Serological survey and risk factors for *Toxoplasma gondii* in domestic ducks and geese in Lower Saxony, Germany

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ABSTRACT

To obtain estimates for the prevalence of *Toxoplasma gondii* infection in ducks and geese in Germany, enzyme-linked immunosorbent assays (ELISA) were established based on affinity-purified *T. gondii* tachyzoite surface antigen 1 (TgSAG1) and used to examine duck and goose sera for *T. gondii*-specific antibodies. The results of 186 sera from 60 non-infected ducks (*Anas platyrhynchos*) and 101 sera from 36 non-infected geese (*Anser anser*) as well as 72 sera from 11 ducks and 89 sera from 12 geese inoculated experimentally with *T. gondii* tachyzoites (intravenously) or oocysts (orally) and positive in a *T. gondii* immunofluorescent antibody test (IFAT) were used to select a cut-off value for the TgSAG1-ELISA. Sera obtained by serial bleeding of experimentally inoculated ducks and geese were tested to analyze the time course of anti-TgSAG1 antibodies after inoculation and to assess the sensitivity of the assays in comparison with IFAT. In ducks, IFAT titres and ELISA indices peaked 2 and 5 weeks p.i with tachyzoites, respectively. Only three of six geese inoculated with tachyzoites at the same time as the ducks elicited a low and non-permanent antibody response as detected by the IFAT. In the TgSAG1-ELISA, only a slight increase of the ELISA indices was observed in four of six tachyzoite-inoculated geese. By contrast, inoculation of ducks and geese with oocysts led to an increase in anti-TgSAG1 antibodies within 1 or 2 weeks, which were still detectable at the end of the observation period, i.e. 11 weeks p.i. Inoculation of three ducks and three geese with oocysts of *Hammondia hammondi*, a protozoon closely related to *T. gondii*, resulted in a transient seroconversion in ducks and geese as measured by IFAT or TgSAG1-ELISA. Using the newly established TgSAG1-ELISA, sera from naturally exposed ducks and geese sampled in the course of a monitoring program for avian influenza were examined for antibodies to *T. gondii*; 145/2534 (5.7%) of the ducks and 94/373 (25.2%) of the geese had antibodies against TgSAG1. Seropositive animals were detected on 20 of 61 duck and in 11 of 13 goose farms; the seroprevalences within positive submissions of single farms ranged from 2.2% to 78.6%. Farms keeping ducks or geese exclusively indoors had a significantly lower risk (odds ratio 0.05, 95% confidence interval 0.01–0.3) of harboring serologically positive animals as compared with farms where the animals had access to an enclosure outside the barn.

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1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infect all warm-blooded animals, i.e. mammals and avian species (Dubey, 2010). *T. gondii* has a clonal population structure with three main clonal lineages designated as types I, II, and III (Howe and Sibley, 1995). These lineages largely predominate in Europe and North America while strains not related to these lineages have been described mainly from other geographic regions (Dardé, 2004, 2008; Sibley et al., 2009). Phylogenetically, *T. gondii* is closely related to *Hammondia hammondi*, a protozoan that – like *T. gondii* – uses felids as definitive hosts (Dubey and Sreekumar, 2003).

T. gondii has been isolated from the tissues of domestic Anseriformes (Literák and Hejlíček, 1993; Dubey et al., 2003, 2007; Bártová et al., 2004; Zia-Ali et al., 2005, 2007). Recent serological studies during the past decade suggest that *T. gondii* infections in domestic Anseriformes may occur worldwide, and are not rare (Literák and Hejlíček, 1993; El Massry et al., 2000; Sroka, 2001; Dubey et al., 2003, 2007; Zia-Ali et al., 2005, 2007; Bártová et al., 2009; Yan et al., 2009; Sroka et al., 2010). Anseriformes may become infected with *T. gondii* by oral ingestion of sporulated *T. gondii* oocysts with feed or water, or of tissue cysts in carcasses of intermediate hosts as demonstrated by experimental studies in domestic ducks (Bártová et al., 2004; Murao et al., 2008). The oocyst is an environmentally resistant stage of the parasite shed by felids such as domestic cats, which are the predominant definitive host of *T. gondii* in Europe. Felids can excrete large numbers of oocysts which become infectious in the environment by sporulation and may remain infectious for several months (Dubey, 2010). Up to 13 million oocysts per gram of feces were reported in naturally infected cats (Schares et al., 2008). Intermediate hosts, such as birds, rodents, domestic mammals or humans can contract the infection by ingesting sporulated *T. gondii* oocysts.

Contamination of drinking water with *T. gondii* oocysts can cause major outbreaks of acute toxoplasmosis in humans (de Moura et al., 2006). However, infection by tissue cysts in raw or undercooked meat is often regarded the predominant route for human infection (Cook et al., 2000). In addition to consumption, handling of raw meat of infected intermediate hosts prior to preparing meals may also pose a risk of infection, as a low standard of kitchen hygiene was demonstrated as a risk factor for primary *T. gondii* infection during pregnancy (Kapperud et al., 1996). To which extent the consumption or handling of poultry meat, including the meat of Anseriformes, contributes to human infections remains to be clarified. The isolation of viable *T. gondii* from the tissue of Anseriforms after acidic pepsin digestions (Zia-Ali et al., 2005, 2007; Dubey et al., 2007) suggests that tissue cysts were present in these tissues.

To assess the potential risk for human infection with *T. gondii* due to exposure to meat from Anseriformes, data on the proportion of infected ducks and geese are needed. Therefore the present study aimed at the development and the validation of serological tests for the detection of *T. gondii*-specific antibodies in ducks and geese. We report for the first time serological data on the *T. gondii* prevalence in ducks and geese in the German federal state of Lower Saxony.

