mazuz. 2022. *Ascaridia galli* adult nematode in chicken egg from a commercial organic farm in Israel. Isr. J. Vet. Med. 77:106–110.