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ORIGINAL ARTICLE

Quantification of parasite shedding and horizontal transmission parameters in *Histomonas meleagridis*-infected turkeys determined by real-time quantitative PCR

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To gain more insight into the within flock transmission of *Histomonas meleagridis*, the shedding of parasites was quantified by a newly developed real-time quantitative (q)PCR and the basic reproduction number (R_0) and the mean number of secondary infections per infectious bird per day in a susceptible population (β) of *H. meleagridis* in the absence of heterakis were assessed. Forty turkeys were divided into two groups of 10 and 30 birds at 14 days of age. Birds of the first group were inoculated with 200,000 histomonads each, the second group served as a susceptible contact group. Cloacal swabs were taken at −1, 1, 4, 7, 9, 11, 14, 18 and 21 days post inoculation (p.i.) to assess the shedding of the parasite by the qPCR (detection limit 330 histomonads/ml droppings). The experiment ended at 28 days p.i. Mortality was 100% in the inoculated birds and started at day 12 p.i., while in the contacts, it was 83% and started at 16 days p.i. Shedding started 1 day after the inoculation in both groups. The mean shedding levels (and 95% CI) expressed as parasite equivalents per gram cloacal content on a $\log_{10}$ scale in the inoculated, contact birds that died and contact birds alive were 2.0 (1.6–2.4), 1.6 (1.4–1.9) and 1.2 (0.5–2.0), respectively. Birds that died shed histomonas more often and were infectious for 13.4 days; in contrast, those that recovered were infectious for 5.7 days. R_0 was estimated to be 8.4 and β 0.70. Simulations made with the parameters obtained were in agreement with the experimental results, confirming their validity.

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Introduction

Histomonosis is a severe and often deadly disease in turkeys caused by the protozoan *Histomonas meleagridis*. *H. meleagridis*-infected turkeys may develop typhlitis and hepatitis as main lesions, which are often fatal. Mortality frequently soars up to 80–100%, both under field and experimental conditions (Hu & McDougald, 2003; McDougald, 2005b; Hess *et al.*, 2006; Callait-Cardinal *et al.*, 2007a). The primary transmission method of histomonas has, for long time, been thought to be its incorporation in the eggs of the small worm *Heterakis gallinarum* (Lund & Burtner, 1957; Ruff *et al.*, 1970). However, recently, it was shown that direct lateral transmission within a flock may occur without the requirement of heterakis eggs (Hu & McDougald, 2003; Hess *et al.*, 2006).

In 2003, all commercial antihistomonal products were banned in the EU due to their suspected carcinogenicity (Byrne, 2001; Fischer Boel, 2002), leaving the industry without effective preventive and therapeutic measures. As a consequence, between April 2003 and June 2006, over 100 outbreaks of histomonosis with mortality up to 80% were reported in France (Callait-Cardinal *et al.*, 2007a). This stresses the need for new intervention strategies. Although in-feed paromomycin proved to be highly effective for the prevention of histomonosis (Bleyen *et al.*, 2009; Hafez *et al.*, 2010; van der Heijden *et al.*, 2011), its registration may be difficult due to increasing concerns of consumers, scientists and legislators about (long-term) antibiotic usage and the development of resistance and its transfer to humans. Consequently, the quest for new intervention strategies has been focused on herbal products and probiotics due to a renewed interest in natural and herbal medicine. Unfortunately, to date, efficacy of these products could not be demonstrated under experimental conditions (van der Heijden & Landman, 2008a, 2008b; Thofner *et al.*, 2012). In parallel, other scientists have chosen a more conventional approach, that is, vaccinology to solve the "histomonas problem" (Hess *et al.*, 2008; Liebhart *et al.*, 2010; Nguyen Pham *et al.*, 2013).

Contrary to herbal products and probiotics, the banned antihistomonal products and paromomycin have showed great efficacy in animal models where birds were intracloacally challenged with relatively high doses of histomonads. It was suggested that a more subtle effect of these "natural" products might have gone unnoticed due to the severity of the challenge. Therefore, an animal model that mimics more closely spontaneous histomonosis outbreaks in the field was developed, that is, a well-characterized transmission model in which birds are infected via lateral spread through contact with inoculated birds.

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This manuscript describes a horizontal transmission model for *H. meleagridis*, based on the previous work by Hu & McDougald (2003), in which the latent period, infectious period, basic reproduction number (R_0) and transmission rate (β) have been determined.