2. Materials and methods

2.1. Parasites

2.1.1. Tachyzoites

The *T. gondii* strains RH (Sabin, 1941), ME49 (Lunde and Jacobs, 1983), and NED (Howe and Sibley, 1995), i.e. representatives of the three main clonal lineages of *T. gondii* (type I, II, and III, respectively), were cultivated at 37 °C, 5% CO_2 in Vero cells for 3–5 days with MEM Dulbecco's medium supplemented with 1% glutamine, 2% fetal calf serum (FCS) and 1% antibiotic solution (10,000 IU penicillin and 10,000 μg streptomycin/ml solution). To harvest *T. gondii* tachyzoites the cells were scraped from the flask and tachyzoites purified by filtration using 5 μm filters (Millipore, Eschborn, Germany), washed five times by centrifugation at $700 \times g$ (4 °C, 8 min) and resuspended in ice-cold phosphate-buffered saline (PBS). For the experimental infection of ducks and geese, the tachyzoites of all three strains were counted using a Neubauer chamber, checked by Trypan Blue exclusion for viability and used immediately after harvest. One day before parasites were harvested for antigen production, the FCS-supplemented medium was removed and infected cells were further cultivated under FCS-free conditions. If used as antigen, RH-strain tachyzoites were centrifuged ($700 \times g$) and dispensed onto IFAT slides or stored as a pellet at −80 °C until used for purification of the *T. gondii* surface antigen 1 (TgSAG1).

2.1.2. Oocysts

T. gondii oocysts were collected from the feces of an experimentally infected cat by flotation using saturated NaCl solution (specific gravidity 1.2). The cat had been orally infected with tissue cysts of the *T. gondii* DX strain (type II; Howe and Sibley, 1995) isolated from a pig (Störmann, 1962). Sporulated oocysts were stored in 2% (v/v) sulfuric acid at 4–8 °C. Prior to use, oocysts were washed with tap water and counted using a Neubauer chamber.

2.2. Experimental infections

All animal experiments reported in this publication were approved by the Ministerium für Landwirtschaft, Umweltschutz und Raumordnung of the German Federal State of Brandenburg or the Lower Saxony State Office for Consumer Protection and Food Safety of the German Federal State of Lower Saxony.

2.2.1. Inoculation of tachyzoites

Ducks (*Anas platyrhynchos*) and geese (*Anser anser*), obtained at the age of 5 weeks from a commercial breeder (Geflügelhof Gaetke, Parchim, Germany) and negative by IFAT, were used to produce positive reference sera. Six geese and six ducks were distributed into three groups of two animals each (Table 1). Two ducks and 2 geese were

inoculated intravenously with 10^4 tachyzoites (in a volume of 0.5 ml MEM Dulbecco's medium supplemented with 1% glutamine) with one of the *T. gondii* strains RH, ME49, or NED. Six ducks and six geese served as negative controls. Sera from all inoculated and control animals were collected at 0, 2, 5, 7, 9 and 11 weeks post-inoculation (p.i.) and stored at −20 °C.

2.2.2. Inoculation of oocysts

A total of 11 ducks and 10 geese (Geflügelhof Gaetke, Parchim, Germany) that had tested negative by IFAT before inoculation were used in this experiment. Five ducks and six geese were inoculated orally with 2 ml of water containing 5×10^5 *T. gondii* DX strain oocysts at the age of 5 weeks. The remaining animals served as controls. Sera from all inoculated and control animals were collected prior to inoculation and at weekly intervals until 11 weeks p.i., and stored at −20 °C until tested by ELISA or IFAT.

Three additional ducks and three geese were orally inoculated with 5×10^5 *H. hammondi* oocysts, strain H.H.34.

2.3. Negative sera

To determine specific cut-offs for the ELISA, 186 sera from 60 IFAT-negative ducks (at the age of 5 weeks to 1 year) and 101 sera from 36 IFAT-negative geese (at the age of 5–16 weeks) were used. This set of negative sera included sera from the animals used in the experiments described above but also sera from other control ducks and geese, used in other non-related experiments. The highest IFAT titre, defined as the reciprocal serum dilution, observed in these sera was 25. In the following, samples from these animals are referred to as negative sera.

2.4. Antigen purification

TgSAG1 was obtained by immunoaffinity chromatography using the mouse monoclonal antibody (mAb) IgG2a P30/3 (ISL, Paignton, UK). This mAb recognizes an epitope on the surface of tachyzoites and was coupled to rProtein A Sepharose 4B (GE healthcare, Heidelberg, Germany) as an immunosorbent. Briefly, cell-culture derived *T. gondii* RH strain tachyzoites were diluted in PBS supplemented with 0.5% Triton X-100 (4 °C) and sonicated on ice for 90 s (50% active cycle, output control level 2; VibraCell, Sonics & Materials Inc., Meryin/Satigny, Switzerland). The suspension was centrifuged at $13,000 \times g$ for 30 min at 4 °C. The supernatant was applied to the immunosorbent, which had been equilibrated with PBS supplemented with 0.1% Triton X-100. After 1 h, the immunosorbent was washed with 100 volumes of PBS supplemented with 0.1% Triton X-100 and eluted with 0.1 M glycin, pH 2.6. The eluent was neutralized using 1 M Tris and 10× PBS. The purity of TgSAG1 was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and India ink staining (Schares et al., 2000; Hosseininejad et al., 2009). The amount of recovered TgSAG1 was determined relative to a standard of bovine serum albumin by silver staining (Heukeshoven and Dernick, 1986). Affinity purification of

Table 1
Serological results of ducks and geese experimentally inoculated with *T. gondii*.

