First Report of *Toxoplasma gondii* in the Woodcock (*Scolopax rusticola*): Preliminary Results

Moustakidis Konstantinos[1], Economou Vangelis[2], Dovas Chrysostomos[3], Symeonidou Isaia[4], Papadopoulos Elias[5], Papazahariadou Margarita[6].

[1] Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece, email: moustakidisdogs@gmail.com.
[2] Laboratory of Hygiene of Food of Animal Origin, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece, email: boikonom@vet.auth.gr.
[3] Diagnostic Laboratory, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, email: dovas@vet.auth.gr
[4] Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece, email: isaia@vet.auth.gr.
[5] Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece, email: eliaspap@vet.auth.gr.
[6] Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece, email: ritap@vet.auth.gr.

Abstract. Toxoplasmosis is one of the most common zoonoses worldwide. It is a systemic infection caused by the protozoan parasite *Toxoplasma gondii*. Felids are the only definitive hosts of *T. gondii* with mammals, humans, poultry, and wild birds serving as intermediate hosts. In this study, the presence of *T. gondii* in woodcocks was investigated. Eighty-six woodcocks were collected from the area of Macedonia and Mesolonghi and examined by PCR for *T. gondii*. Four samples were tested positive and the prevalence rate was 4.76%. Therefore, woodcocks carrying *T. gondii* tissue cysts can contaminate animals and humans feeding on them. This is the first report of the detection of *T. gondii* in woodcocks. Further study is needed to investigate the isolation and genetic characterization of *T. gondii* in woodcocks in Greece in order to elucidate the role of this bird in the transmission of *T. gondii* and to safeguard public health.

Keywords: *Toxoplasma gondii*, protozoan, woodcock, *Scolopax rusticola*, game meat, polymerase chain reaction.

1 Introduction

Toxoplasmosis is one of the most common zoonosis worldwide caused by the protozoan parasite *Toxoplasma gondii*, which occurs in domestic and wild mammals, humans, poultry, and wild birds also (Cabezón et al., 2011; Salant et al., 2013). This protozoan has heteroxenous life cycle with the sexual development occurring only in the intestine of felines, (definitive hosts) and asexual replication occurring extraintestinally into the tissues (tissue cysts) in homeothermic vertebrate hosts,

Proceedings of the 8th International Conference on Information and Communication Technologies in Agriculture, Food and Environment (HAICTA 2017), Chania, Greece, 21-24 September, 2017.

(intermediate hosts) (Gennari et al., 2013; Halova et al., 2013; Sandström et al., 2013). Cats or other members of the family Felidae may become infected with *T. gondii* via predation on infected birds and rodents when feeding on food scraps containing meat of livestock or by ingesting sporulated oocysts of the parasite from the environment (Darwich et al., 2012; Molina-López et al., 2012).

Toxoplasmosis is of veterinary and medical importance, because it may cause abortion or congenital disease and even death in its intermediate hosts (Darwich et al., 2012; Huang et al., 2012). Concerning public health, the parasite causes an asymptomatic infection in most healthy people; however, the infection can be fatal for a fetus during pregnancy or for immuno-compromised individuals. In humans, toxoplasmosis is a benign illness associated with mild clinical symptoms. However, congenitally infected children can exhibit blindness and mental retardation. The current global estimated incidence of congenital toxoplasmosis is 190,100 cases a year in humans (Lopes et al., 2011; Huang et al., 2012; Matsuo et al., 2014). In immuno-compromised individuals *T. gondii* infection is ranked as the leading cause of death (Huang et al., 2012; Tian et al., 2012). Humans acquire *T. gondii* through the consumption of undercooked meat containing tissue cysts or through the ingestion of sporulated oocysts in soil and water, or on vegetables (Cabezón et al. 2011; Yu, L et al., 2013). Still, oocysts have been found both in water and in soil samples around human dwellings contaminating among others marine mammals and filter feeding fish and bivalves (Sandström et al., 2013). A European multicenter case–control study found that between 30% and 63% of *T. gondii* infections in humans could be attributed to meat consumption (including cured meat) (Halova et al., 2013).