Materials and methods

Experimental design

Forty day-old BUT BIG 7 turkeys (50% male, 50% female) were purchased from a commercial turkey breeder in Germany (Heidemark Mästerkreis GmbH & Co. KG, Ahlhorn, Germany) and placed in a 1.8 × 3.2 m floor pen with wood shavings. The birds were fed a standard turkey feed (metabolizable energy of 9.6 MJ/kg and crude protein of 258 g/kg) without antihistomonals *ad libitum* throughout the experiment and had unlimited access to non-chlorinated drinking water. Light was given continuously for 16 hours per day.

At 14 days of age 10 birds (five females and five males) were individually inoculated intracloacally with 200,000 histomonads (*H. meleagridis* isolate Turkey/Netherlands/AL306/03 type I) and commingled with the other birds (15 females and 15 males) that were not inoculated or treated otherwise. The intracloacal inoculations (volume 1 ml) were performed using a stainless steel curved knobbed cannula (1.8 mm × 80 mm, product no. 1135523, Instruvet, Cuijk, the Netherlands). Before inoculation, each turkey was placed in a cardboard box until a dropping was produced (generally within a minute). Subsequently, they were held in inverted position during inoculation and 5–10 min afterwards in order to prevent voiding of the inoculum. No birds were seen producing droppings shortly after returning them to their pen.

Preparation of the inocula for the animal experiment was done as described (Van der Heijden & Landman, 2007).

All birds were subjected to gross post-mortem examination after they died during the experiment or when the experiment ended at 28 days post inoculation (p.i.). Lesions in the liver and caeca were scored on a 0 to 4 scale as described (McDougald & Hu, 2001).

H. meleagridis real-time quantitative (q)PCR

At day −1, 1, 4, 7, 9, 11, 14, 18 and 21 p.i., a cloacal swab was taken from every turkey poult. The swabs were stored at −80°C until further analysis with a qPCR for histomonas, which was developed for this purpose. Primers (Eurogentec, Seraing, Belgium) and a minor groove binding probe (Life Technologies, Bleiswijk, the Netherlands) (see Table 1) were designed based on 31 *H. meleagridis* 5.8S rRNA DNA sequences (GenBank) originating from four different research groups. The qPCR yielded an 81 bp product. DNA was extracted from samples using the AB MagMax Total Nucleic Acid Isolation Kit (Applied Biosystems, Bleiswijk, the Netherlands). Real-time PCR was performed using the Quantifast Pathogen +IC kit (Qiagen, Venlo, the Netherlands), using either the ABI 7500 fast cycler (Applied Biosystems) or the LC480 cycler (Roche, Woerden, the Netherlands). The PCR protocol consisted of 15-minute incubation at 95°C, followed by 40 alternating cycles of 15 seconds at 95°C and of 30 seconds at 60°C. One PCR per sample was performed.

The sensitivity was assessed by analysing 17 Dutch *H. meleagridis* isolates, including the inoculation strain, with the qPCR. These isolates were obtained earlier from poultry farms by culture and had been identified by morphological examination of live parasites, which showed the characteristic "rocking motion". A *Tetratrichomonas gallinarum* strain was analysed to assess the specificity. The specificity was also tested *in silico* based on alignments with GenBank sequences.

The calibration curve, detection limit, as well as the repeatability and reproducibility of the qPCR for both the LC480 cycler and the ABI7500 cycler were determined by testing samples of suspensions of droppings spiked with histomonads. Therefore, a 10-fold serial dilution (10^0 to 10^{-6}) was prepared from a *H. meleagridis* culture with 3.3×10^5 histomonads/ml. Subsequently, 10% suspensions of droppings from specified pathogen free birds in phosphate-buffered saline (pH 7.2) were spiked in triplicate by adding 60 µl of diluted culture to 540 µl of suspension. From each of the three series of spiked suspensions (ranging from 3.3×10^4 to 3.3×10^{-2} histomonads/ml) DNA was isolated and stored at −80°C until each series was assayed by qPCR on different days in triplicate.

Data on the animal experiment including qPCR results and data on the validation of the qPCR can be found in the supplementary data.

Shedding levels

In order to estimate the transmission parameters (see below), an assumption was necessary on whether shedding levels in inoculated, and contact birds are different on average. To make a proper assumption, log-shedding levels were compared among three groups: (1) inoculated birds (ino, $n = 10$), (2) contact birds that were alive at the end of the experiment, clinically healthy and showed no lesions at post-mortem (ctc-alive, $n = 5$), and (3) contact birds that showed clinical signs and lesions at post-mortem, of which all but one died (ctc-dead, $n = 25$).

