Retrospective study of severe outbreaks of *Histomonas meleagridis* in Dutch turkey flocks from 2010-2015

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ABSTRACT

Histomonas meleagridis (*H. meleagridis*) is a flagellated protozoa and the causative agent of a severe disease, histomonosis, that harms the worldwide turkey industry. It emerged in Europe since the ban on nitroimidiazoles and nitrofurans in 2003. Due to rapid horizontal spread in flocks and severe mortality in affected barns it poses a serious financial threat to the Dutch turkey industry. The aim of this study was to create an overview of possible routes of introduction for *H. meleagridis*, for 13 individual outbreaks of histomonosis selected by the Turkey Sales Cooperation Holland (BAV) in the years 2010 through 2015, and to create an insight in the course of infection for these outbreaks. Selected farms were visited and a survey was conducted to collect information on possible routes of introduction and quantitative and descriptive data were collected for analysis. No correlation could be demonstrated for possible routes of introduction for *H. meleagridis*. All the outbreaks affected commercial meat flocks and the most outbreaks occurred in the warmer months from spring until fall. Flock size ranged from 10.500 up to 31.000 animals, comprising of both hens and toms, or just hens or toms. In only one case all the barns were affected, in the other cases multiple barns remained unaffected. Cumulative mortality ratio ranged from 22,96% up to 82,55% with a mean of 52,80%. Daily mortality ratio ranged from 2,16% up to 29,98% with a mean of 9,90%. Animals of affected barns that survived the histomonosis did not differ significantly, in weight and condemnation ratio of net carcass weight, from unaffected turkey flocks. Since multiple factors contribute to the severity of an outbreak there should be a benchmark of data under 'normal' conditions for reference. Furthermore, if an outbreak occurs all original data of the entire production period must be preserved to collect as many variables as possible. To elucidate difference in mortality and susceptibility under field conditions, isolates obtained from different outbreaks should be analysed to rule out or confirm a difference in pathogenicity between isolates.

INTRODUCTION

Histomonas meleagridis (*H. meleagridis*) is a single cell parasite which is infectious for several species of poultry and is the initiator of blackhead disease. The disease is also known as histomoniasis. However, in 1990 it was decided that the suffix –osis is solely used for parasitic diseases (Kassai, 2006). Therefore, histomonosis is the adopted term in this study. *H. meleagridis* is a flagellated protozoa which affects the caecum and eventually migrates to the liver of the host. When histomonas arrives in the liver it causes tissue damage due to cell dividing which results in liver necrosis (McDougald & Fuller, 2005). Due to the liver necrosis sulphur colour pigment will appear in the faeces (Styles & Phalen, 1998). Infection of a flock of turkeys could lead to a mortality ratio of 100% (Hauck & Hafez, 2013). During the last century, histomonosis was no threat for commercial meat turkey flocks because of prophylactic use of antihistomonads such as nitrofurans (Liebhart, Ganas, Sulejmanovic, & Hess, 2017). Furthermore, histomonosis was highly treatable with dimetridazole, morbid birds were able to recover from near death (Liebhart et al., 2017). A ban in 2003 of the antihistomonads, due to concerns of residues in food, toxicity and carcinogenicity (EC No 1756, 2002), used to prevent histomonosis contributed to an increased incidence of histomonosis (Callait-Cardinal et al., 2007).

Research on histomonosis has been resumed since the diseases is re-emerging since the ban. However, no (chemo)therapy has been established to treat turkeys infected with *H. meleagridis* nowadays. Research on different therapies such as Protophyt™ B and Enteroguard™ showed promising results in vitro but, could not provide prevention or curative results in vivo (Hauck & Hafez, 2009; van der Heijden & Landman, 2008a; Van Der Heijden & Landman, 2008b). Use of paromomycin as a prophylactic feed additive showed promising results (Hafez, 2010), despite these results usage of antimicrobials as a prophylaxis is not permitted in Europe. Other studies confirmed the prophylactic effect of paromomycin but questioned the

application as a curative medicament (Bleyen et al., 2009; Lindquist, 1962; Van Der Heijden, De Gussem, & Landman, 2011).

Since there is no allowed preventive or curative treatment for histomonosis, another field of interest is risk factors for introduction of *H. meleagridis* into a flock. Knowledge of predisposition factors for *H. meleagridis* could result in effective biosecurity measurements for turkey farmers reducing infection.

In the early years it was assumed that the host for *H. meleagridis* were earthworms (Ackert, 1917). Afterwards, it was demonstrated that histomonosis was transmitted via eggs of *Heterakis gallinarum* (Wehr, 1954), and that earthworms harboured larvae of *H. gallinarum* (Lund, Wehr, & Elli, 1966). However, *H. meleagridis* spreads rapid through a flock and this would not be possible with just *H. gallinarum* as a route of transmission. Ingestion of *H. meleagridis* could not result in infection of turkeys (Lund, 1956). Hu and McDougald (2003) suggested another route of infection via the cloaca with fecal matter containing *H. meleagridis*. Via antiperistatical contractions of the cloaca, cloacal drinking, turkeys become infective for other poults two days post inoculation (McDougald & Fuller, 2005). It has been demonstrated that transmission via cloacal drinking contributes to the rapid spread through a flock resulting in high morbidity and mortality (Hu & McDougald, 2003; Landman, ter Veen, van der Heijden, & Klinkenberg, 2015; McDougald & Fuller, 2005). Histomonal trophozoites survive only up to nine hours, in favourable conditions, in fecal matter and therefore it is suggested that the limited time of survival outside the turkey prevents reinfection of a new flock via trophozoites (Lotfi, Abdelwhab, & Hafez, 2012).

Due to rapid horizontal spread within flocks of turkeys, combined with high mortality of the disease, histomonosis is a serious financial issue for the turkey sector (Callait-Cardinal et al., 2007). Moreover, it is a severe risk for animal health of galliformes (Hess & McDougald, 2013). Therefore the turkey sector in the Netherlands has appointed histomonosis as an objective for research for the following years.

Routes of introduction for *H. Meleagridis* have not been clearly identified and therefore data regarding possible introduction routes should be collected (Clark & Kimminau, 2017; Liebhart et al., 2017). Experimental infections have demonstrated high mortality and morbidity for turkeys (Hauck & Hafez, 2013; Hu & McDougald, 2003; Landman et al., 2015; McDougald & Fuller, 2005). However, the extent of morbidity and mortality of clinical infections in the Netherlands has not been described. The Turkey Sales Cooperation Holland (BAV) has requested the Animal Health Centre Deventer (GD) to cooperate in a study regarding *H. meleagridis* and predisposition factors. The aim of the present study was to analyse infected turkey farms of the past five years and create an overview of possible introduction routes for *H. meleagridis* in the flocks. Furthermore, data were gathered to create an insight in the course of infection in the different flocks.

MATERIAL AND METHODS

Selection of farms

The study focused on routes of introduction for *H. meleagridis* outbreaks in the Netherlands. With a small population of 30 turkey farmers, all commercial meat flocks, with 40 locations in 2016 (BAV, 2016) there were not many registered outbreaks. A list of farms which endured an outbreak of *H. meleagridis* was supplied by the BAV. Members of the BAV are obliged to report infections of *H. meleagridis*. The BAV recorded the disease in the Netherlands and some associated farms in Belgium. At the start of the study only 9 farms endured an outbreak with severe mortality ratio. Severe mortality ratio was classified by the BAV as >10% mortality ratio per infected barn. To enhance the sample size, farms who endured an outbreak were included as well. The outbreaks had to be in the past five years to ensure the reliability and quantity of the collected data for each outbreak. In total 13 farms were visited in the Netherlands and one in Belgium. Some farms encountered two outbreaks in the selected period, different outbreaks on farms were processed as individual outbreaks since there was no connection between flocks. Altogether, data of 19 outbreaks were collected.