Host species	Type of inoculation			Route of inoculation[a]	IFAT				TgSAG1-ELISA		
	Stage	Strain	Clonal lineage		Sero-positive (x/n)	Sero-conversion (weeks p.i.)[b,c]	Maximum titre[c]	Duration of seropositivity (weeks p.i.)[b,c]	Sero-positive (x/n)	Sero-conversion (weeks p.i.)[b,c]	Duration of seropositivity (weeks p.i.)[b,c]
Duck	Tachyzoite	RH	I	i.v.	2/2	2	1600	9–≥11	2/2	2	≥11
		ME49	II	i.v.	2/2	2	3200	9–≥11	2/2	2–5	≥11
		NED	III	i.v.	2/2	2	800	9–≥11	2/2	2	≥11
	Oocyst	DX	II	p.o.	5/5	1	12800	≥11	5/5	1–2	≥11
Goose	Tachyzoite	RH	I	i.v.	1/2	2	200	2	1/2	9	9
		ME49	II	i.v.	0/2	–	–	–	1/2	11	≥11
		NED	III	i.v.	2/2	2	800	2–9	2/2	5, 11	≥11
	Oocyst	DX	II	p.o.	6/6	1	3200	≥11	6/6	1–2	≥11

[a] i.v., intravenously; p.o., per os.
[b] p.i., post-inoculation.
[c] Data refer to seropositive animals only.

TgSAG1 resulted in an antigen preparation that was free from detectable contaminating components.

2.5. Serology

2.5.1. Indirect fluorescent antibody test (IFAT)

Ten μl of a suspension of cell culture-derived *T. gondii* RH strain tachyzoites ($5 \times 10^6 \, ml^{-1}$) in PBS were used to sensitize IFAT slide wells. Slides were air-dried and stored frozen at $-20\,°C$ until used. The slides were fixed with ice-cold acetone for 10 min and then incubated in PBS for 10 min. Duck and goose sera were titrated in PBS in 2-fold steps starting at a dilution of 1:25. The test was performed as described for *N. caninum* (Schares et al., 1998) but with the following modification: anti-duck IgY (whole molecule) produced in rabbit and coupled to FITC [Nordic immunology/Tebu-bio, RADu/IgG (H + L) POD, Offenbach, Germany] diluted 1:50 in PBS, 0.2% Evans Blue was used to detect primary antibodies. This conjugate also specifically reacts with goose IgY as indicated by the supplier. The slides were examined using an Axiovert fluorescence microscope (AHBT3, Olympus, Hamburg, Germany). Only complete peripheral fluorescence of the tachyzoite was considered specific. A titre of 100 was used as the positive cut-off titre.

2.5.2. Quantification of TgSAG1-specific antibodies in ELISA

To examine the humoral immune responses against *T. gondii* in ducks and geese, the affinity-purified TgSAG1 was diluted in coating buffer (0.1 M sodium bicarbonate, pH 8.3) and used to sensitize Polysorp ELISA plates (Nunc GmbH & Co. KG, Langenselbold, Germany) at $37\,°C$ for 1 h (150 ng antigen/well). Serum samples were diluted 1:200 in PBS-T/1% casein (PBS, pH 7.2, supplemented with 0.05% Tween 20 and 1% casein from bovine milk; Sigma–Aldrich, Deisenhofen, Germany). Anti-duck IgY (whole molecule) produced in rabbit and coupled to peroxidase [Nordic immunology/Tebu-bio, RADu/IgG (H + L) POD, Offenbach, Germany] diluted 1:2000 in PBS-T/1% casein was used to detect primary antibodies. This conjugate also specifically reacts with goose IgY as indicated by the supplier. After each incubation step, the plates were incubated at $37\,°C$ for 30 min and washed three times with PBS-T (PBS, pH 7.2, supplemented with 0.05% Tween 20). After the final step, the wells were washed three times with PBS and twice with distilled water. They were then incubated with a substrate solution containing 100 μg/ml 3,3′,5,5′-tetramethylbenzidine and 0.004% hydrogen peroxide in 0.2 M sodium acetate and 0.2 M citric acid at $37\,°C$. The reaction was stopped after 15 min by adding 2 N sulphuric acid and the optical density (OD) was measured at 450 nm (reference filter: 620 nm) on a 12 channel ELISA reader SunriseTM (TECAN Deutschland GmbH, Crailsheim, Germany).

All sample and control sera were examined in duplicate. On each ELISA plate, one positive and one negative control serum was tested. To avoid inter-plate variation, index values for each sample were recorded as the arithmetic mean of two index values, SI_1 and SI_2. These index values were calculated using the formula $SI_n = (S_n - N)/(P - N)$, where SI_n is one of two individual index values and S_n is one of the two individual OD values obtained for a sin-

Table 2
Serological results of domestic ducks and geese kept on 71 farms in Lower Saxony, Germany, obtained in the TgSAG1-ELISA.

Host species	Type of animals examined	No. of animals examined (n)	Sero-positive animals (x/%)	No. of farms examined (n)	Seropositive farms (x/%)	No. of submissions examined (n)	Seropositive submissions (x/%)	Prevalence in submission[a] Range (%)	Average (%)
Duck	Fattening ducks	1471	50/3.4	38	11/28.9	41	11/26.8	2.5–30	13.2
	Breeding ducks	127	26/20.5	4	2/50	4	2/50	2.2–62.5	32.4
	Unknown	936	69/7.4	27	8/29.6	28	8/28.6	2.5–78.6	23.3
	Total	2534	145/5.7	61[b,c]	20/32.8	73	20/27.4	2.2–78.6	18.3
Goose	Fattening geese	277	63/22.7	9	8/88.9	9	8/88.9	2.5–63	25.4
	Breeding geese	40	19/47.5	1	1/100	1	1/100	–	47.5
	Unknown	56	12/21.4	3	2/66.7	3	2/66.7	15.8–52.9	22.9
	Total	373	94/25.2	13[b]	11/84.6	13	11/84.6	2.5–63	29.0

[a] Data refer to seropositive submissions only.
[b] Including three farms that kept ducks and geese.
[c] Including farms for which more than one submission but with animals of different or unknown types had been examined.

gle sample on an ELISA plate. *N* is the arithmetic mean of two OD values obtained for the negative control sera. The negative control sera used in the duck TgSAG1-ELISA had been taken from one of the control group ducks at 11 weeks p.i. In case of the goose TgSAG1-ELISA, a serum from one of the control group geese at 11 weeks p.i. was used as a negative control serum. *P* represents the arithmetic mean of two OD values obtained for the positive control sera. Positive control sera had been taken at 11 weeks p.i. from a duck inoculated with 10^4 RH strain tachyzoites (duck TgSAG1-ELISA) or from a goose inoculated with 10^4 NED strain tachyzoites (goose TgSAG1-ELISA), respectively.