Comparison of shedding levels between the three groups was done by Bayesian analysis in WinBUGS, by estimating the mean $\log_{10}$-shedding level in each group and constructing 95% credible intervals for differences between group means; if intervals contained 0, group means were assumed to be equal in the transmission analysis. Individual observations were modelled as the sum of the group mean, a random animal effect and a measurement error. Samples below the detection limit before the first positive sample of a bird was detected by the qPCR were not included as it was assumed that shedding had not started yet, whereas samples below the detection limit after the first positive sample were included as 1 (= log(10)). Prior distributions for means and sd were uniform between 0–100 (means) and 0–50 (sd). After a burn-in of 10,000 updates, posterior distributions of size 1000 were obtained (with thinning of 100).

Transmission parameters. A susceptible – latently infected – infectious – recovered (SLIR) model (Höhle *et al.*, 2005) was used to estimate the following transmission parameters:

- mean and standard deviation of the latent period (L and sd_L), which is the period between becoming infected and starting of shedding.
- probability to die from the infection (p_{dead}).
- mean and standard deviation of the infectious period, separately for birds that died and birds that recovered (D_{dead}, D_{alive}, sd_{Ddead} and sd_{Dalive}), which is the duration of shedding.
- mean transmission rate β, which is the number of birds that is on average infected by one infectious bird during one day in a susceptible population.
- basic reproduction ratio R_0, which is the mean number of birds infected by one infected bird during its entire period of infection in a susceptible population. In terms of the previous parameters, $R_0 = \beta(p_{dead} \times D_{dead} + (1 - p_{dead}) \times D_{alive})$.

In more detail, it was assumed that the latent period L_i, the infectious period D_i and the transmission rate β_i were different for each bird i, and that all were gamma-distributed across the birds. The qPCR data were assumed to reflect infectiousness: only if positive, was the bird in its infectious period, and β_i was proportional to the mean qPCR level in bird i. Susceptible birds were infected at rate $\lambda(t)$, the force of infection, that is, the probability per day to become infected; $\lambda(t)$ was equal to the sum of all β_i of birds that were infectious at time t, divided by initial population size N (equal to 40).

Estimation was done by Bayesian analysis, by Markov chain Monte Carlo using of the Metropolis–Hastings algorithm. All prior distributions were uniform, for parameters as well as unobserved time points of infection and recovery. The analysis was programmed in statistical software R (www.r-project.org; the code is available upon request). Program data used for the estimation of transmission parameters, simulations of the experiment and simulations of a flock with histomonosis can be found in the supplementary data.

The results were used to carry out stochastic simulations using the same SLIR model as described above, with parameter values sampled from the posterior distribution. First, the experiment itself was simulated (100 replications) and compared to the actual data (prevalence of shedding and mortality in time). Second, 100 simulations were done for a flock of 10,000 birds, starting with 10 infected birds, to extrapolate our results to realistic population sizes. For this extrapolation, the same stocking density was assumed, allowing extrapolation of the transmission rate by frequency-dependent transmission (β_i independent of population size).

Ethics. The study was approved by the Institutional Experimental Committee, DEC-Consult Foundation, according to Dutch law on experimental birds (Wet op de dierproeven).

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Table 1. Name and sequence of the primers and minor groove binding probe used in the qPCR.

	Name	Sequence
Forward primer	Hm5.8F2	5′- CTTAAACAACggATgTCTTggCTCT – 3′
Reverse primer	Hm5.8R	5′- TTKAgCTAATCTAgTCgCTTATCAC – 3′
MGB probe	Hm5.8P	5′- CAATgTTCTTCTTCgTg – 3′

Results

Mortality and lesions. Mortality in the inoculated group started at 12 days p.i., all turkeys in this group died before 22 days p.i. (Figure 1). The mean death time in this group was 15.1 ± 2.7 days. All birds that were inoculated had a caecum lesion score of 4, four of 10 inoculated birds had a liver lesion score of 3 and the remaining six birds in this group had a liver lesion score of 4 (Table 2). Mortality in the contact group started at 16 days p.i. and increased fast, at the end of the experiment (28 days p.i.) 24 of 30 contact poults in the susceptible group had died. One surviving bird showed clinical signs of histomonosis (drowsiness, reluctance to move, ruffled feathers, inappetence, drooping wings (McDougald, 2005b)), while the other survivors were clinically healthy. The mean death time in the contact group was 19.8 ± 2.4 days. All poults that died during the experiment, and the surviving poult with clinical signs showed typical lesions of histomonosis at post-mortem, although one bird had lesions in the liver only. Of the contact poults that died during the experiment one, two, three and 18 birds had caecum lesion scores of 0, 2, 3 and 4, respectively, of the six poults that survived until 28 days p.i. five had a caecum lesion score of 0 and the remaining poult had a caecum lesion score of 4. There were eight and 16 poults in the contact group that died during the experiment with liver lesion scores of 3 and 4, respectively; five of the surviving birds had a liver lesion score of 0 while the remaining bird had a liver lesion score of 4.