T. gondii infections are prevalent in many avian species, and can cause mortality in some of them, including poultry, game and other species in the wild (Cabezón et al., 2011). There are a lot of wild birds found infected with this parasite worldwide such as, Tawny owls, Galapagos penguins, Flightless Cormorants, Ostrich ,griffon vulture, Spanish Imperial eagle common buzzard, Egyptian vulture, cinereous vulture, black kite ,bearded vulture, common kestrel ,short-toed snake-eagle, Bonelli's eagle and many others (Hove et al., 2005; Deem et al., 2010; Alvarado-Esquivel et al., 2011; Cabezón et al., 2011; Gondim et al., 2011; Darwich et al., 2012; Gennari et al., 2014). The importance of wild birds as intermediate hosts of *T. gondii* lies on the predation of them by felines, the consumption of birds by humans and the dissemination of the parasite to distant places through migration. In addition, ground-feeding birds are considered sentinels for soil contamination with *T. gondii* oocysts (Cabezón et al., 2011; Gennari et al., 2014). Among the ground-feeding birds, the Eurasian woodcock (*Scolopax rusticola*) is a migratory bird that is of importance since it is a highly-prized prey consumed in large numbers. Woodcocks nest in Russia, Ukraine, Latvia and Finland, and migrate among other countries to Greece from late October to mid-November, where they spend their winter (Legakis, 2008).

The information on toxoplasmosis in woodcocks among other food wild birds is very useful for evaluating the risk it poses to public health. Because there is no data on toxoplasmosis in woodcocks according to literature, the aim of this study was to determine the prevalence of infection of the birds with *T. gondii* using polymerase chain reaction.

2 Materials and Methods

2.1 Sample collection and DNA extraction

Eighty-six hunted woodcocks were collected from local hunters from the prefecture of Macedonia (n=40) and the area of Mesolonghi (n=46), Greece. Samples were collected from October 2014 to February 2015. The heads of the hunted birds were transported to the Laboratory of Parasitology and kept at -30^{o}C until examination. For DNA analysis, the brain was aseptically removed and DNA extraction was performed according to the phenol – ethanol protocol of Psifidi et al. (2010). In brief, 1 ml of lysis reagent SLB [10 mM Tris–HCl (PH=7.5), 1 mM EDTA, 50 mM NaCl, 0.2% SDS] and 1 mg of proteinase K were used to digest brain tissue. The lysate was extracted twice with 1 ml of phenol:chloroform (1:1). The aqueous phase was transferred and the DNA was precipitated at -20°C for 3 hours after the addition of 2.5 volumes of ethanol and 0.1 volume of sodium acetate 3 M (pH=5.2). The DNA was recovered after centrifugation at 12,000 g for 20 min, the supernatant was discarded and the DNA pellet was washed with 70% ethanol. After a final centrifugation, the DNA pellet was dried and finally re-suspended in 100 μl elution buffer (10 mM Tris–HCl, pH=8.0).

2.2 PCR analysis

Two primers based on Reischl et al. (2003), were modified (Table 1) so as to increase amplification efficiency and sensitivity of detection. The target was a 529 bp repetitive fragment (AF487550) of *T. gondii,* identified in the *Toxoplasma* genome by Homan et al. (2000) in over 300 copies. PCR assays targeting the 529 bp repeat are 10–100 fold more sensitive than the B1 marker (Su et al., 2010). Because of this high sensitivity, the 529 bp fragment is a preferred marker for the detection of *T. gondii* in human and animal tissues (Su and Dubey, 2009). The primer sequences were evaluated in silico using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The melting temperature (T_m) of the primers was calculated using the "OligoAnalyzer 3.1" software (http://eu.idtdna.com/calc/analyzer) developed by IDT (Integrated DNA Technologies, Coralville, IA).