2.6. Field study

Sera from ducks and geese were collected in the course of a monitoring program for avian influenza in the Federal State of Lower Saxony during 2007 and 2008. Lower Saxony was selected because 66% of the duck and 31% of the goose production in Germany are located in this state (https://www.genesis.destatis.de/genesis/online). Sampling was performed non-randomly by the local veterinary authorities. Holdings were preferentially selected if they were believed to be at an increased risk of infection with avian influenza virus (e.g. free-ranging poultry; farms located close to water bodies or to breeding places of wild birds). The samples were stored frozen at −20 °C until submitted to the Friedrich-Loeffler-Institut, Wusterhausen, where they were tested for antibodies to *T. gondii*.

As far as ducks were concerned, 73 submissions (consisting of 2–40 [mean 28.7] samples per submission) from 61 farms were available for analysis by the TgSAG1-ELISA. With regard to geese, 13 submissions (consisting of 10–40 [mean 34.7] samples per farm) from 13 farms were examined. Three of the latter farms were identical with three farms from which duck samples had been submitted.

Within the monitoring program for avian influenza, the veterinarians who took the samples, were asked to complete a standardized questionnaire that included questions on farm size, type of farming (e.g. organic farming, free-ranging poultry and poultry kept exclusively indoors) and management (e.g. more than one poultry species on the premise, more than one age group at the same time, more than one type of specialisation [breeding, fattening] at the same time and replacement regime [all in-all out, continuous]). If the birds had access to areas outside stables, we also asked for the wild bird species seen in the enclosures and their abundance. We also asked whether pigs were kept on the farm. Although this questionnaire was not specifically designed for *T. gondii* epidemiology the information gained was partially applicable to the present study. However, questions specifically addressing the *T. gondii* infection risk (e.g. questions to gather information on the presence of cats) were missing. Filled-in questionnaires were available for 46 submissions from 42 duck farms and for 10 submissions from 10 goose farms.

2.7. Logistic regression to detect risk factors

An explorative and retrospective data analysis was done to find relationships between the *T. gondii* seropositivity on farms and variables addressed in the questionnaire. Data were first analyzed by univariate and later by multivariate logistic regression using the statistic software S-Plus (Mathsoft Inc., Seattle, USA). Multivariate logistic regression was applied to those input variables which were statistically significant in the univariate analysis. A *P* (Wald's test) < 0.05 was regarded as statistically significant.

Farms were categorized into large (size ≥ median of all farm sizes) and small (size < median of all farm sizes).

3. Results

3.1. TgSAG1-ELISA validation and cut-off determination

A cut-off was established for the TgSAG1-ELISA based on the ELISA indices obtained for 186 sera from 60 IFAT-negative (titre < 100) ducks and 101 sera from 36 IFAT-negative (titre < 100) geese as well as the indices for 72 sera from 11 ducks and 89 sera from 12 geese that were infected experimentally with either tachyzoites or oocysts and had IFAT titres ≥ 100. To ensure optimal specificity, cut-offs were selected for the duck and the goose Tg-SAG1-ELISAs based on the following criterion. The cut-off was equal to the lowest index value observed for sera from IFAT-positive inoculated animals, which – at the same time – exceeded all index values observed for IFAT-negative, non-inoculated animals. By this protocol we selected for both, duck and goose sera, a cut-off of 0.14. At this cut-off all sera from non-inoculated, IFAT-negative animals were judged negative, and sera of inoculated and IFAT-positive ducks and geese were judged positive at a proportion of 95.8% (69/72) or 94.4% (84/89), respectively.

3.2. Time course of antibody responses in experimentally infected animals

3.2.1. Time course of antibody responses after tachyzoite inoculation

All ducks inoculated with tachyzoites seroconverted in the IFAT (Fig. 1A) and had IFAT titres > 200 (range 400–3200) at 2 weeks p.i. Five weeks p.i., the ducks had IFAT titres ranging from 100 to 1600. At the end of the observation period, i.e. 11 weeks p.i., titres had decreased to the range of 25–100 (Table 1).

In the TgSAG1-ELISA, all ducks (except one ME49-inoculated bird), had positive reactions at 2 weeks after inoculation (Fig. 1B). On the remaining sampling dates, all ducks were ELISA-positive, although the indices decreased markedly in all animals until the end of the observation period (Table 1).

In contrast to ducks, only one goose, which was inoculated with NED tachyzoites, seroconverted in the IFAT and had IFAT titres ≥ 100 at more than one of 10 sampling dates p.i. (Fig. 2A). Two other geese had IFAT titres ≥ 100 only once, i.e. 2 weeks p.i. (Table 1). As judged by the TgSAG1-ELISA, three geese, of which two were inoculated with NED and one with RH tachyzoites, seroconverted and had posi-
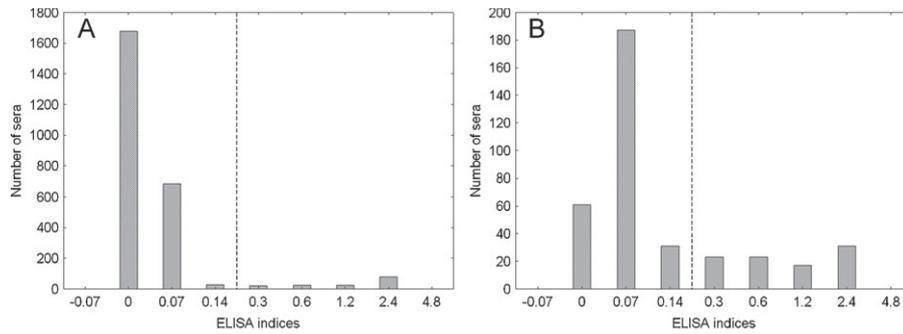
Fig. 1. Comparison of the time course of antibody responses in ducks as determined by IFAT (A and C) and the TgSAG1-ELISA (B and D). Ducks were inoculated experimentally with *T. gondii* tachyzoites (A and B) or oocysts (C and D) (—). Control ducks (...). For reasons of clarity, the data for inoculated animals were plotted left and those for control animals right of the respective time point.