Survey

The survey existed of closed-ended questions and open questions (Supplemental material II). It was divided in seven different categories. The first two categories were designed to obtain information about biosecurity regarding *H. meleagridis*. Therefore variables such as flock size, geographical location, farm layout, poultry activities in a close proximity and riskful contacts were examined. The other questions in the survey focused on general hygiene, general management and use of pharmaceutics or other measurements before and during the outbreak.

Data individual outbreaks

Data were collected from July to September 2016 by means of a physical survey. After completing the survey quantitative and descriptive data were copied from original logbooks from the farmers. Collected data included mortality, slaughter reports, water and

Table 1. Main data of the selected flocks with histomonosis.

Flock[a]	Month and year of outbreak	Breed	Total flock size	Barns affected / total barns	Sex		Number of turkeys per affected barn
					Reared at farm	In barn	
A	Oct-11	B.U.T.6	16.000	3/3	♂	♂ ♂ ♂	5.333 5.334 5.333
B1	Aug-12	B.U.T.6	33.040	2/6	♂/♀	♂ ♀	17.160 15.880
B2	Aug-13	B.U.T.6	31.040	1/6	♂/♀	♂	15.520
C	Nov-13	B.U.T.6	36.080	2/7	♂/♀	♀ ♀	8.000 3.750
D	Mar-11	B.U.T.6	10.640	1/2	♂/♀	♂	5.200
E	Aug-11	B.U.T.6	17.000	2/4	♂	♂ ♀	5.629 8.400
F	June-12	B.U.T.6	20.202	1/2	♂/♀	♂	5.520
G1	July-15	B.U.T.6	30.640	1/6	♂/♀	♂	14.000
G2	Dec-15	B.U.T.6	28.900	1/6	♂/♀	♂	13.003
H1	Nov-13	B.U.T.6	13.960	1/3	♂	♂	6.700
H2	Dec-15	B.U.T.6	14.105	2/3	♂	♂ ♂	6.812 2.925
I	Aug-15	B.U.T.6, TP7, Hybrid Converter	22.000	1/10	♀	♀	5.500
J	Sept-12	Hybrid XL	17.464	3/5	♂/♀	♂ ♂ ♂	5.516 2.688 2.880

Notes:

[a] Letters indicate individual farms, numbers indicate different outbreaks

feed uptake, veterinary logbooks, diagnosis and administration of pharmaceutics.

Despite the selected period some farms could not provide the required data or the data were inaccurate. Consequently 10 farms were selected to take part in this study providing, in total, data of 13 outbreaks.

The duration of an outbreak is defined as the time between the day of diagnosis histomonosis and the day the daily mortality ratio was below the reference of 0.04% for three consecutive days. Since there is no documentation off norm mortality ratio for turkeys or information from performance objectives it was calculated based on information collected by the Wageningen University of the Dutch turkey sector (KWIN, 2019). Norm mortality ratio for both toms and hens was set on 0,04% daily at any time during the production period.

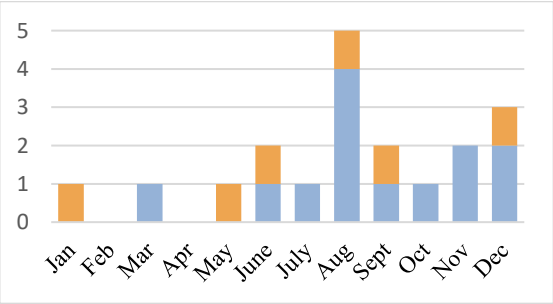


Figure. 1. Monthly number of cases with *H. meleagridis* diagnosed between 2011 through 2015. Histomonosis cases included in this study are displayed in blue. Cases displayed in orange could not provide the required data and therefore are not included in the analysis.

The variables of the data relating to weight and condemnation ratio were ranked and tested according to the Wilcoxon-rank-sum since the data were uneven distributed between collected and reference data test (Rey & Neuhäuser, 2011). All descriptive data were analysed via Excel 2016

RESULTS

Survey

No significant outcome was demonstrated for the survey due to the small amount of outbreaks, identical answers amongst the participants and the lack of a control group.

Individual outbreaks

The number of cases varied each year. Most outbreaks (12/19) occurred in the summer and fall. There was an apparent peak in August regarding the month of outbreak (Fig. 1). Main data of the nine affected Farms (A-I) and subsequent outbreaks (1-2) are given in Table 1. Flocks range in size from 10.640 to 36.080 animals. Most farms reared both toms and hens (6/9) whereas three farms only reared toms or hens. On most farms toms and hens were reared in the same barn for the first weeks. Around four weeks hens and toms were relocated in different

Table 2. Data of mortality ratio and infection of the selected flocks with histomonosis.

Flock[a]	Barn	Mortality (%)					Infection (days)	
		Total mortality during production period	Average daily mortality before outbreak	During outbreak			Age when diagnosed with histomonosis	Duration of infection
				Cumulative mortality	Max daily mortality	Average daily mortality		
A[b]	1	50,76	nd	50,76	7,25	1,93	42	36
	2	47,22		47,22	6,24	1,74	42	36
	3	50,82		50,82	7,39	1,93	42	36
B1	1	60,17	0,13	56,46	16,06	7,09	25	18[d]
	2	59,23	0,16	56,13	23,70	4,60	25	11[d]
B2	1	44,11	0,06	39,06	2,16	0,57	50	81
C	1	27,08	0,13	22,96	4,94	1,40	30	44
	2	56,13	0,13	54,77	7,38	1,77	30	44
D	1	32,85	0,08	27,71	4,11	0,39	66	87[e]
E	1	37,50	0,13	33,08	5,21	0,79	62	50
	2	85,73	0,10	82,55	13,85	3,39	33	54
F	1	63,61	0,23	53,22	29,98	8,94	48	8[d]
G1	1	53,46	0,10	49,93	3,95	0,83	38	87
G2	1	84,93	0,11	81,03	15,46	2,87	35	56
H1[c]	1	77,01	0,03	76,49	9,07	1,54	47	94
H2[c]	1	80,01	0,05	78,58	14,49	3,26	63	46
	2	77,78	0,08	74,94	13,45	3,06	63	46
I	2	47,64	0,05	47,13	6,64	1,46	62	43
J	1	30,16	0,11	27,77	3,02	0,70	76	45
	2	68,83	0,14	55,51	7,92	1,78	76	43
	3	43,89	0,11	42,64	5,59	2,14	76	26[d]
Max		85,73	0,23	82,55	29,98	8,94	76	94
Min		27,08	0,03	22,96	2,16	0,39	25	8
Mean		56,14	0,11	52,80	9,90	2,48	48	47
Median		53,46	0,11	50,82	7,38	1,78	48	44

Notes:

nd - no data available

[a] Letters indicate individual farms, numbers indicate different outbreaks

[b] Only data during the 36 days of outbreak were available.

[c] Poults were reared on an external farm for 28 days, no data was available for this period.

[d] Flocks were culled due to high mortality.

[e] Mortality ratio peaks at two moments during the outbreak. At day one for 3 days and at day 26 for 18 days.

barns. Only on one farm toms and hens shared the same barn during the outbreak (F). B.U.T. 6 is the most frequently used breed at the selected farms (8/9). One farm only reared Hybrid-XL and one farm complements the B.U.T. 6 turkeys with TP7 and Hybrid Converter hens since no hatchery can provide 22.000 hens at once.