***H. meleagridis* qPCR**. The qPCR detected all *H. meleagridis* isolates, while negative results were found after analysis of the *T. gallinarum* strain. The detection limit of the qPCR was 330 histomonads/ml suspension of droppings. The regression squared values of the calibration curves were 0.987 and 0.961 for the ABI7500 and LC840, respectively.

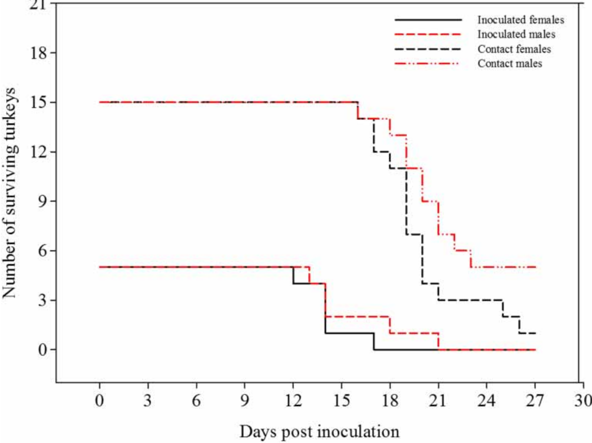

Figure 1. A Kaplan–Meier graph shows the number of surviving *H. meleagridis* inoculated and contact poults with time. The starting numbers of turkeys were five females and five males that had been inoculated intracloacally with histomonas and were thereafter commingled with 15 contact females and 15 contact males.

The repeatability and reproducibility were calculated using a suspension of 3.3×10^4 histomonads/ml as for this concentration a C_t-value below 40 was obtained. The average repeatability (CVr%) and reproducibility (CVR%) for the LC480 cycler were 1.6% and 10.0%, respectively, and for the ABI7500 cycler, 2.9% and 10.4%, respectively.

The PCR efficiency was calculated from the results of the third test run, with C_t values below 40 for four *H. meleagridis* concentrations (three replicates) available. The efficiency for the LC480 and ABI7500 cyclers was 115.8% and 95.4%, respectively.

The day before inoculation (day −1 p.i.), *H. meleagridis* concentrations in samples of cloacal content of all poults were below the detection limit. The inoculated group started shedding parasites at one day p.i. (7/10). Thereafter, shedding with time (of surviving birds) was as follows: at four days p.i. 7/10, at seven days p.i. 8/10, at nine days p.i. 9/10, at 14 days p.i. 3/3 and at 18 days p.i. 0/1. In the contact birds, shedding of parasites started at one day p.i., but only concerned 1/10. Subsequently, shedding with time (of surviving birds) was as follows: at four days p.i. 6/30, at seven days p.i. 7/30, at nine days p.i. 21/30, at 14 days p.i. 25/30, at 18 days p.i. 13/24 and at 21 days p.i. 6/11. The percentage of turkeys per group and gender shedding parasites with time has been outlined in Figure 2.

Shedding levels. The mean shedding levels (and 95% CI) expressed as parasite equivalents per gram cloacal content on a $\log_{10}$ scale in the ino, ctc-dead and ctc-alive groups were 2.0 (1.6–2.4), 1.6 (1.4–1.9) and 1.2 (0.5–2.0), respectively. The 95% CIs of the differences between the groups were −0.2 and +0.9 between ino and ctc-dead, −0.1 and +1.6 between ino and ctc-alive, and −0.4 and +1.2 between ctc-dead and ctc-alive. All intervals included 0, so we assumed an equal mean shedding level for the three groups in the subsequent analysis.

An overview of the $\log_{10}$ parasite equivalents shed per bird and gram cloacal content in histomonas inoculated and contact infected turkeys is shown in Table 3.

Transmission parameters. Results of the transmission analysis are presented in Table 4. It appears that birds that died from the infection (83% (including one surviving bird with severe histomonas lesions)) are infectious for more than twice as long as birds that survive: 13.4 and 5.7 days on average, respectively. The overall mean infectious period, therefore, is 12.0 days. The mean transmission rate β is estimated at 0.70, which is the mean number of secondary infections per infectious bird per day in a susceptible population. Overall, this results in a basic reproduction ratio R_0 of 8.4.

The prevalence, the shedding levels and the survival of inoculated and contact birds, deduced from both data and

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Table 2. Lesion scores of caecum and liver in inoculated and contact poults that died or survived during the experiment.