Table 1. Description of primers used for PCR detection.

Primer	Sequence (5'-3')	T_m (^{o}C)
Tox-9upAu	TCTTGGAGGAGAGATATCAGGACTGTAG	65.7
Tox-11doAu	AGCGTCGTCTCGTCTAGATCGCA	68.3

The PCR (25 μl) was comprised of 1x F-517 Optimized DyNAzymeTM EXT Buffer Detergent-free [Composition: 50 mM Tris–HCl, 1.5 mM MgCl2, 15 mM (NH4)2SO4; Thermo Fischer Scientific, Vantaa, Finland], 0.2 mM of each dNTP, 1.5 mM MgSO4 (New England Biolabs, Ipswich, MA), 3 U of HotStartTaq DNA

polymerase (Qiagen, Hilden, Germany), 0.2 μM of each primer and 2 μl of DNA extract. Amplification was carried out in an automatic DNA thermal cycler (Perkin-Elmer, California). The initial denaturation (15 min at 94°C) was followed by 45 cycles of amplification (denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 10 sec), ending with a final extension at 72°C for 3 min. Positive control samples to *T. gondii* generously provided by Dr J.P. Dubey (ARS, USDA, Beltsville, USA) were included in all PCR analyses. PCR products were examined by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light (Fig. 1).

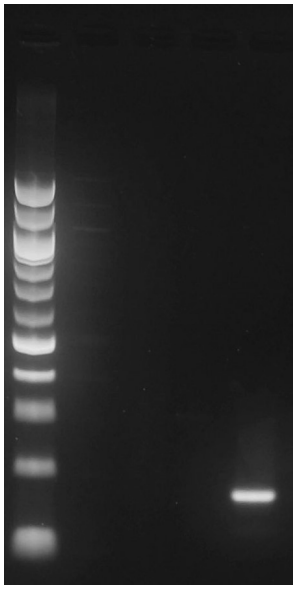

Fig. 1. PCR detection of *T. gondii*. Lane 1, 100-bp DNA ladder; lane 4, positive sample (product size=170 bp).

3 Results and Discussion

In this survey, of the 86 examined birds, 4 of them were tested positive by PCR for *T. gondii* (prevalence=4.76%). This is the first report of the occurrence of *T. gondii* among the woodcock population. Three of the positive samples were collected from Central Macedonia (7.5%) whereas the one positive sample from South Western Greece (2.2%). Since no data are available in the literature the present results are discussed relating to findings from other wild birds with similar habits. The prevalence rate observed in our study is lower than the one reported by Mancianti et al. (2013) (2.91%) who have investigated the occurrence of the parasite in wild waterfowls from Italy. It should be noted that although 9 of the 103 birds sampled were seropositive, only 3 out of 9 of the positive birds were tested positive by PCR. Even lower prevalence was found by Huang et al. (2012) who among 178 wild birds

(pheasants and sparrows), only 4 (2.25%) were tested positive. Alvarado-Esquivel et al (2011) also reported that in Mexico, 17 (2.6%) of the 653 pigeons were seropositive, although viable *T. gondii* was isolated from only 1 of the 7 seropositive pigeons, interestingly belonging to an atypical genotype. On the contrary, Zhang et al. (2015) noticed that among 249 waterfowls from China, 7.2% were tested positive, a rate that is higher than the one observed in the present study. Also, according to Darwich et al. (2012), among wild birds from Spain, 6% was positive for *T. gondii*.