Mortality ratio and infection characteristics are presented in Table 2. Diagnosis was made by a veterinarian based on gross lesions in liver and caeca for three outbreaks. Two outbreaks of *H. meleagridis* were confirmed by polymerase chain reaction (PCR). For eight outbreaks no documentation of a diagnosis was available. At the time of diagnosis the age of the poults varied between 25 and 76 days, the median age was 47 days. The duration of an outbreak ranged from 8 till 94 days with a mean of 47 days and a median of 44 days.

In all flocks, cumulative mortality ratio ranged between 22,96% and 82,55% with a mean of 52,80% and a median of 50,82%. Average daily mortality ratio during this period ranged between 0,39% and 8,94% with a mean of 2,48% and a median of 1,78%. Maximum daily mortality ratio during the outbreak ranged between 2,16% and 29,98% with a mean of 9,90% and a median of 7,38%. There was no correlation between sex or age when diagnosed or mortality ratio.

Table 3. Economic results of eight production periods with histomonosis

Flock[a]	Sex	Age at slaughter (days)	Amount of slaughtered turkeys	Net slaughter weight (kg)	Dead and condemned turkeys (%)	Condemned animal parts (%)[b]	Total condemned[c] (%)
A	♂	138	7.174	17,17	0,70%	2,56%	3,16%
B2	♂	146	5.175	21,32	0,52%	2,36%	2,73%
	♀	113	7.446	10,84	0,13%	2,03%	2,12%
	♀	112	7.436	10,49	0,24%	2,23%	2,36%
C	♀	112	3.688	10,83	nd	nd	2,47%
D	♂	147	3.336	20,43	1,43%	2,07%	3,48%
E[d]	♂	147	3.519	20,73	3,27%	3,59%	6,57%
	♀	96	1.488	9,91	0,72%	1,58%	2,03%
H2	♂	136	5.085	21,41	1,97%	1,74%	3,58%
	♂	136	776	21,38	1,19%	1,92%	2,98%
I	♀	109	6.170	10,64	0,21%	0,79%	1,01%
		♂	Max	21,41	3,27%	3,59%	6,57%
			Min	17,17	0,52%	1,74%	2,73%
			Mean	20,41	1,51%	2,38%	3,75%
			Median	21,03	1,31%	2,21%	3,32%
		♀	Max	10,84	0,72%	2,23%	2,47%
			Min	9,91	0,13%	0,79%	1,01%
			Mean	10,54	0,32%	1,66%	2,00%
			Median	10,64	0,22%	1,80%	2,12%

Notes:

nd- no data available

[a] Letters indicate individual farms, the numbers indicates subsequent outbreaks.

[b] Condemned animal parts contain: Heart, liver, stomach, leg, wing and filet.

[c] As a ratio of total carcass weight.

[d] E consists of approximately 2500 affected and 1000 unaffected toms.

[e] Supplied data were supplied as a monthly average of all flocks slaughtered, since it is an average it can only be used as reference for the means of hens and toms.

Supplemental figures 1 till 13 are included in the supplemented data to give a more graphic representation of the outbreaks. They display the course of daily mortality ratio, antimicrobial use, transfer to the farm or relocation between barns and the start of the outbreak.

Slaughter results

Slaughter results of the affected flocks are listed in Table 3. Economic results were available for seven affected flocks. All hens were slaughtered between 96 and 113 days of age with an average weight ranging from 9,91 kg to 10,84 kg with a mean of 10,64 kg and a median of 10,64 kg. The weight of the hens from affected barns is higher than the reported weight of unaffected hens (9,0 kg to 10,5 kg) (KWIN, 2019). The toms were slaughtered between 136 and 147 days of age with an average weight of 17,17 kg to 21,41 kg with a mean of 20,41 kg and a median of 21,03 kg. Consequently, the weights of the toms, from affected flocks, did not differ from the weight of unaffected toms ranging from 19 kg till 21 kg (KWIN, 2019).

Condemned turkeys, animal parts and organs are expressed as a ratio of the carcass weight and ranged from 1,01% up to 2,47% for hens with a mean of 2,00% and a median of 2,12%. For toms it ranged from 2,73% up to 6,57% with a mean of 3,75% and a median of 3,32%. The reference for these ratios ,monthly averages from farms associated with the BAV for the years 2015 and 2016, were provided by the BAV. These data were supplied as a monthly average of all flocks slaughtered, since it is an average it can only be used as reference for the means of hens and toms. The means of the supplied data (data not shown) were 2,01% and 4,03% respectively for hens and toms and did not differ statistically from the turkeys that outlived histomonosis on the affected farms.

DISCUSSION

Data

In this study, surveys were conducted and data were collected to create insights in possible routes of introduction for *H. meleagridis* for turkey flocks with a severe outbreak. The BAV also documented outbreaks (data not shown) with less mortality ratio (<10%). The cases with low mortality ratio are in accordance to findings in turkeys in France and Austria (Callait-Cardinal et al., 2007; Sulejmanovic et al., 2017). Therefore, the selected outbreaks in this paper do not reflect all outbreaks of histomonosis in the Dutch turkey sector.

No control farms, to compare with the surveys of the affected farms ,were included in this study due to a lack of time. Because of the lack of a control group and the similarity between the conducted surveys, no possible routes of introduction could be appointed. However, it is highly doubtful that with a reference group the survey would have had significant positive outcomes as discussed in the next section.

All conducted surveys indicated that the general biosecurity measurements on most of the farms had been taken into account, e.g., chemical and mechanical disinfection, overalls and boots per barn, washing hands between barns etc. Therefore, according to the surveys, almost all farms took similar biosecurity measures which also could be expected in a references group. Furthermore, it should be mentioned that when the barns were visited, after conducting the survey, not all biosecurity measures, which were enunciated in the surveys, were observed. Farmers are aware of the suitable biosecurity measures to enhance biosecurity on their farm and therefore, inadvertent, misreport some data creating bias. This type of bias is known as social desirability bias (Krumpal, 2013), and to some extent the survey was subjected to this.

Of the 19 submitted outbreaks only 13 cases were able to provide the requested data of the outbreaks i.e., diagnosis, feed and water uptake or mortality. This data were not available, had been discarded due to termination of the farm or data were overwritten in automatic systems. To ensure the quality and quantity of the data, data should be preserved during or shortly after an outbreak. In addition, farmers should be informed on forehand how to collect unprocessed and original data, and which data is feasible, i.e., mortality, original diagnosis, feed and water uptake, treatments and dosage, veterinary logbooks, slaughter records. Furthermore, there not many production parameters to use as a reference. For mortality ratio, no reliable data could be supplied by neither the turkey sector or breeding companies. Turkey production on other regions differ in production systems, production periods and turkeys are marketed at different weights. The production parameters form other regions are not reliable to use as references (Hauck, Stoute, Chin, Sentíes-Cué, & Shivaprasad, 2018).

In our study an outbreak ended when the daily mortality ratio was below 0,04% for three consecutive days. However, in a small flock, for example 1.000 animals, the daily mortality ratio of 0,04% is already exceeded with one dead bird a day. So in small flocks of turkeys the duration of the outbreak was estimated longer than the actual duration of the outbreak. A minor deviation at the start of an outbreak is reasonable, not every dead bird has been examined so the first bird that actually died due to a *H. meleagridis* infection could not been diagnosed.

Course of infection

12 of the 19 outbreaks occurred during the summer and fall , which is in accordance with other studies (Hauck et al., 2018; Sulejmanovic et al., 2017; Sulejmanovic, Grafl, Bilic, Jaskulska, & Hess, 2019).