Fig. 2. Comparison of the time course of antibody responses in geese as determined by IFAT (A and C) and the TgSAG1-ELISA (B and D). Geese were inoculated experimentally with *T. gondii* tachyzoites (A and B) or oocysts (C and D) (—). Control geese (...). For reasons of clarity, the data for inoculated animals were plotted left and those for control animals right of the respective time point.

Fig. 3. Reactions of field sera from 2534 ducks (A) and 373 geese (B) in the TgSAG1-ELISA. ELISA cut-off (- - -).

tive ELISA reactions at 2–4 sampling dates p.i. (Fig. 2B). The first positive ELISA reactions were observed 5 weeks p.i. Another goose, which was inoculated with ME49 tachyzoites, reacted positively in the ELISA only on the last sampling date (Table 1).

Control ducks and geese had *T. gondii* IFAT titres < 100 and were ELISA-negative during the entire observation period.

3.2.2. Time course of antibody responses after oocyst inoculation

Four of the five ducks inoculated with oocysts were positive in the TgSAG1-ELISA at 1 week p.i. (Fig. 1D). From 2 weeks p.i. onwards, all five ducks were ELISA-positive. All ELISA-positive ducks had IFAT titres > 200 (Fig. 1C). No marked decrease of the antibody response was observed until the end of the observation period. None of the negative control ducks reacted positive in the TgSAG1-ELISA. All negative control ducks had IFAT titres < 100 (Table 1).

Four of the six geese inoculated with oocysts were positive in the TgSAG1-ELISA at 1 week p.i. (Fig. 2D). From 2 weeks p.i. onwards, all six geese were ELISA-positive. All ELISA-positive geese had IFAT titres > 200 (Fig. 2C). Until the end of the observation period, no marked decrease of the antibody response was observed (Table 1). None of the four control geese reacted positively. All control geese had IFAT titres < 25.

3.3. Reactions in ducks and geese inoculated with Hammondia hammondi oocysts

Thirty-six sera obtained from ducks ($n = 3$) or geese ($n = 3$) inoculated with 5×10^5 oocysts of *H. hammondi* did not produce positive results in the TgSAG1-ELISA, except for one goose serum obtained 1 week p.i. By contrast, one duck and two geese inoculated with *H. hammondi* occasionally showed *T. gondii*-IFAT titres of 100 (one duck: 1 week p.i.; one goose 1 and 2 weeks p.i.; another goose 5 weeks p.i.).

3.4. Field study

3.4.1. Seroprevalence in ducks

A total of 73 sample sets were examined comprising sera of 2534 ducks from 61 different farms. In the TgSAG1-ELISA, 145 (5.7%) of the duck samples from 20 farms tested posi-

tive (Fig. 3A). The seroprevalence in the submissions from different positive farms varied between 2.2% and 78.6% (mean prevalence in positive submissions: 18.3%).

Eight duck farms had been sampled in the year 2007 and again in 2008. On one initially positive farm, positive animals were detected again when sampled for the second time. One initially negative farm was tested positive, when sampled a second time. Six negative farms remained negative at further sampling dates.

For 42 duck farms, the purpose for which the sampled animals were used, i.e. fattening or breeding, had been recorded. On 38 farms with fattening ducks, 3.4% (50/1471) of the samples and on four farms with breeding ducks 20.5% (26/127) of the samples reacted positively in the ELISA (Table 2).

3.4.2. Seroprevalence in geese

A total of 373 goose samples from 13 different farms were serologically examined. Ninety-four (25.2%) of the goose samples from 11 farms were positive in the TgSAG1-ELISA (Fig. 3B). The seroprevalence among animals on different positive farms varied between 2.5% and 63% (mean prevalence in submissions from positive farms 29.0%).

For 10 goose farms, the purpose for which the sampled animals were used, i.e. fattening or breeding, was known; 22.7% (63/277) of the fattening geese from nine farms were serologically positive. All breeding geese came from a single farm with a seroprevalence of 47.5% (19/40) (Table 2).

3.5. Risk factors

The following variables showed *P* values (Wald's test) < 0.05 in the univariate logistic regression analysis: (1) geese had a higher risk of being serologically positive (odds ratio 13.62 [95% CI, 2.78–66.76]) than ducks; (2) poultry kept exclusively indoors were protected from exposure to *T. gondii* (odds ratio 0.05 [95% CI, 0.01–0.3]) (Table 3). Multivariate logistic regression using input variables which were statistically significant in the univariate analysis revealed "barn without outside access for sampled animals" as the only statistically significant input variable in this model (adjusted odds ratio 0.09 [95% CI, 0.02–0.57]) (Table 4).

Fourteen submissions for which a filled-in questionnaire was available came from 14 duck farms, where the birds had access to enclosures outside the barn where they

Table 3

Univariate logistic regression analysis of potential associations of farm-related variables addressed in a questionnaire with *T. gondii* seropositivity at the farm level.

