Title: Investigation of systemic isosporosis outbreaks in an aviary of Greenfinch (*Carduelis chloris*) and Goldfinch (*Carduelis carduelis*) and a possible link with local wild Sparrows (*Passer domesticus*)

Matthew Gosbell[a,b]*, Olusola M. Olaogun[a], Kathy Luk[a], Amir H. Noormohammadi[a]

[a]Faculty of Veterinary and Agricultural Sciences, the University of Melbourne, Werribee, Victoria, 3030, Australia.

[b]Melbourne Bird Veterinary Clinic, Scoresby, Victoria 3179, Australia

***Corresponding Author**: Matthew Gosbell

[b]Melbourne Bird Veterinary Clinic, Scoresby, Victoria 3179, Australia

Ph: +61 (3) 9546 5022, Email: birdvetmatt@gmail.com

Keywords: ***Isospora, atoxoplasmosis, finches, coccidiosis, systemic isospora, systemic coccidiosis***

Abstract

An outbreak of systemic isosporosis caused mortalities in greenfinches (*Carduelis chloris*) and goldfinches (*Carduelis carduelis*) kept in an aviary in the western suburbs of Melbourne. The following year, a further outbreak in the same aviary occurred in a different flock of goldfinches. At the time of the second outbreak, dead and sick common sparrows (*Passer domesticus*) discovered near the aviary were also found to have systemic isosporosis. The systemic isosporosis was investigated and described using histopathology, electron microscopy and sequence analysis of the 18s gene. *Isospora* spp. infecting the greenfinch and the goldfinch caused significant thickening of the duodenal lamina propria. Measurements in the goldfinches showed an inverse correlation coefficient between the thickening of the duodenum and the weight of the birds. Electron microscopy confirmed the presence of *Isospora* spp. within lymphocytes migrating into the lamina propria of the duodenum. Analysis of the 18s sequence discovered two different gene sequences across the three species of birds that didn't completely match any sequences previously deposited in GenBank. Although the sparrows were found to have died from causes other than systemic *Isospora*, molecular studies of samples from their liver revealed the presence of an *Isospora* with 18s gene sequence identical to that found in the captive greenfinches.

Introduction

Systemic isosporosis, also called atoxoplasmosis or visceral coccidiosis, is a disease that affects a range of bird species. It is a condition caused by *Isospora* spp that can invade circulating lymphocytes and therefore can affect internal organs and intestines.[1] *Isospora* spp are protozoan parasites in the Apicomplexia phylum along with members of the genera *Eimeria, Cryptosporidium*, *Toxoplasma* and other members of the suborder Eimeriorina. The name Apicomplexa refers to a set of organelles composed from spirally arranged microtubules, polar rings, and secretory bodies, such as rhoptries and mircronemes. Apical complex structures mediate entry of the parasite into the host cells, where they usually survive inside parasitophorous vacuoles.[2] The life cycle includes both a sexual and asexual multiplication in the parasitised host, forming an environmentally resilient oocyst.[2] Transmission of

the parasite occurs by ingestion of sporulated oocysts. *Isospora* spp. that become systemic have a complex life cycle involving the invasion of the reticuloendothelial system and the intestinal epithelium. Merogony occurs in intestinal and lymphoid-macrophage cells. Merozoites in lymphocytes are disseminated within the blood stream to the viscera where merogony continues. This is considered a latent stage of the life cycle exhibited by some species.[3] Gametogenesis occurs in the intestinal epithelial cells.[4-5] Oocytes are then released into the environment where they sporulate and become infectective.[2].

Coccidia species can be differentiated based on the structure of their sporulated oocyst stage. *Isospora* spp are defined by sporulated oocysts that have two sporocyst each with four sporozoites.[6] The size, shape, colour, texture, and type of internal contents of the oocytes are features that can be used to distinguish between species of *Isospora*.[7, 8] Different species of *Isospora* invade different sites and different levels of the bowel, some species invade the mucosal cells of the villi and some the crypts.[2, 9]

Isospora spp is most commonly described in passerine species with approximately 500 species of isospora reported from this order of birds.[10] Generally, *Isospora* spp. are highly host specific, many being significantly pathogenic to their host, sometimes with multiple species infecting a host at any one time. A study of twelve species of *Isospora* that are found in sparrow, are considered highly specific, because transmission of infection could not be repeated in several captive species.[11] Another study of *Isospora michaelbakeri* from russet sparrows *(Passer rutilans)* failed to infect spotted munias *(Lonchura punctulate)*, canaries *(Serinus canaria)*, Java sparrows *(Padda oryzivora)*, chickens *(Gallus domesticus)* or ducks *(Anas platyrhynchos).*[12] Prevalence studies and case studies, indicate that *Isospora* has a worldwide distribution, with a high prevalence (up to 90%) reported in greenfinches in Estonia,[13] blackbirds *(Turdus merula)* in Germany,[14] sparrows in Israel[15] and a range of passerines in New Zealand.[16]

In this communication, we report outbreaks of systemic isosporosis causing death in consecutive years in captive greenfinches and goldfinches and a possible relationship in a local population of sparrows. Histopathology, electron microscopy and molecular studies were used to identify the presence of *Isospora* and describe the infection it caused. A relationship between the degree of histological change found in the intestine and the weight of the goldfinches was also investigated.