62 percent of the outbreaks occurred on farms rearing/fattening both hens and toms. There is no data available on the number of farms rearing mixed or single sex flocks in the Netherlands. Therefore, the data are insufficient to suggest that mixed flocks are more susceptible to *H. meleagridis*. In only one case, Flock A, all the barns of the farm were affected. In flock E the outbreak started in the hens at 33 days of age and 31 days later an outbreak occurred in the toms. This is the only outbreak, in this study, with a large interval between an outbreak in different. In the majority of the cases multiple barns were affected, but in the remaining barns histomonosis was not diagnosed. This is in agreement with reports in Europe and the U.S.A. (Hauck et al., 2018; Popp, Hauck, Blazey, Hänel, & Hafez, 2011; Sulejmanovic et al., 2017). Hence, it seems that with biosecurity measures spreading of *H. meleagridis* can be limited between barns during an outbreak.

Severity and duration of mortality varied widely between the selected outbreaks in this study as is showed in table 2 and the supplemental material. The duration of the outbreaks in this paper ranged from eight days, due to culling at 63%, to 94 days of age with a mean of 47 days which is 1-2 weeks longer than previously reported (Hauck et al., 2018).

Previously reported mortality ratios varied, both high and low mortality ratios were reported (Aka, Hauck, Blankenstein, Balczulat, & Hafez, 2011; Hauck et al., 2018; Sulejmanovic et al., 2017). In our study the maximum daily mortality ratio ranges from

2.16% up to 29,98% and cumulative mortality ratio during the affected period ranges from 22,96% up to 82,55%, which is in accordance with the publications of Hauck et al. (2018) and Sulejmanovic et al. (2017) Turkeys that survived the infection or showed no clinical signs of histomonosis reached market weight and the condemnation ratio was not increased. These results are in contrast to a report in California. where in half of the cases the turkeys were marketed with a body weight lower than the optimal weight. (Hauck et al., 2018).

Mortality of an outbreak comprises of an equilibrium of three elements e.g., environmental factors, host resistance and virulence of *H. meleagridis* (Callait-Cardinal et al., 2010). Since these conditions differ from farm to farm, production parameters and the clinical appearance of histomonosis can vary for each outbreak. In the environment, outside of a host, *H. meleagridis* can only survive for a short period under specific conditions (Lotfi et al., 2012). However, at the same time this period is long enough to ensure rapid horizontal spread within a barn (Hu & McDougald, 2003; Landman et al., 2015; McDougald & Fuller, 2005). In barns with wet litter, infrequent litter regeneration or infrequent supply of fresh shavings there is probably more contact between animals and infectious *H. meleagridis*, increasing the risk of infection (Callait-Cardinal et al., 2010). Moreover, spread of *H. melaegridis* before the diagnosis of histomonosis could be fierce compared to barns with clean and dry shavings. Furthermore, in wet contaminated litter the amount of trophozoites can accumulate, causing an increase of the infectious dose, resulting in an increased morbidity and mortality ratio (Liebhart, Grabensteiner, & Hess, 2008; Van Der Heijden & Landman, 2008).

In some flocks, paromomycin is administered as a therapy to minimize the impact of an outbreak. However, paromomycin has solely a prophylactic effect (Bleyen et al., 2009; Hafez, Hauck, Gad, de Gussem, & Lotfi, 2010; Van Der Heijden et al., 2011). When paromomycin is administered at the start of an outbreak, the day the first mortality due to histomonosis is noticed, there is no significant difference in mortality with untreated groups of animals (Bleyen et al., 2009). Since an outbreak of histomonosis is diagnosed as early as the first dead turkey due to histomonosis, the effect of therapeutic use of paromomycin is questionable (Manders, Fischer, & Landman, 2018). The flocks discussed in this paper that were treated with paromomycin resemble these findings with cumulative mortality ratio ranging from 27,71% up to 78,81% (see supplemental figures A, B1, B2, D, H1, H2, I and J).

In one flock, toms and hens were reared in the same barn, the toms got infected but the hens showed no morbidity or increased mortality due to *H. meleagridis* (see supplemental material, Flock F). Under experimental conditions no difference in susceptibility was demonstrated between hens and toms (Liebhart et al., 2008; Van Der Heijden & Landman, 2008). Similar cases, describing the large difference in the mortality between hens and toms reared in the same barn, are reported twice under field conditions (Popp et al., 2011; Sulejmanovic et al., 2019).

Cloacal swaps of hens in the study of Sulejmanovic et al. (2019) tested positive for *H. meleagridis* DNA via real-time PCR. No live histomonads could be cultured from the faeces of these hens, so it is unclear to what extent they were affected by *H. meleagridis*. PCR samples of dust from 65 turkey barns tested positive for histomonal DNA. Only six flocks developed clinical histomonosis and the other flocks remained clinical unaffected (Sulejmanovic, Turblin, Bilic, Jaskulska, & Hess, 2019). So the clinical value of positive (dust) samples for DNA of *H. meleagridis* is unclear. To ensure that the rise of antibodies in unaffected hens is due to an infection with the causative agent and not just antigens from the environment. Actual trophozoites should be detected in the ceca or cloaca.

Under field conditions, there seems to be some sort of difference in susceptibility and pathogenicity between sexes and between outbreaks. To get a better understanding of the different clinical appearances of histomonosis and its underlying host-pathogen interaction. Genotyping would be helpful to determine difference between isolates and their pathogenicity (Hess, Liebhart, Bilic, & Ganas, 2015).

CONCLUSSION

It turned out that the collected surveys and descriptive data were not feasible to create an insight in possible introduction routes for *H. meleagridis* for the selected farms. However, the descriptive data created an insight in the severity and the losses due to histomonosis for the selected farms. For example the rapid spread within barns, that resulted in severe cumulative mortality ratios up to 82%. The impact of *H. meleagridis* for affected farms can be enormous.

The exact epidemiology of *H. meleagridis* remains unclear and need to be further investigated to understand the differences in mortality and susceptibility under field conditions. Retrospective data collection in the form of a survey appeared to be unfit to determine the introduction route for *H. meleagridis*. For further research, when an outbreak occurs biosecurity should be physically inspected

and all original production parameters of the entire production period must be preserved. Although pathological lesions of histomonosis appear to be pathognomonic, those pathological findings should always be confirmed via PCR or immunohistochemistry. Animals in affected barns without clinical signs should be tested for the presence of trophozoites of *H. meleagridis*. Determination of the different strains of histomonas for each outbreak could contribute to create insight in the difference in pathogenicity of *H. meleagridis* strains.

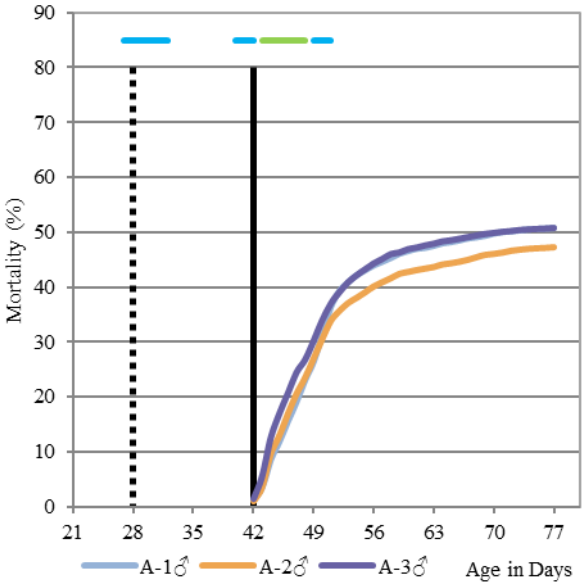
Since many factors can contribute to the severity of an outbreak and no clear benchmark for production parameters are available. The Dutch turkey sector should establish their own benchmark of production parameters. With such a clear benchmark, flocks with for example histomonosis can be compared with the benchmark.