Input variables	Odds ratio (95% CI)	*P* (Wald's test)
Species (geese *vs* ducks)	13.62 (2.78–66.76)	0.001[*]
Purpose the sampled animals are used for (fattening *vs* breeding)	0.41 (0.06–2.67)	0.35
No. of animals at the premise >500 (yes *vs* no)	0.65 (0.17–2.41)	0.516
Barn without outside access for sampled animals (yes *vs* no)	0.05 (0.01–0.3)	<0.001[*]
Poultry species per farm (one *vs* several)	1.19 (0.26–5.34)	0.825
Pigs on farm (yes *vs* no)	4.41 (0.77–25.25)	0.095
All in–all out (yes *vs* no)	2.1 (0.68–6.47)	0.196
Continuous replacement (yes *vs* no)	0.24 (0.03–2.13)	0.199
More than one age group, specialisation or species per enclosure (yes *vs* no)	1.44 (0.38–5.55)	0.592
More than one age group per enclosure (yes *vs* no)	0.5 (0.09–2.76)	0.426
More than one specialisation per enclosure (yes *vs* no)	0 (0–Inf)	0.992
More than one species per enclosure (yes *vs* no)	0.64 (0.11–3.65)	0.611
Only a single species kept in the free-range enclosure (yes *vs* no)	4.5 (0.85–23.8)	0.077
Wild birds seen on free-range enclosure (often *vs* seldom)	2.33 (0.51–10.78)	0.278

[*] Statistically significant ($P < 0.05$).

Table 4

Multivariate logistic regression analysis of potential associations of farm-related variables addressed in a questionnaire with *T. gondii* seropositivity at the farm level.

Input variables	Adjusted odds ratio (95% CI)	*P* (Wald's test)
Species (geese *vs* ducks)	17.6 (1.93–160.29)	0.132
Barn without outside access for sampled animals (yes *vs* no)	0.09 (0.02–0.57)	0.01[*]

[*] Statistically significant ($P < 0.05$).

were kept; eight of these farms had seropositive ducks. Of 18 submissions from 17 farms, where the questionnaire indicated that ducks had no outside access, two farms were seropositive. For the remaining submission from duck farms data on outside access was not available. On all nine goose farms, where this information was available, the birds sampled for this study had outside access.

On two of three farms where both, ducks and geese had been sampled, both the submissions of geese and ducks were seropositive. Among the submissions of the remaining farm only the submission of geese was tested positive.

4. Discussion

In the present study, we used native TgSAG1 that was affinity-purified from tachyzoite lysates as a diagnostic antigen. This molecule was first described by Nagel and Boothroyd (1989) and belongs to the superfamily of glycophosphatidylinositol-linked surface antigens (termed SAG) (Lekutis et al., 2001). A strong antibody response against this antigen could be detected after infection in a number of animal species including mice, dogs, cats, sheep, goats (Bessieres et al., 1992; Kimbita et al., 2001; Sager et al., 2003; Velmurugan et al., 2008; Santos de Azevedo et al., 2010) and humans (Partanen et al., 1984; Bessieres et al., 1992). Thus, TgSAG1 seems to be extremely useful for establishing diagnostic ELISAs for various host species. Most immunodominant B-cell epitopes of TgSAG1 are conformational (Graille et al., 2005). For diagnostic purposes, the native antigen, purified from parasite lysates, may therefore be superior to a recombinant TgSAG1 protein.

Recombinant protein based ELISAs were found to have low diagnostic efficiency for *T. gondii* infection in pigs (Gamble et al., 2000). In consequence, it was not surprising that most, i.e. about 95%, of the IFAT positive sera from inoculated ducks and geese showed also positive reactions in the TgSAG1-ELISA. Inoculation with *H. hammondi*, a parasite species closely related to *T. gondii* elicited only a transient response in the TgSAG1-ELISA in one of three inoculated geese in the first week after inoculation. A similar observation was also made by IFAT, where one duck and two geese had transient low titres. Such minor cross-reactions may be due to the close relationship of *T. gondii* and *H. hammondi*, which is also manifested at the antigen level (Riahi et al., 1998, 2000).

When we inoculated ducks and geese with *T. gondii* oocysts, all animals developed a strong and long lasting (≥ 11 weeks p.i., i.e. the end of the observation period) antibody response. However, inoculation of ducks and geese with tachyzoites led to an unexpected result. While all of the six ducks were susceptible to *T. gondii* infection via this stage, only four of the six geese seroconverted, suggesting that they may be less susceptible to infection with tachyzoites.

In the present study, most field samples were accompanied by data collected as part of the avian influenza monitoring program. Among all variables and potential risk factors for which data had been collected on the farms, only the exclusive maintenance of the birds in barns was significantly associated ($P < 0.001$) with the *T. gondii* serological status of these farms and had a strong protective effect. This finding is not surprising since a similar association has been described in pig production (Kijlstra et al., 2004; Van der Giessen et al., 2007). Nevertheless, the information is important as it demonstrates the effect for the first time for domestic Anseriformes. It also clearly indicates that infections of ducks and geese with *T. gondii* can be prevented by keeping the animals indoors, and thus minimizing exposure to oocysts.

When we analyzed field sera, we found a much higher overall seroprevalence in geese (25.2%) than in ducks (5.7%) and in the univariate logistic regression the species of Anserimores was a statistically significant input variable.

This finding is in contrast to the results of our tachyzoite inoculation experiment, which suggested a higher susceptibility of ducks for an infection at least by this stage of *T. gondii*. However, higher prevalences in geese than in ducks were also reported from the Czech Republic (Literák and Hejlíček, 1993; Bártová et al., 2009).

The reasons for these observations are not fully understood. In the present study, the species of Anseriformes was no longer a statistically significant input variable when examined in multivariate logistic regression. Only the variable "barn without outside access for sampled animals" remained statistically significant in the multivariate model. Therefore our study suggests that free-ranging Anseriformes – independent of their species – are at an increased risk of exposure to *T. gondii*. This may be explained by more frequent access of free-ranging animals to materials contaminated with *T. gondii* oocysts (e.g. soil, fodder and water) or tissue cysts (e.g. carcasses of small rodents or insectivores).