Information on the prevalence of contamination of wild birds is useful for assessing the threat to public health and the oocyst environmental contamination (Lopes et al., 2011). The results of several investigations show that *T. gondii* infection is widespread among wild birds, with large variation among different species, orders, geographical regions and feeding behaviour (Salant et al., 2009; Tian et al., 2012; Gennari et al., 2014). The warm and humid climate of Central Macedonia and Mesolonghi, Greece, favours the survival of *T. gondii* oocysts and the transmission of the parasite. The main risk factors associated with wild birds carrying *T. gondii* are age and feeding behaviour, with higher rates of contamination reported in older animals and in species with a meat-based diet (Cabezón et al., 2011; Lopes, et al., 2011). Specifically, birds dwelling in the forest floor, such as woodcocks, are more prone to *T. gondii* contamination (Gennari et al., 2014). Woodcocks are regarded omnivorous birds, feeding mainly on earthworms, adult insects and their larvae (e.g. beetles, scissors and centipedes), spiders, slugs, slips, and plant material such as grains, fruits, cereals (e.g., oats and corn), grasses and leaves (del Hoyo et al., 1996). Contamination of woodcocks by feeding on insects cannot be ruled out since it has been reported that *T. gondii* oocysts can survive up to 10 days in cockroaches, whereas flies have been identified as vectors of the parasite (Graczyk et al., 2005). Particularly during migration, woodcocks also feed on small freshwater bivalves, molluscs and crustaceans (Johnsgard, 1981). Since molluscs may act as vectors for the transmission of *T. gondii* to humans (Robertson, 2007), they can possibly contaminate also the feeding woodcocks.

The role of woodcocks as intermediate hosts is rather interesting since woodcocks are migratory birds, travelling to Greece from regions of Russia, Latvia and Finland and passing through Ukraine. It is evident that the carriage of the parasite by woodcocks and generally migratory birds can disseminate different types of the parasite to quite distant areas (Gennari et al., 2014). Also, woodcock is a highly-prized catch among hunters, because of its savoury meat and the remarkable ability to evade catch. Annually, 3-4 million woodcocks are reported to be hunted in Europe. (Ferrand & Gossmann, 2001). Undercooked or cured wild game meat can be a potential source of infection for humans and other animals. Consumers of woodcock meat should be aware of the possibility of *T. gondii* infection and should be advised to handle meat properly (Karatepe et al., 2011; Lopes et al., 2011; Halova et al., 2013; Matsuo et al., 2014). Further study is needed to investigate the isolation and genetic characterization of *T. gondii* in woodcocks in Greece in order to elucidate the role of this bird in the transmission of *T. gondii* and to safeguard public health.

4 Conclusions

This study presents the first report of the occurrence of *T. gondii* among the woodcock population. Woodcocks carrying *T. gondii* tissue cysts can contaminate animals and humans feeding on them. More precisely the consumption of undercooked woodcock meat can be a potential source of infection for humans. Still, further investigation is needed so as to elucidate the role of this migratory bird in the epizootiology of toxoplasmosis.

Acknowledgements. The authors would like to thank Theologos Papadopoulos for his expert technical assistance.