Since the ban of the last chemotherapeutics no effective therapy is available combined with the lack of knowledge on all of the possible introduction routes for *H. meleagridis*, the only advice is to retain strict biosecurity measures on the farms. In terms of veterinary medicine, we are 120 years back in time and we have to keep searching for new approaches to conquer *H. meleagridis*.

SUPPLEMENTAL MATERIAL

Description of the outbreaks

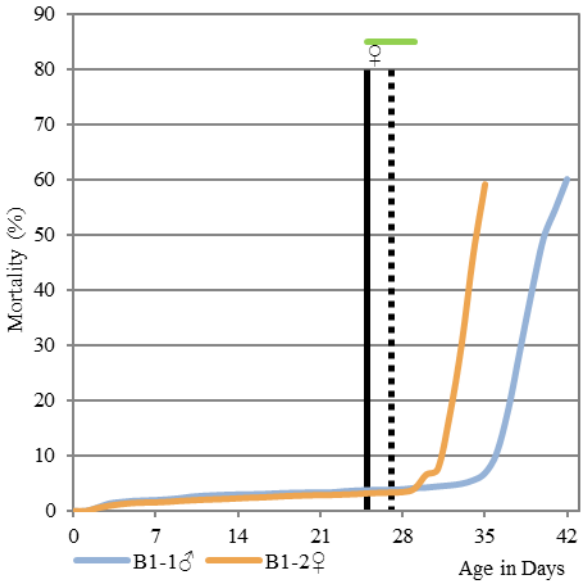
Supplemental figures 1 till 13 are included to illustrate the course of daily mortality ratio, antimicrobial use, transfer to or on the farm and start of the outbreak. As much detail of treatments and measurements are given as provided.



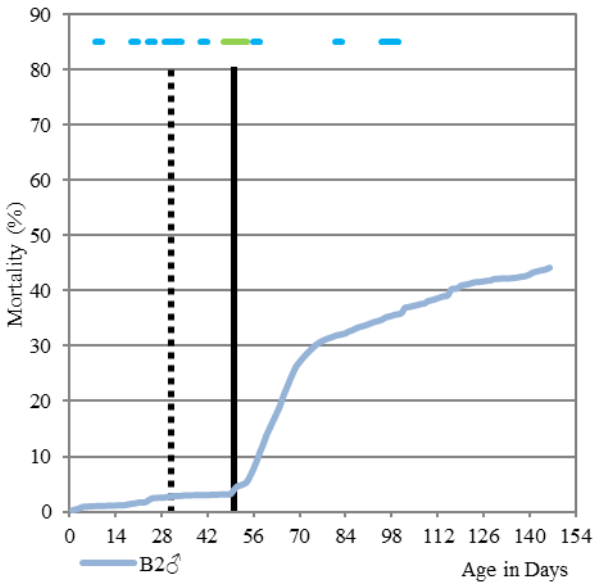
Supplemental Figure 1.- Cumulative mortality ratio Flock A. Numbers denote different barns. The solid line indicates the diagnosis of histomonosis (42 days of age). The dotted line indicates the transfer from an external brooding farm (28 days of age). The blue and green lines represent the start and length of respectively an antibiotic or paromomycin treatment in the flock.

Description outbreak-Flock A

At 27 days of age treatment started for three days for coccidiosis with Esb®3 on the brooding farm. The 16.000 toms arrived at 28 days of age at the farm and were housed in three barns. At 39 days of age the toms were treated with Paracilline®, exact indication unknown. At 42 days of age *H. Meleagridis* was diagnosed, how the diagnosis is established is unclear. For six days Gabrovet® 70 was administered (1 kg/1000 l) in all barns. Subsequently the toms were treated for three days with Phenoxypen® WSB for necrotic enteritis, and concurrent treatment with Sulfaquinoxaline Natrium® was administered for a respiratory infection. Besides the antimicrobials, salt, Presan®-FY and a Virkon™ S solution were applied to the bedding on a daily base. Despite the (antimicrobial)therapies, cumulative mortality ratio reached over 47% for all barns. At the age of 138 days the toms were slaughtered. Total condemnation ratio at the slaughter line reached 3,16% of the total carcass weight.



Supplemental Figure 2.- Cumulative mortality ratio Flock B1.
Numbers denote different barns. The solid line indicate the diagnosis of histomonosis in the barns with hens and toms (25 days of age). The dotted line indicates the transfer from the brooding barn to the finishing barn (27 days of age). Due to high mortality all toms (41 days of age) and hens (34 days of age) were culled.



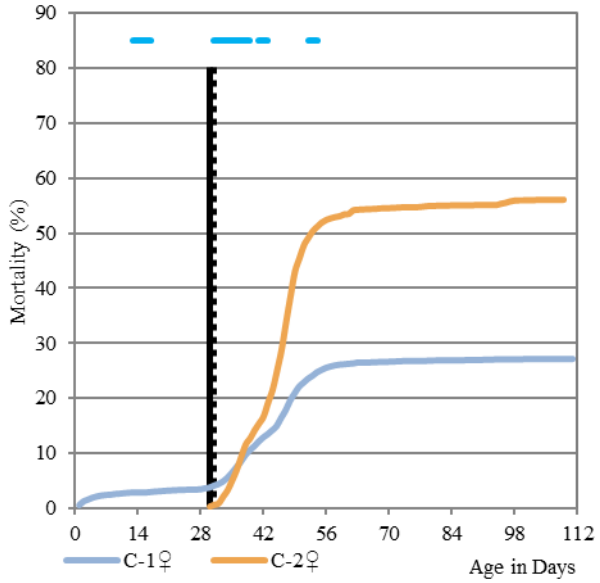
Supplemental Figure 3.- Cumulative mortality ratio Flock B2.
The solid line indicates the diagnosis of histomonosis (50 days of age). The dotted line indicates the transfer from the brooding barn to finishing barn (31 days of age). The blue and green lines represent the start and length of respectively an antibiotic or paromomycin treatment in the flock.

Description outbreak-Flock B1

The farm consisted of six barns of which two were used as brooder barn. Hereafter, the poults were reared in two larger barns for the remaining production period. The two remaining barns were used as an infirmary. The farm reared around 16.000 toms and 17.000 hens in different barns at all time. Previous to the first outbreak the farm endured an infection with Avian Influenza (type unknown) and all animals were culled. Due to the infection the farm was intensively cleaned and disinfected. Sentinel poults were reared for several weeks and in the last week *H. meleagridis* was diagnosed in one tom. All barns were thoroughly disinfected again and new poults arrived on the farm for rearing. The hens and toms were both diagnosed with histomonosis at 25 days of age via macroscopic lesions. All poults received Gabrovet® 70 for two days (2 kg/1000 l) and subsequently two days at a dose of 1 kg/1000 l. Although infected, the poults were transferred at 27 days of age to a finishing barn due to limited space in the brooder barn. Besides the antimicrobials salt and a chlorine solution were applied five times a day over the bedding. Daily mortality ratio reached 23,70% and 16,06% for hens and toms respectively. Hens and toms were all culled at respectively 34 and 41 days old.