	Caecum			Liver		
	Inoculated[a]	Contact		Inoculated[a]	Contact	
Lesion score	Died	Died	Survived	Died	Died	Survived
0	0	1	5	0	0	5
1	0	0	0	0	0	0
2	0	2	0	0	0	0
3	0	3	0	4	8	0
4	10	18	1	6	16	1

[a]All inoculated poults died during the experiment.

simulations, are shown in Figure 3(a–c). Predominantly, the data lie in the 90% band of simulated results (100 stochastic simulations with parameter sets from the posterior distributions), showing that the model reliably reproduced the experimental data.

The same parameter sets were used to simulate outbreaks in a flock of 10,000 turkeys, starting with 10 infected turkeys. Figure 4(a–c) shows the median and 90% bands of prevalence, shedding level and survival, plus three individual simulations selected to represent the variability in outcome. It appears that the outbreak peaks 3–5 weeks after introduction of the 10 infected birds, and that the peak prevalence is about 7300 infected birds. The amount of shedding by the flock as a whole is highly variable between individual outbreaks as can be seen from the intervals and the three example simulations: there was a fourfold difference between the fifth highest shedding outbreak and the fifth lowest shedding outbreak. Most turkeys die in weeks 3–7, and total mortality directly reflects the estimated probability to die from the infection (p_{dead}; tables 3 and 4).

Discussion

In this study, histomonosis was successfully induced in both inoculated and contact turkey poults with 85% total mortality and lesion scores of mainly 3 and 4 for both the liver and the caecum. This is in agreement with other studies where this disease was induced by horizontal transmission (Hu & McDougald, 2003; Hess *et al.*, 2006; Liebhart & Hess, 2009) although small differences in first day of death and mortality were noted probably due to differences in histomonas strain (McDougald, 2005a). Horizontal

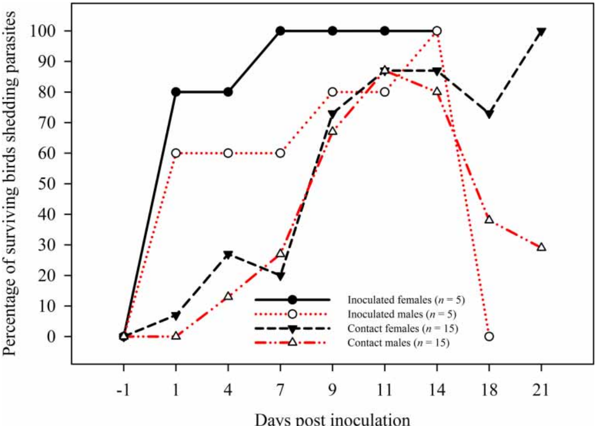

Figure 2. Shedding of *H. meleagridis* in the inoculated and contact poults as a percentage of survivors with time.

transmission likely occurs via the cloacal route (Hu *et al.*, 2004) as retrograde transport of cloacal fluids to the caecum is fast (Akester *et al.*, 1967), while oral inoculation with lumen stages of the parasite, probably due to the acidity of the gizzard, often fails to result in disease (Farmer & Stephenson, 1949; Horton-Smith & Long, 1956; Lund, 1956). The non-vector-mediated transmission is supported by the survival of *H. meleagridis* for up to 9 hours in non-chlorinated water or faecal matter (Lotfi *et al.*, 2012). In addition, a possible role for cyst-like structures, which have recently been identified in histomonas cultures (Munsch *et al.*, 2009; Zaragatzki *et al.*, 2010) in horizontal transmission, cannot be discarded. These “cysts” might cause infection by both, the oral and cloacal route.

In addition to the recording of the mortality and lesion scores, the shedding of parasites was determined by a newly developed qPCR targeting the 5.8S ribosomal gene of *H. meleagridis*. This gene is part of the large subunit of the ribosomal gene of which multiple copies are present as tandem repeats in each cell. The detection limit of 330 histomonads/ml droppings was lower than that of another *H. meleagridis* PCR described before, which had a detection limit of 10^3–10^5 histomonads/ml droppings (Huber *et al.*, 2005). Reduced sensitivity of PCR in faecal samples has been described, and various strategies to reduce the negative effects of inhibitors present in these samples are available (Radstrom *et al.*, 2004). While other research groups used Flinders Technology Associates (FTA™) cards (Huber *et al.*, 2005) to bypass this problem, we found that the AM1840 extraction kit (Ambion) is suitable to extract high-quality DNA from faecal samples (data not shown). An internal control was included to make sure negative samples are not due to inhibition of the PCR process. The specificity of the qPCR was not assessed extensively (with a *T. gallinarum* strain negative results were obtained), but this is not very important in this study where cloaca swabs of birds yielded negative results at the start of the experiment and were subsequently inoculated with a *H. meleagridis* strain.