References

1. Alvarado-Esquivel, C., Rajendran, C., Ferreira, L.R., Kwok, O.C., Choudhary, S., Alvarado-Esquivel, D., RodríguezPeña, S., Villena, I., Dubey, J.P. (2011). Prevalence of *Toxoplasma gondii* infection in wild birds in Durango, Mexico. Journal of Parasitology, 97(5), pp. 809-12.
2. Cabezón, O., García-Bocanegra, I., Molina-López, R., Marco, I., Blanco, J.M., Höfle, U., Margalida, A., Abach-Raich, E., Darwich, L., Echeverría, I., Obón, E., Hernández, M., Lavín, S., Dubey, J.P., Almería, S. (2011). Seropositivity and risk factors associated with *Toxoplasma gondii infection* in wild birds from Spain. PLoS One, 6(12), e29549.
3. Casartelli-Alves, L., Boechat, V.C., Macedo-Couto, R., Ferreira, L.C., Nicolau, J.L., Neves, L.B. Millar, P.R., Vicente, R.T., Oliveira, R.V.C., Muniz, A.G., Bonna, I.C.F., Amendoeir, M.R.R. Silva, R.C., Langoni, H., Schubach, T.M.P., Menezes, R.C. (2014). Sensitivity and specificity of serological tests, histopathology and immunohistochemistry for detection of *Toxoplasma gondii* infection in domestic chickens. Veterinary Parasitology, 204(3-4), pp. 346-51.
4. Darwich, L., Cabezón, O., Echeverria, I., Pabón, M., Marco, I., Molina-López, R., Alarcia-Alejos, O., López-Gatius, F., Lavín, S., Almería, S. (2012). Presence of *Toxoplasma gondii* and *Neospora caninum* DNA in the brain of wild birds.Veterinary Parasitology, 183(3-4), pp. 377-81.
5. Deem, S.L., Merkel, J., Ballweber, L., Vargas, F.H., Cruz, M.B., Parker, P.G. (2010). Exposure to *Toxoplasma gondii* in Galapagos Penguins (*Spheniscus mendiculus*) and Flightless Cormorants (*Phalacrocorax harrisi*) in the Galapagos Islands, Ecuador. Journal of Wildlife Diseases, 46(3), pp. 1005-1011.
6. Del Hoyo, J., Elliott, A., Sargatal, J. (1996). Handbook of the Birds of the World, vol. 3: Hoatzin to Auks. Barcelona: Lynx Edicions.
7. Gennari, S.M., Ogrzewalska, M., Soares, H.S., Saraiva, D.G., Pinter, A., Labruna, M.B., Dubey, J.P. (2013). Occurrence of *Toxoplasma gondii* antibodies in birds from the Atlantic Forest, state of São Paulo, Brazil. Veterinary Parasitology, 200

(1-2), pp. 193-7.

8. Gondim, L.S., Abe-Sandes, K., Uzêda, R.S., Silva, M.S., Santos, S.L., Mota, R.A., Vilela, S.M., Gondim, L.F. (2010). *Toxoplasma gondii* and *Neospora caninum* in sparrows (*Passer domesticus*) in the Northeast of Brazil. Veterinary Parasitology, 168 (1-2), pp. 121-4.
9. Graczyk, T.K., Knight, R. Tamang, L. (2005). Mechanical transmission of human protozoan parasites by insects. Clinical Microbiology Reviews, 18(1), pp. 128-132.
10. Halova, D., Mulcahy, G., Rafter, P., Turc, L., Grant, T., de Waal, T. (2013). *Toxoplasma gondii* in Ireland: Seroprevalence and novel molecular detection method in sheep, pigs, deer and chickens. Zoonoses Public Health, 60(2), pp. 168-73.
11. Homan, W.L., Vercammen, M., De Braekeleer, J., Verschueren, H. (2000). Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. International Journal of Parasitology, 30(1), 69-75.
12. Hove, T., Mukaratirwa, S. (2005). Seroprevalence of *Toxoplasma gondii* in farm-reared ostriches and wild game species from Zimbabwe. Acta Tropica, 94(1), p.49-53.
13. Huang, S.Y., Cong, W., Zhou, P., Zhou, D.H., Wu, S.M., Xu, M.J., Zou, F.C., Song, H.Q., Zhu, X.Q. (2012). First report of genotyping of *Toxoplasma gondii* isolates from wild birds in China. Journal of Parasitology, 98(3), pp. 681-2.
14. Karatepe, M., Kılıç, S., Karatepe, B., Babür, C. (2011). Prevalence of *Toxoplasma gondii* antibodies in domestic (*Columba livia domestica*) and wild (*Columba livia livia*) pigeons in Niğde region,Turkey. Türkiye Parazitoloji Dergisi 35(1), pp.23-6.
15. Legakis, A. (2008). Study on the migratory birds in Greece and South Ukraine. Final report (in Greek). http://users.uoa.gr/~alegakis/index_el_files/PDFfiles/Teliki Anafora.pdf (accessed 15 May 2017).
16. Lopes, A.P., Sargo, R., Rodrigues, M., Cardoso, L. (2011). High seroprevalence of antibodies to *Toxoplasma gondii* in wild animals from Portugal. Parasitology Research, 108(5), pp. 1163-9.
17. Mancianti, F., Nardoni, S., Mugnaini, L., Poli, A. (2013). *Toxoplasma gondii* in waterfowl: The first detection of this parasite in *Anas crecca* and *Anas clypeata* from Italy. Journal of Parasitology, 99(3), pp. 561-563.
18. Matsuo, K., Kamai, R., Uetsu, H., Goto, H., Takashima, Y., Nagamune, K. (2014). Seroprevalence of *Toxoplasma gondii* infection in cattle, horses, pigs and chickens in Japan. Parasitology International, 63(4), pp. 638-9.
19. Molina-López, R., Cabezón, O., Pabón, M., Darwich, L., Obón, E., Lopez-Gatius, F., Dubey, JP., Almería, S. (2012). High seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in the common raven (*Corvus corax*) in the Northeast of Spain. Research in Veterinary Science, 93(1), pp. 300-2.