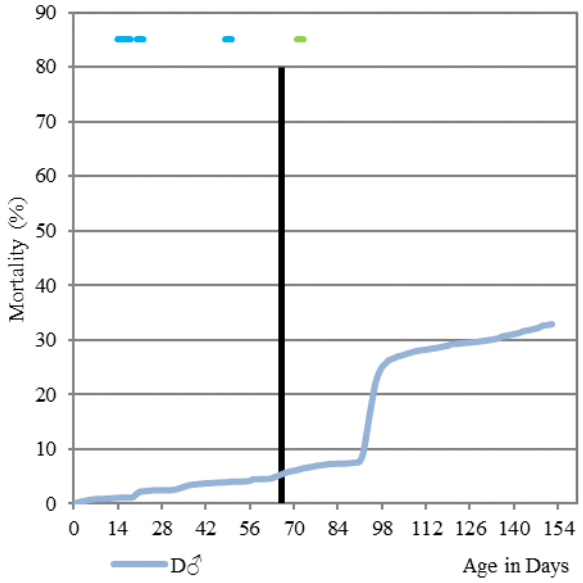
Description outbreak-Flock B2

Subsequently after the culling of the poults (B1) the barns were disinfected. After several weeks of halted production new poults, around 15.500 hens and 15.500 toms, were reared for economic reasons. Despite the intensive cleaning and disinfection of the barns, histomonosis reoccurred at 49 days of age in the barn with toms and diagnosis was confirmed via PCR. Previous to the diagnosis the hens received multiple treatments with Paracilline® for gastrointestinal infections and Sulfaquinoxaline Natrium® for respiratory infections. Gabrovet® 70 was administered for nine days (1 kg/1000 l) subsequent on diagnosis of *H. meleagridis*. A Virkon™ S solution and salt were applied on a daily base over the bedding. Cumulative mortality ratio reached 39,06% during the affected period for the hens. The separate housed toms remained unaffected. Total condemnation ratio at the slaughter line reached 2,73% and 2,63% in toms (unaffected) and hens respectively of total carcass weight.



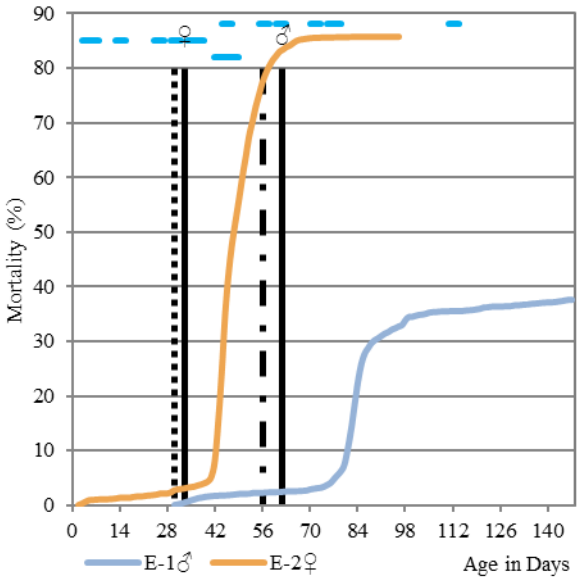
Supplemental Figure 4.- Cumulative mortality ratio Flock C.
Numbers denote different barns. The solid line indicates the diagnosis of histomonosis (30 days of age). The dotted line indicates the transfer of 50% of the hens from barn C1 to C2 (31 days of age). The blue lines represent the start and length of an antibiotic treatment in the flock.

Flock C - The farm consisted of seven barns in which around 18.000 hens and 18.000 toms were reared. Hens and toms were reared separate in 4 barns for 28 days. Hereafter, the toms were transferred to the three unoccupied barns for the remaining production period. The hens were divided over their two barns and the two former tom barns. At 30 days of age two barns with hens were diagnosed with *H. meleagridis*. Previous of the transfer the hens were reared together in the same barns. When diagnosed, Neosol® 100% (10 mg/kg) was administered in all the barns to the turkeys for nine days. A cresol solution was applied over the bedding every day for five days in the barns of the hens. Gabrovet® 70 was administered for four days, 5kg/day/barn, to the toms despite being unaffected. Cumulative mortality ratio reached 54,77% and 22,96% for the affected barns. The other five barns remained unaffected by histomonosis. Total condemnation ratio at the slaughter line reached 2,47% of total carcass weight.



Supplemental Figure 5.- Cumulative mortality ratio Flock D.
The solid line indicates the diagnosis of histomonosis (66 days of age). The blue and green lines represent the start and length of respectively an antibiotic or paromomycin treatment in the flock.

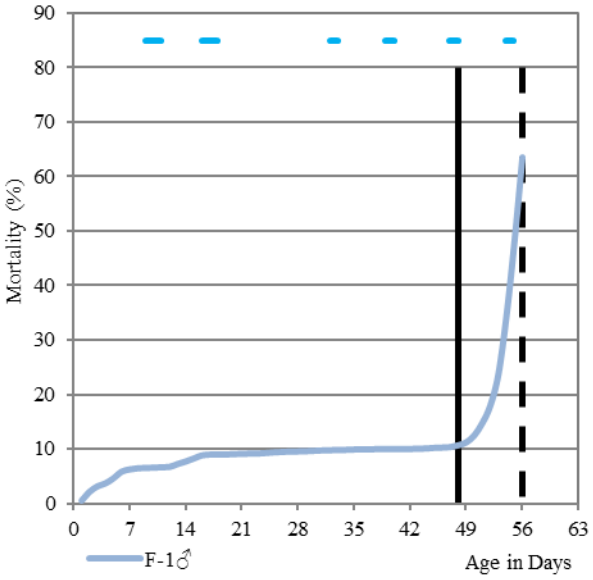
Flock D - The farm reared around 5.100 toms and 5.500 hens in two separate barns. *H. meleagridis* was diagnosed at 66 days of age, diagnosis was confirmed via post-mortem investigation by the GD. Gabrovet® 70 was administered, dose and duration unknown. Concurrently, twice a day quicklime was applied over the bedding. The first 25 days of the outbreak daily mortality ratio remained low with a mean of 0,11%. Hereafter, daily mortality ratio increased suddenly up to 4.11% and resulting in a cumulative mortality ratio during infection of 27,71%. Total condemnation ratio at the slaughter line reached 3,48% of total carcass weight.



Supplemental Figure 6.- Cumulative mortality ratio Flock E.
Numbers denote different barns. The solid lines indicate the diagnosis of histomonosis in the barns with hens (33 days of age) and toms (62 days of age). The dotted line indicates the separation of the toms and the hens from the brooding barn. The dashed line indicates selection and transfer of the toms by 29% to another barn (56 days of age). The blue lines represent the start and length of an antibiotic treatment in the flock. After separation the light blue line represents the toms and the dark blue line represents the hens.

Flock E - The farm reared around 8.000 hens and 9.000 toms in four barns. The largest barn was used as brooder barn for both hens and toms. During the brooding period all poults were treated for pericarditis and coccidiosis with enrofloxacin and doxycycline respectively. After 28 days the toms were transferred to two of the other barns and the hens were finished in the brooder barn. At 33 days of age one dead hen was diagnosed with Histomonosis, how the diagnosis is established is unclear. Mortality increased after diagnosis and cumulative mortality ratio reached over 85,73%. Total condemnation of the total carcass weight of the hens was 2,03%.