The first *H. meleagridis* shedding poults were found 1 day p.i., which is 1 day earlier than reported by others (Hess *et al.*, 2006). This may have been caused by the lower detection limit of our qPCR. All poults in the experiment shed parasites, and no statistical difference was found in the level of shedding between inoculated poults, contact poults that died from infection and contact poults that recovered. However, assessment of transmission parameters showed that the infectious period of recovered poults was shorter than that of poults that died from infection. Although the histomonads detected in recovered poults could be transient parasites resulting from cloacal drinking, the level and duration of shedding suggest

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Table 3. Log_{10} parasite equivalents shed per bird and g cloacal content in histomonas inoculated and contact infected turkeys.

Group	Gender	Parasite equivalents per gram cloacal content at day								
		–1	1	4	7	9	11	14	18	21
Inoculated	F	0.0[a]	0.0	0.0	2.3	4.2	4.6	3.0	X[b]	X
Inoculated	F	0.0	1.6	3.0	3.4	2.6	3.4	X	X	X
Inoculated	F	0.0	1.6	2.9	2.4	2.8	2.5	X	X	X
Inoculated	F	0.0	4.3	3.1	2.6	1.9	2.9	X	X	X
Inoculated	F	0.0	2.8	3.5	3.5	3.6	1.7	X	X	X
Inoculated	M	0.0	0.0	3.7	3.8	3.2	4.1	X	X	X
Inoculated	M	0.0	2.5	3.9	2.5	4.8	5.1	X	X	X
Inoculated	M	0.0	2.7	0.0	0.0	0.0	4.1	2.1	0.0	X
Inoculated	M	0.0	1.6	2.1	1.9	2.0	0.0	X	X	X
Inoculated	M	0.0	0.0	0.0	0.0	3.4	6.1	1.5	X	X
Contact	F	0.0	0.0	0.0	0.0	3.2	3.2	1.2	X	X
Contact	F	0.0	0.0	0.0	0.0	2.8	1.9	4.8	0.0	X
Contact	F	0.0	0.0	2.2	1.9	2.1	3.2	0.0	X	X
Contact	F	0.0	0.0	0.0	0.0	2.7	3.5	2.0	0.0	X
Contact	F	0.0	0.0	0.0	0.0	2.8	3.7	2.3	3.2	X
Contact	F	0.0	0.0	0.0	1.7	0.0	3.7	1.5	X	X
Contact	F	0.0	0.0	1.2	0.0	4.7	3.6	2.3	4.1	X
Contact	F	0.0	0.0	1.5	2.5	1.3	2.4	1.5	1.8	X
Contact	F	0.0	0.0	0.0	0.0	0.0	3.3	2.8	2.1	3.1
Contact	F	0.0	0.0	0.0	0.0	0.0	0.0	1.8	X	X
Contact	F	0.0	0.0	0.0	0.0	1.7	3.8	1.4	1.6	2.9
Contact	F	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0	3.4
Contact	F	0.0	0.8	0.0	0.0	2.4	3.2	2.4	2.7	X
Contact	F	0.0	0.0	0.0	0.0	3.7	3.7	2.1	0.0	X
Contact	F	0.0	0.0	0.0	0.0	2.4	2.6	1.4	0.0	1.7
Contact	M	0.0	0.0	0.0	0.0	0.0	3.1	3.2	2.0	1.7
Contact	M	0.0	0.0	0.0	3.1	3.3	2.9	1.8	X	X
Contact	M	0.0	0.0	0.0	0.0	0.0	3.0	0.0	0.0	0.0
Contact	M	0.0	0.0	0.0	2.4	3.5	3.3	0.0	0.0	X
Contact	M	0.0	0.0	0.0	0.0	4.2	3.3	2.3	X	X
Contact	M	0.0	0.0	0.0	0.0	3.4	2.3	1.4	0.0	X
Contact	M	0.0	0.0	1.5	0.0	0.0	3.2	1.6	4.9	X
Contact	M	0.0	0.0	0.0	2.4	2.2	1.8	0.0	0.0	0.0
Contact	M	0.0	0.0	0.0	2.2	2.2	0.0	1.4	0.0	X
Contact	M	0.0	0.0	0.0	0.0	2.0	0.0	2.1	0.0	0.0
Contact	M	0.0	0.0	1.7	0.0	1.8	1.6	2.7	2.4	X
Contact	M	0.0	0.0	0.0	0.0	0.0	1.6	1.6	0.0	0.0
Contact	M	0.0	0.0	0.0	0.0	2.9	4.0	2.8	2.3	3.6
Contact	M	0.0	0.0	0.0	0.0	0.0	2.5	1.8	0.0	0.0
Contact	M	0.0	0.0	0.0	0.0	5.1	2.1	2.2	3.5	X

[a]Below the detection limit.
[b]Dead.