20. Psifidi, A, Dovas, C, and Banos, G. (2010). A comparison of six methods for genomic DNA extraction suitable for PCR-based genotyping applications using ovine milk samples. Molecular and Cellular Probes, 24 (2), pp. 93-98.
21. Quist, C.F., Dubey, J.P., Luttrell, M.P., Davidson, W.R. (1995). Toxoplasmosis in wild turkeys: a case report and serologic survey. Journal of Wildlife Diseases. 31(2), pp. 255-258.
22. Reischl, U., Bretagne, S., Krüger, D., Ernault, P., Costa, J.-M. (2003). Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. BMC Infectious Diseases, 3, pp. 7.
23. Robertson, L.J. (2007). The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: A review. International Journal of Food Microbiology, 120(3), pp. 201-216.
24. Salant, H., Landau, D.Y., Baneth, G. (2009). A cross-sectional survey of *Toxoplasma gondii* antibodies in Israeli pigeons. Veterinary Parasitology, 165 (1-2), pp. 145-9.
25. Sandström, C.A., Buma, A.G., Hoye, B.J., Prop, J., van der Jeugd, H., Voslamber, B., Madsen, J., Loonen, M.J. (2013). Latitudinal variability in the seroprevalence of antibodies against *Toxoplasma gondii* in non-migrant and Arctic migratory geese. Veterinary Parasitology, 194(1), pp. 9-15.
26. Sedlak, K., Franti, I.L. (2000). High susceptibility of partridges (*Perdix perdix*) to toxoplasmosis compared with other gallinaceous birds. Avian Pathology, 29(6), pp. 563-9.
27. Su, C., Dubey, J.P. 2009. *Toxoplasma gondii*. In: Liu, D. editor. Molecular Detection of Foodborne Pathogens. Boca Raton, Florida: CRC Press. pp. 741-753.
28. Su, C., Shwab, E.K., Zhou, P., Zhu, X.Q., Dubey, J.P. (2010). Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitology, 137(1), pp. 1-11.
29. Tian, Y.M., Dai, F.Y., Huang, S.Y., Deng, Z.H., Duan, G., Zhou, D.H., Yang, J.F., Weng, Y.B., Zhu, X.Q., Zou, F.C. (2012). First report of *Toxoplasma gondii* seroprevalence in peafowls in Yunnan Province, Southwestern China. Parasites & Vectors, 19(5), p. 205.
30. Yu, L., Shen, J., Su, C., Sundermann, C.A. (2013). Genetic characterization of *Toxoplasma gondii* in wildlife from Alabama, USA. Parasitological Research, 112(3), pp. 1333-6.
31. Zhang, F.-., Wang, H.-., Qin, S.-., Wang, Z.-., Lou, Z.-., Zhu, X.-., Liu, Q. (2015). Molecular detection and genotypic characterization of *Toxoplasma gondii* in wild waterfowls in Jilin Province, Northeastern China. Parasitology International, 64(6), pp. 576-578.