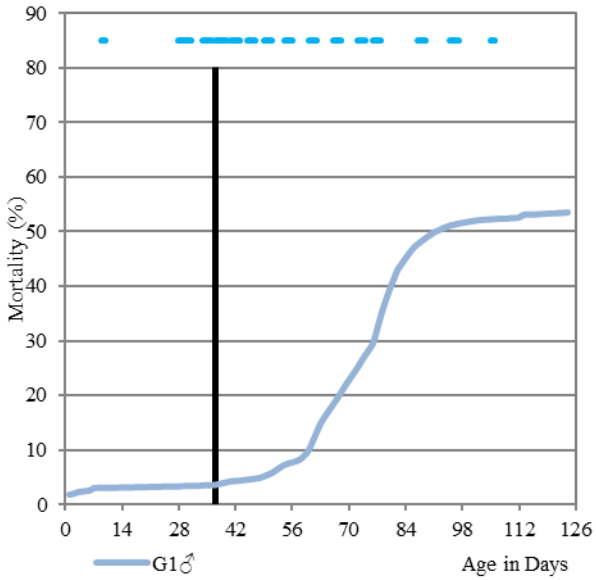
Meanwhile, the toms had been treated with several antimicrobials (doxycycline, sulfaquinoxaline and tylosin), for different infections, but none of which was histomonosis. At 56 days of age 29% of the toms from barn 2 were transferred to the remaining barn and six days after transfer there was an outbreak of histomonosis in barn 2. In barn 3 there was no increase in mortality whilst the toms also originated from barn 2. Cumulative mortality ratio for the toms reached 33%. Total condemnation ratio of the total carcass weight of the toms was 6,57%. Of the 3.500 slaughtered toms around 1.000 originated from an barn which was unaffected by *H. meleagridis.* Salt and Stalosan® F was applied over the bedding during both infections on a daily basis.



Supplemental Figure 7.- Cumulative mortality ratio ratio Flock F.
The solid line indicates the diagnosis of histomonosis (48 days of age). The dashed line indicates culling of the toms and removing of the separation fence (56 days of age). The blue lines represent the start and length of an antibiotic treatment in the flock.

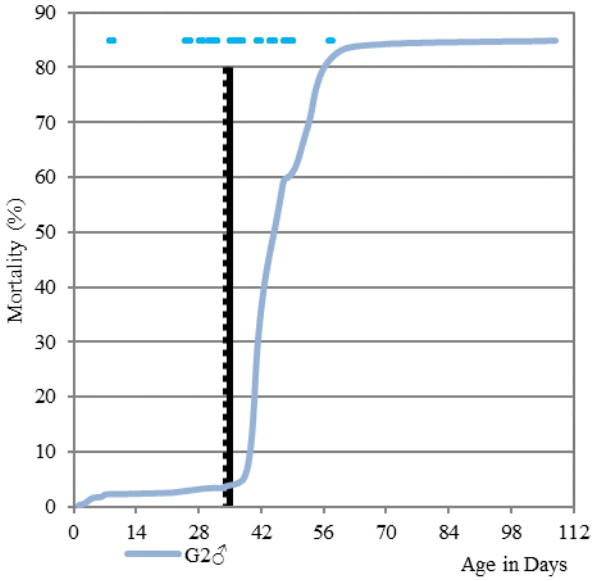
Flock F - The farm reared 15.000 hens and 5.500 toms. The farm contained two barns, one for 10.000 hens and one for 5.000 hens and 5.500 toms. The hens and toms were separated by a wooden fence, but direct contact was possible through the fence at the gate, waterlines and feedlines. Previous of the infection both barns were treated twice with enrofloxacin for *Escherichia coli* (*E. coli)* and twice with amoxicillin for Clostridial infections. At 48 days of age the toms were diagnosed with *H. meleagridis*. Diagnosis was based on post-mortem investigation, no data is collected of the post-mortem investigation. No antimicrobial therapy was applied, but a chlorine solution was applied on a daily base over the bedding during the outbreak. After eight days, at 52,90% cumulative mortality ratio, all remaining toms were culled.

After culling the litter was removed and new wood shavings were added. The fence was removed and the entire barn became accessible for the hens. No increased mortality was noticed for the hens during the outbreak or subsequent to removal of the fence.



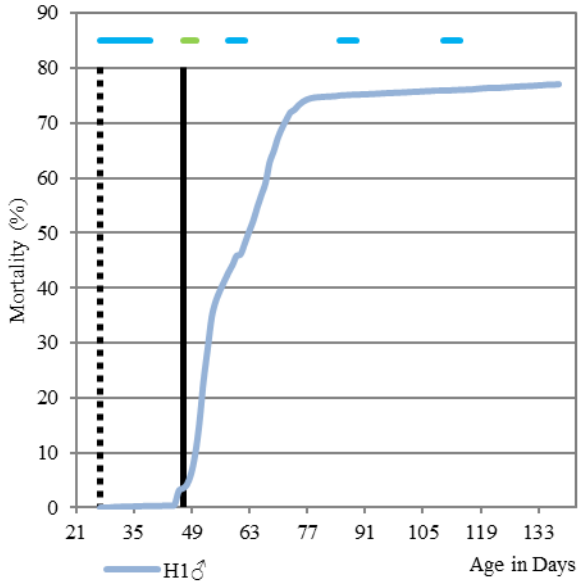
Supplemental Figure 8.- Cumulative mortality ratio Flock G1.
The solid line indicates the diagnosis of histomonosis (37 days of age). The blue lines represent the start and length of an antibiotic treatment in the flock.

Flock G1 - The farm reared around 14.000 toms and 17.000 hens in seven barns. All toms were reared in one barn. The infection with *H. meleagridis* was diagnosed in the barn with the toms at 37 days of age, how the diagnosis was established is unclear. Throughout the entire production period the toms were treated recurrently with Suramox®, Sulfaquinoxaline Natrium® and Tylan® WO for gastrointestinal infections, however no specific treatment for histomonosis was applied. Cumulative mortality ratio reached 53,46% during the entire production period.



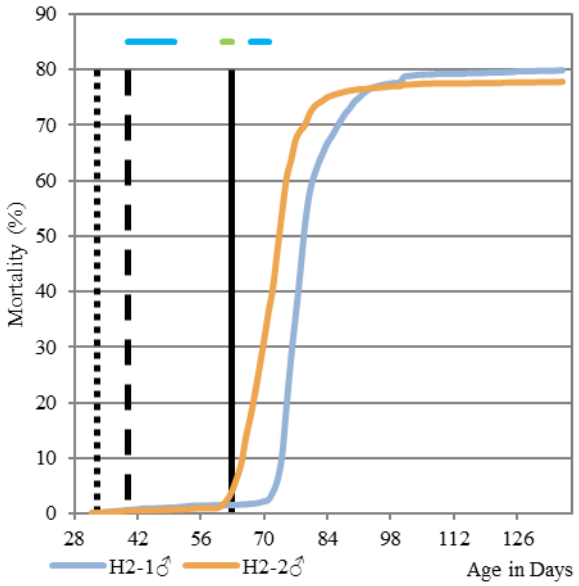
Supplemental Figure 9.- Cumulative mortality ratio Flock G2.
The solid line indicates the diagnosis of histomonosis (35 days of age). The dotted line indicates the transfer of turkeys from the brooding barn to the finishing barn (34 days of age). The blue lines represent the start and length of an antibiotic treatment in the flock.

Flock G2 - During the subsequent production period the farm reared 13.000 toms and 15.700 hens. Before the outbreak several antimicrobial therapies had been applied for digestive problems. At 34 days of age all the toms were transferred to another barn. One day later *H. meleagridis* was diagnosed in the toms, via post-mortem investigation by a veterinarian (data not collected). Daily mortality ratio reached up to 15,46% toms a day resulting in 81,03% cumulative mortality ratio during the affected period. No specific antimicrobial therapy was used for the *H. meleagridis* infection, but enrofloxacin was administered for a respiratory infection for one day.