Bártová et al. (2009) suggested that age may also influence the *T. gondii* seroprevalence. Our data on ducks are in accordance with this hypothesis, as fattening ducks had a much lower seroprevalence (3.4%) than breeding ducks (20.5%). Unfortunately, our sera were not accompanied with precise data on the age of the sampled animals. A thorough examination of this putatively important input variable in logistic regression was therefore not possible.

To our knowledge, this is the first study in which the presence of antibodies against *T. gondii* in ducks and geese from Germany was investigated. The results suggest that free-ranging Anseriformes are more often infected than birds kept exclusively indoors. Although a number of studies demonstrated the presence of tissue cysts in tissues of Anseriformes (Zia-Ali et al., 2005, 2007; Dubey et al., 2007) it remains to be elucidated to which extent *T. gondii* seropositive ducks and geese carry infectious tissue cysts and thereby represent an infection risk for consumers.

Conflict of interest statement

Acknowledgements

References

Bártová, E., Dvořáková, H., Bárta, J., Sedlák, K., Literák, I., 2004. Susceptibility of the domestic duck (*Anas platyrhynchos*) to experimental infection with *Toxoplasma gondii* oocysts. Avian Pathol. 33, 153–157.

Bártová, E., Sedlák, K., Literák, I., 2009. Serologic survey for toxoplasmosis in domestic birds from the Czech Republic. Avian Pathol. 38, 317–320.

Bessieres, M.H., Le Breton, S., Seguela, J.P., 1992. Analysis by immunoblotting of *Toxoplasma gondii* exo-antigens and comparison with somatic antigens. Parasitol. Res. 78, 222–228.

Cook, A.J.C., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of toxoplasma infection in pregnant women: European multicentre case–control study. Br. Med. J. 321, 142–147.

Dardé, M.L., 2004. Genetic analysis of the diversity in *Toxoplasma gondii*. Ann. Ist. Super. Sanita 40, 57–63.

Dardé, M.L., 2008. *Toxoplasma gondii* "new" genotypes and virulence. Parasite 15, 366–371.

de Moura, L., Bahia-Oliveira, L.M., Wada, M.Y., Jones, J.L., Tuboi, S.H., Carmo, E.H., Ramalho, W.M., Camargo, N.J., Trevisan, R., Graca, R.M., da Silva, A.J., Moura, I., Dubey, J.P., Garrett, D.O., 2006. Waterborne toxoplasmosis, Brazil, from field to gene. Emerg. Infect. Dis. 12, 326–329.

Dubey, J.P., Sreekumar, C., 2003. Redescription of *Hammondia hammondi* and its differentiation from *Toxoplasma gondii*. Int. J. Parasitol. 33, 1437–1453.

Dubey, J.P., Graham, D.H., Dahl, E., Hilali, M., El Ghaysh, A., Sreekumar, C., Kwok, O.C., Shen, S.K., Lehmann, T., 2003. Isolation and molecular characterization of *Toxoplasma gondii* from chickens and ducks from Egypt. Vet. Parasitol. 114, 89–95.

Dubey, J.P., Webb, D.M., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C., Su, C., 2007. Endemic avian toxoplasmosis on a farm in Illinois: clinical disease, diagnosis, biologic and genetic characteristics of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*), and a goose (*Anser anser*). Vet. Parasitol. 148, 207–212.

Dubey, J.P., 2010. Toxoplasmosis of Animals and Humans. CRC Press, Boca Rotan, FL, 313 pp.

El Massry, A., Mahdy, O.A., El Ghaysh, A., Dubey, J.P., 2000. Prevalence of *Toxoplasma gondii* antibodies in sera of turkeys, chickens, and ducks from Egypt. J. Parasitol. 86, 627–628.

Gamble, H.R., Andrews, C.D., Dubey, J.P., Webert, D.W., Parmley, S.F., 2000. Use of recombinant antigens for detection of *Toxoplasma gondii* infection in swine. J. Parasitol. 86, 459–462.

Graille, M., Stura, E.A., Bossus, M., Muller, B.H., Letourneur, O., Battail-Poirot, N., Sibai, G., Gauthier, M., Rolland, D., Le Du, M.H., Ducancel, F., 2005. Crystal structure of the complex between the monomeric form of *Toxoplasma gondii* surface antigen 1 (SAG1) and a monoclonal antibody that mimics the human immune response. J. Mol. Biol. 354, 447–458.

Heukeshoven, J., Dernick, R., 1986. In: Radola, B.J. (Ed.), Elektrophorese Forum 1986. TU München, pp. 22–27.

Hosseininejad, M., Azizi, H.R., Hosseini, F., Schares, G., 2009. Development of an indirect ELISA test using a purified tachyzoite surface antigen SAG1 for sero-diagnosis of canine *Toxoplasma gondii* infection. Vet. Parasitol. 164, 315–319.

Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. 172, 1561–1566.

Kapperud, G., Jenum, P.A., Straypedersen, B., Melby, K.K., Eskild, A., Eng, J., 1996. Risk factors for *Toxoplasma gondii* infection in pregnancy: results of a prospective case-control study in Norway. Am. J. Epidemiol. 144, 405–412.

Kijlstra, A., Eissen, O.A., Cornelissen, J., Munniksma, K., Eijck, I., Kortbeek, T., 2004. *Toxoplasma gondii* infection in animal-friendly pig production systems. Invest. Ophthalmol. Vis. Sci. 45, 3165–3169.

Kimbita, E.N., Xuan, X., Huang, X., Miyazawa, T., Fukumoto, S., Mishima, M., Suzuki, H., Sugimoto, C., Nagasawa, H., Fujisaki, K., Suzuki, N., Mikami, T., Igarashi, I., 2001. Serodiagnosis of *Toxoplasma gondii* infection in cats by enzyme-linked immunosorbent assay using recombinant SAG1. Vet. Parasitol. 102, 35–44.