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Table 4. Parameter estimates of the transmission model.

Parameter symbol	Description (dimension)	Estimate (posterior distribution)	
		Median	95% CI
L	latent period: mean (days)	2.1	1.2–3.6
sd_L	latent period: sd (days)	2.0	1.2–3.7
D_{dead}	infectious period of birds that died: mean (days)	13.4	12.1–14.8
sd_{Ddead}	infectious period of birds that died: sd (days)	3.8	3.0–5.3
D_{alive}	infectious period of birds that survived: mean (days)	5.7	3.9–8.6
sd_{Dalive}	infectious period of birds that survived: sd (days)	1.8	0.84–4.9
p_{dead}	probability to die from the infection (–)	0.82	0.66–0.93
β	mean transmission rate (per day)	0.70	0.25–2.3
$\log(PE)_{mean}$	parasite equivalents per gram cloacal content: mean ($\log_{10}$(count))	1.9	1.5–2.2
$\log(PE)_{sd}$	parasite equivalents per gram cloacal content: sd ($\log_{10}$(count))	0.14	0.0045–0.46
R_0	basic reproduction ratio [count] $= \beta(P_{dead}D_{dead} + (1 - P_{dead})D_{alive})$	8.4	4.7–16.9

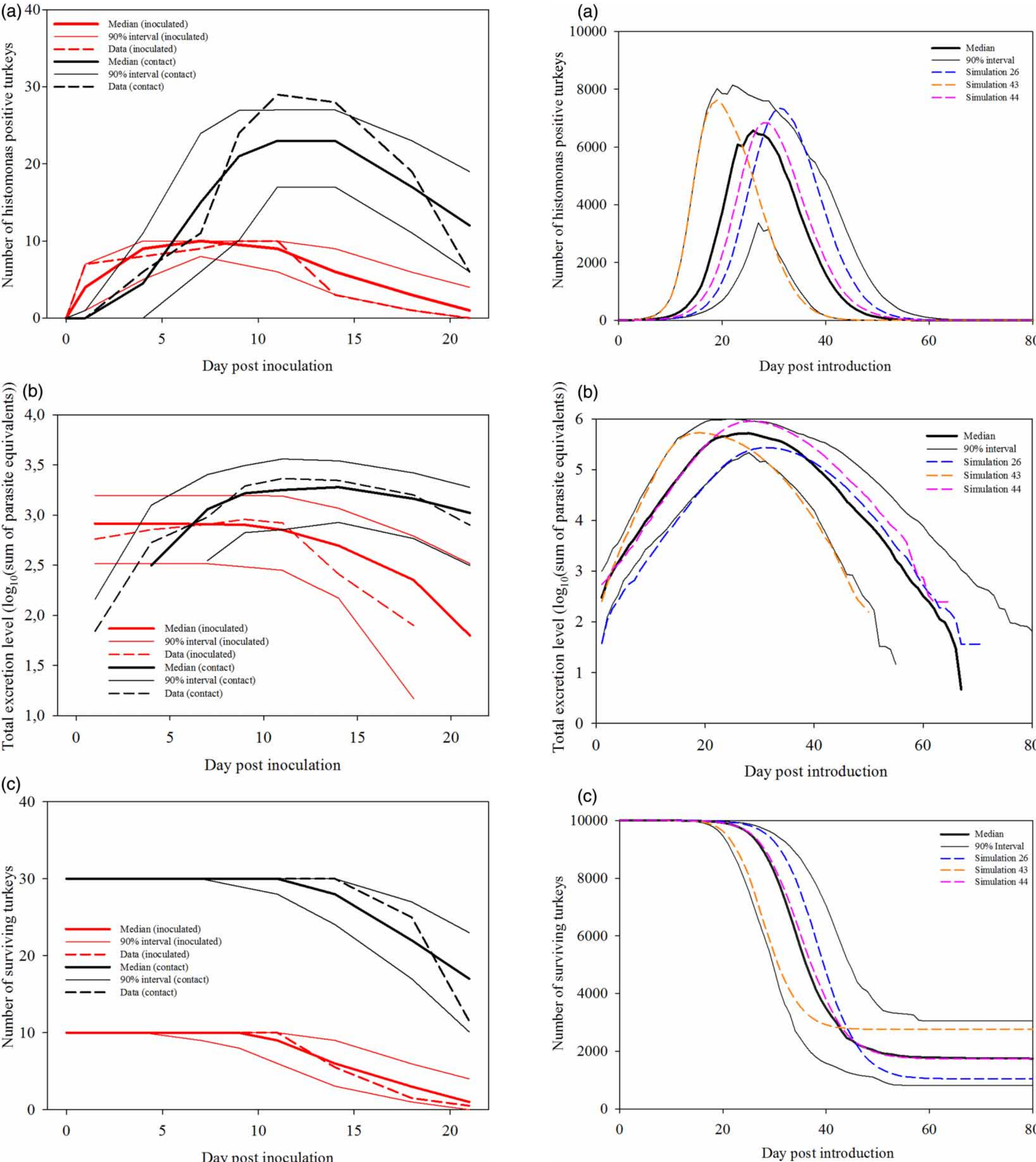

Figure 3. *(a)* The median number of histomonas positive inoculated and contact turkeys and 90% interval (prevalence) after 100 simulations of the experiment. Dotted lines denote data from the experiment. *(b)* The median total $\log_{10}$ excretion of histomonads and 90% interval of inoculated and contact turkeys after 100 simulations of the experiment. Dotted lines denote data from the experiment. *(c)* The median number and 90% interval of surviving inoculated and contact turkeys after 100 simulations of the experiment. Dotted lines denote data from the experiment.

Figure 4. *(a)* The median number and 90% interval of histomonas positive turkeys (prevalence) after 100 simulations of an outbreak in a flock of 10,000 birds. Three single simulations are also depicted. *(b)* The median $\log_{10}$ total excretion of histomonads and 90% interval after 100 simulations of an outbreak in a flock of 10,000 birds. Three single simulations are also depicted. *(c)* The median number and 90% interval of surviving turkeys after 100 simulations of an outbreak in a flock of 10,000 birds. Three single simulations are also depicted.

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an active role of these birds in the transmission of *H. meleagridis*. Detection of transient parasites could be a possible explanation for the *H. meleagridis* positive contact poult at day one.

The pattern of histomonas shedding was used to estimate parameters of a SLIR model. Simulation of the experiment with estimated parameters showed that the model could well reproduce the experimental results. By using Bayesian statistics and obtaining posterior distributions by Markov chain Monte Carlo sampling, we were able to allow for more realistic distributions for latent and infectious periods (gamma distributions instead of the standard exponential distributions),