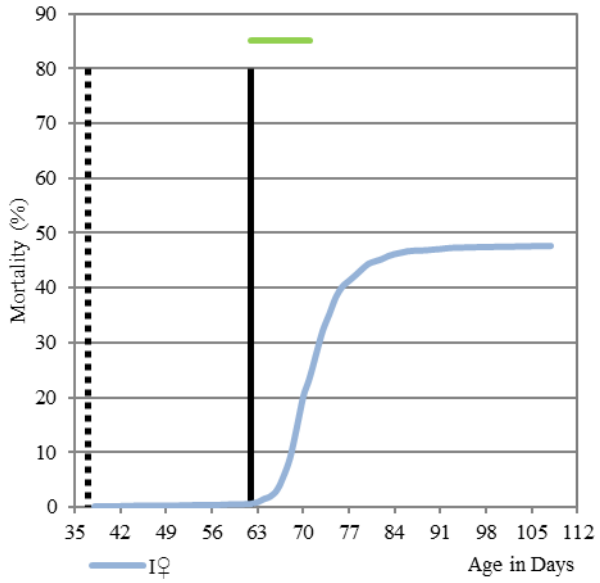
Supplemental Figure 10.- Cumulative mortality ratio Flock H1.
The solid line indicates the diagnosis of histomonosis (47 days of age). The dotted line indicates the transfer from an external brooding farm (27 days of age). The blue and green lines represent the start and length of respectively an antibiotic or paromomycin treatment in the flock.

Flock H1 - The farm reared 14.000 toms in three barns. The first 27 days the toms were housed on a brooding farm. After arrival, they received Sulfadimidine-NA® for Coccidiosis and subsequently paracillin and Phenoxypen® WSP for Clostridial infections for five, four and four days respectively. At 47 days of age *H. meleagridis* was diagnosed in one barn, how the diagnosis is established is unclear. Subsequent of the diagnosis all the toms received Gabrovet® 70 and Neosol® 100% for four days. From day 58 till day 62 they also received enrofloxacin for *H. meleagridis*. Salt was scattered over the bedding daily Despite the treatment total cumulative mortality ratio reached 77,01%. The other two barns remained unaffected by *H. meleagridis*.



Supplemental Figure 11.- Cumulative mortality ratio Flock H2.
Numbers denote different barns. The solid line indicates the diagnosis of histomonosis (63 days of age). The dotted line indicates the transfer from an external brooding farm (33 days of age). The blue and green lines represent the start and length of respectively an antibiotic or paromomycin treatment in the flock

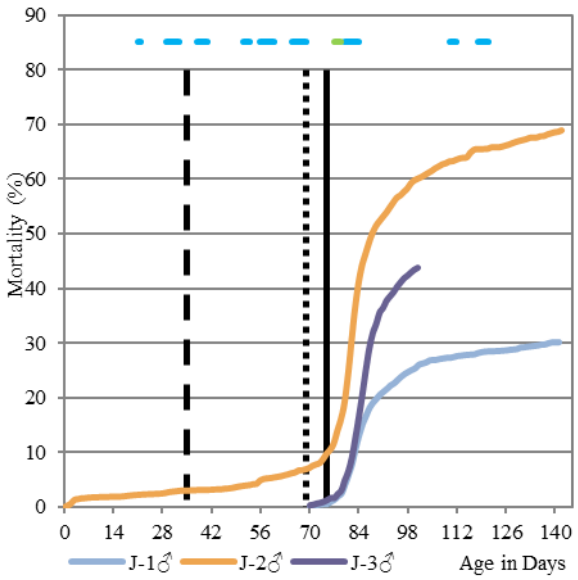
Flock H2 – Around 14.000 tom poults arrived at 32 days of age at the farm. After eight days they received Suramox®, Tylan® WO, Doxycycline-hyclaat® 50% and Neosol® 100% for Clostridial subsequently and concurrent for eleven days. At 53 days of age a mild-acute typhlitis associated with the presence of clostridial-like bacteria, and an enteritis-like lesions caused by a virus in the intestines was seen at post-mortem investigation by the GD. Furthermore, *Eimeria meleagridis* and some non-invasive flagellates were present in the intestines. Ten days later post-mortem investigation of five toms, by a veterinarian, due to a sudden increase in mortality, revealed a severe-necrotizing-typhlitis and necrotic-hepatitis in all five animals. A swap sample, origin unknown, was made and tested PCR positive for *H. meleagridis*. All animals received Gabrovet® 70 for three days. Only two barns were affected during the outbreak and total mortality ratio was 80,01% and 77,78% for the barns. The market weight of the toms that survived the outbreak, in the two affected barns, was on average 21,4 kg and the condemnation ratio of the total carcass weight was 3,5%. During the outbreak Suramox® was administered in all barns for *Mycoplasma Synoviae*.



Supplemental Figure 12.- Cumulative mortality ratio Flock I.
The solid line indicates the diagnosis of histomonosis (62 days of age). The dotted line indicates the transfer from the brooding barn to the finishing barns (37 days of age). The green line represent the start and length of a paromomycin treatment in the flock.

Flock I – The farm reared only hens and consisted of ten barns. Around 22.000 poults were reared each round in one large brooder barn. After 5 weeks the hens were transferred and divided over five barns. The farm reared three age groups at once, and therefore up to 66.000 hens could be present. Since no hatchery could supply such amounts of hens, the farm uses three different breeds during each round.

At 37 days of age the hens were transferred to the five finishing barns. At 62 days of age *H. meleagridis* was diagnosed in barn 5, how the diagnosis is established is unclear. No data of antimicrobials during the rearing period and the period subsequent to the outbreak is available. The day before the outbreak the bedding of the barns was regenerated and the litter regenerator was used from barn 5 till 1 respectively. After the diagnosis, barn 1 till 4 were treated with Parofor® 70 2 kg/1000 l for two days and eight days with 1,2 kg/1000 l subsequently. Barn 5 was treated with Suramox® for three days. Barn 1 till 4 showed no significant increase in mortality ratio during the outbreak with exception when the Parofor® was ended and a minor increase in mortality ratio occurred (data not shown). However, barn 5 reached a total mortality ratio of 47,13% during the affected period. The hens of barn 5 were complemented during slaughter with hens of barn 1. The condemnation ratio of the net carcass weight was 1,01%.



Supplemental Figure 13.- Cumulative mortality ratio Flock J.
Numbers denote different barns. The solid line indicates the diagnosis of histomonosis (76 days of age). The dotted line indicates the transfer between the brooding barn and the finishing barns (69 days of age). The dashed line indicates the transfer of all the poults to a larger brooding barn. The hens of barn 3 were divided amongst barn 1 and 2 (101 days of age). The blue and green lines represent the start and length of respectively an antibiotic or paromomycin treatment in the flock.

Flock J – The farm reared around 12.000 toms and 5.400 hens in seven barns. Hens and toms were reared in one barn. At day 35 all toms were transferred to barn 1 and 2 and the hens were transferred to barn 4. At day 70 some toms from both barns were transferred to barn 3. In the period before the outbreak the turkeys were treated with various antimicrobials such as Pulmotil®, sulfaquinoxaline natrium, enrofloxacin, doxycycline, phenoxymethyl-penicillin and polymyxin E. At 76 days of age *H. meleagridis* was diagnosed, how the diagnosis is established is unclear. For three days the toms received Parofor®, dose is unknown. Subsequent phenoxymethyl-penicillin and sulfaquinoxaline natrium was administered for five days, dose is unknown. Concurrently a chlorocresol solution and BreCalSan® was applied over the bedding over the bedding on a daily base. The toms housed in barn 3 were divided over barn 1 and 2 at 101 days of age. The hens remained unaffected during the outbreak

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