Lekutis, C., Ferguson, D.J., Grigg, M.E., Camps, M., Boothroyd, J.C., 2001. Surface antigens of *Toxoplasma gondii*: variations on a theme. Int. J. Parasitol. 31, 1285–1292.

Literák, I., Hejlíček, K., 1993. Incidence of *Toxoplasma gondii* in populations of domestic birds in the Czech republic. Avian Pathol. 22, 275–281.

Lunde, M.N., Jacobs, L., 1983. Antigenic differences between endozoites and cystozoites of *Toxoplasma gondii*. J. Parasitol. 69, 806–808.

Murao, T., Omata, Y., Kano, R., Murata, S., Okada, T., Konnai, S., Asakawa, M., Ohashi, K., Onuma, M., 2008. Serological survey of *Toxoplasma gondii*

in wild waterfowl in Chukotka, Kamchatka, Russia and Hokkaido. Jpn. J. Pharmacol. 94, 830–833.

Nagel, S.D., Boothroyd, J.C., 1989. The major surface antigen, P30, of *Toxoplasma gondii* is anchored by a glycolipid. J. Biol. Chem. 264, 5569–5574.

Partanen, P., Turunen, H.J., Paasivuo, R.T., Leinikki, P.O., 1984. Immunoblot analysis of *Toxoplasma gondii* antigens by human immunoglobulins G, M, and A antibodies at different stages of infection. J. Clin. Microbiol. 20, 133–135.

Riahi, H., Bouteille, B., Darde, M.L., 1998. Antigenic similarity between *Hammondia hammondi* and *Toxoplasma gondii* tachyzoites. J. Parasitol. 84, 651–653.

Riahi, H., Leboutet, M.J., Labrousse, F., Bouteille, B., Darde, M.L., 2000. Monoclonal antibodies to *Hammondia hammondi* allowing immunological differentiation from *Toxoplasma gondii*. J. Parasitol. 86, 1362–1366.

Sabin, A., 1941. Toxoplasmic encephalitis in children. J. Am. Med. Assoc. 116, 801–814.

Sager, H., Gloor, M., Tenter, A., Maley, S., Hässig, M., Gottstein, B., 2003. Immunodiagnosis of primary *Toxoplasma gondii* infection in sheep by the use of a P30 IgG avidity ELISA. Parasitol. Res. 91, 171–174.

Santos de Azevedo, S., Pena, H.F., Alves, C.J., Guimarães Filho, A.A., Oliveira, R.M., Maksimov, P., Schares, G., Gennari, S.M., 2010. Prevalence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies in swine from Northeastern Brazil. Rev. Bras. Parasitol. Vet. 19, 80–84.

Schares, G., Peters, M., Wurm, R., Bärwald, A., Conraths, F.J., 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet. Parasitol. 80, 87–98.

Schares, G., Rauser, M., Söndgen, P., Rehberg, P., Bärwald, A., Dubey, J.P., Edelhofer, R., Conraths, F.J., 2000. Use of purified tachyzoite surface antigen p38 in an ELISA to diagnose bovine neosporosis. Int. J. Parasitol. 30, 1123–1130.

Schares, G., Vrhovec, M.G., Pantchev, N., Herrmann, D.C., Conraths, F.J., 2008. Occurrence of *Toxoplasma gondii* and *Hammondia hammondi* oocysts in the faeces of cats from Germany and other European countries. Vet. Parasitol. 152, 34–45.

Sibley, L.D., Khan, A., Ajioka, J.W., Rosenthal, B.M., 2009. Genetic diversity of *Toxoplasma gondii* in animals and humans. Trans. R. Soc. B 364, 2749–2761.

Sroka, J., 2001. Seroepidemiology of toxoplasmosis in the Lublin region. Ann. Agric. Environ. Med. 8, 25–31.

Sroka, J., Wojcik-Fatla, A., Szymanska, J., Dutkiewicz, J., Zajac, V., Zwolinski, J., 2010. The occurrence of *Toxoplasma gondii* infection in people and animals from rural environment of Lublin region—estimate of potential role of water as a source of infection. Ann. Agric. Environ. Med. 17, 125–132.

Störmann, F., 1962. Zum Problem der Übertragung von *Toxoplasma gondii* auf den Menschen durch Schlachtfleisch. Dissertation, Rheinische Friedrich-Wilhelms-Universität, Bonn.

Van der Giessen, J., Fonville, M., Bouwknegt, M., Langelaar, M., Vollema, A., 2007. Seroprevalence of *Trichinella spiralis* and *Toxoplasma gondii* in pigs from different housing systems in The Netherlands. Vet. Parasitol. 148, 371–374.

Velmurugan, G.V., Tewari, A.K., Rao, J.R., Baidya, S., Kumar, M.U., Mishra, A.K., 2008. High-level expression of SAG1 and GRA7 gene of *Toxoplasma gondii* (Izatnagar isolate) and their application in serodiagnosis of goat toxoplasmosis. Vet. Parasitol. 154 (3–4), 185–192.

Yan, C., Yue, C.L., Yuan, Z.G., He, Y., Yin, C.C., Lin, R.Q., Dubey, J.P., Zhu, X.Q., 2009. *Toxoplasma gondii* infection in domestic ducks, free-range and caged chickens in southern China. Vet. Parasitol. 165, 337–340.

Zia-Ali, N., Keshavarz-Valian, H., Rezaian, M., Khorramizadeh, M.R., Kazemi, B., Fazaeli, A., Darde, M., 2005. Molecular characterization of *Toxoplasma gondii* from bird hosts. Iranian J. Public Health 34, 27–30.

Zia-Ali, N., Fazaeli, A., Khoramizadeh, M., Ajzenberg, D., Darde, M., Keshavarz-Valian, H., 2007. Isolation and molecular characterization of *Toxoplasma gondii* strains from different hosts in Iran. Parasitol. Res. 101, 111–115.