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and to include shedding levels as a measure of infectiousness of individual birds instead of assuming equal infectiousness for all birds. Whereas the former (realistic distributions) may affect outbreak dynamics significantly in terms of peak prevalence and outbreak duration, the latter (variable infectiousness) is probably less essential. However, it does allow the estimation of variability in shedding between outbreaks in flocks, which turns out to be large. Differences of 10-fold can easily occur, which are not reflected by peak prevalence or mortality. It must be noted that it is unclear what this implies for the transmission of *H. meleagridis* between flocks, because higher shedding may be associated with lower transmissibility per histomonad or shedding level may not be at all relevant for transmission between flocks.

In order to assess intervention strategies in a transmission model the probability of a minor outbreak due to a too small number of seeder birds and a low R_0 should be avoided (Velthuis *et al.*, 2007). The set-up of this experiment virtually excludes this possibility, with 10 seeder birds and a lower limit R_0 of 4.7. Indeed, all simulations of the experiment resulted in major outbreaks. Based on power calculations for much smaller groups (Velthuis *et al.*, 2007), a single repetition of this experiment for both a control group and a vaccine group should be enough to assess effectiveness of a vaccine if the vaccine can reduce R_0 to a value below 1. Observation of the poults for 28 days after inoculation seems to be enough, because all contact poults had been shedding parasites at 14 days p.i., leaving enough time to infect the maximum number of birds if the β is lower. However, since one bird was ill at the day the experiment was ended and two contact birds had died in the previous three days, the observation period may be extended in order to reach maximum mortality.

In this paper, for the first time quantitative shedding of *H. meleagridis* in the inoculated and contact poults measured by qPCR is described. Furthermore, the shedding patterns have been used to calculate the latent period, the infectious period, R_0 and β of the *H. meleagridis* strain used. These data can be used to assess the efficacy of potential antihistomonal compounds, vaccines and other intervention strategies against histomonosis.

Using less virulent *H. meleagridis* strains, for example, strains obtained from subclinical or mild cases of histomonosis (Callait-Cardinal *et al.*, 2007b; Aka *et al.*, 2011), will likely result in a less efficient transmission of the disease and other parasite transmission parameters (R_0 and β). In such a model, intervention strategies would be insufficiently challenged and their robustness overestimated.

In conclusion, no significant differences were observed in mortality, lesion scores and shedding of the parasites between inoculated and contact birds. Indicating that an intracloacal challenge model using a similarly virulent strain as used here would not have yielded signifcant different results regarding the effect of 'natural' therapeutic products.

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Supplemental data

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