Materials and methods

Some birds were euthanised during this study, these birds failed to respond to treatments and were euthanised with consent and according to the procedure outlined in the Melbourne University Animal Ethics approval 1714337.

Case history

A condition where birds had reduced activity and fluffed feathers followed by death (usually within two days), was reported in birds of an aviary housing a mixture of greenfinches (*Carduelis chloris*) and goldfinches (*Carduelis carduelis*) in 2017, and a flock of goldfinch in 2018. Finches were all under one year of age. The aviary was a new purpose-built facility that had concrete flooring, part covered roof and protection from the weather on all sides. The aviary was cleaned weekly and fresh clean food and water supplied daily. During 2017, four out of 20 greenfinches died after a short illness and were subjected to gross and microscopic pathological examinations. Two of the birds (accessions W599-17 and W600-17) were also subjected to molecular examinations using a combination of 18S rRNA polymerase chain reaction (PCR) and sequencing of the resultant amplicons which confirmed the presence of an *Isospora* sp.

After a thorough clean and before restocking of the aviary with 19 captive bred goldfinches in 2018, the aviary was left unoccupied for a period of approximately six months. All finches from both years came from the same breeder. In February of 2018, a goldfinch was found to be sick and died shortly after. Three months later, a second goldfinch was found fluffed but still eating, after being separated

into a hospital cage. On suspicion of *Isospora* causing the sickness, all birds were than treated with toltrazuril at 50mg/l in water medication for five days (Baycox, Bayer Australia Ltd). Faecal swabs were taken from the sick bird and the apparently healthy birds in the aviary before and after treatment and subjected to *Isospora* 18S rRNA PCR. The clinically sick finch was later euthanised due to lack of response to treatment and examined for gross and microscopic lesions. Another finch was found dead and another fluffed up. The dead finch was found to be too autolysed for diagnosis and therefore was not examined further. The sick finch was euthanised and subjected to gross and microscopic examinations.

For welfare reasons, the remaining finches were euthanised, ten of which were subjected to thorough gross and microscopic examinations. At the same time six common sparrows (*Passer domesticus*) were found dead within 100 meters of the aviary where the finches were kept. These birds were also subjected to gross, microscopic examinations and PCR testing.

Microscopic pathology

Small pieces of liver, upper and lower intestine were collected and fixed in 10% buffered formalin imbedded in paraffin, sliced and mounted on slides then stained with Hematoxylin and Eosin. Some samples were further stained with PAS (periodic acid-schiff) or Giemsa for better visualisation of some stages of *Isospora*.

Statistical analysis

Cross sections of the upper intestine of the 10 goldfinches from 2018 (accession W509-18) were examined microscopically and the thickness of the lamina propria was measured in six different axes. The averages of the six measurements were then analysed against the body weights using correlation coefficient function in Microsoft excel 365.

Extraction of genomic DNA extraction

Total genomic DNA was extracted from faecal samples and tissues as described previously.[17] Briefly, swabs from faecal samples and tissues were placed into 500 µl of RLT lysis buffer (Qiagen, Doncaster, Victoria, Australia), containing 1% of β mercaptoethanol and incubated for 4 hours at room temperature or at 4°C overnight. Lysate was centrifuged using multispin MSK-100 columns (Axygen Inc., Hayward, CA, USA) bedded with Qiaex®II suspension matrix beads (Qiagen, Victoria, Australia). The column was washed once with 600 µl buffer RW1 (Qiagen, VIC, Australia), twice with 500µl buffer RPE (Qiagen, VIC, Australia), and eluted in 50µl diethylpyrocarbonate (DEPC)-treated water. Extracted DNA stored at −20°C until required.

PCR amplification of 18S ribosomal gene

A nested polymerase chain reaction (PCR) was used to amplify a 457 bp portion of the 18S ribosomal RNA (18S rRNA) gene common to all species of *Eimeria* and *Isospora* as previously described.[18 4] The PCR products were analysed on a 1.5% agarose gel containing GelRed™ nucleic acid gel stain (Biotium, CA, USA) in 0.5 × TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0) using 50-2,000 bp PCR Marker (Sigma-Aldrich USA) as molecular weight marker. Stained gels were visualized using a UV transilluminator (Bio-Rad Laboratories, USA).

Sequencing of PCR amplicons and sequence analysis

Bands of expected size from the secondary PCR reaction were excised from the gel and purified using FavorPrepTM Gel/PCR Purification Kit (Favorgen Biotech Corp, Taiwan) following the manufacturer's instructions. Purified DNA was quantified using a NanoDrop spectrophotometer ND-1000 v3.7 (Thermo Fisher Scientific). DNA sequencing reaction was performed using BigDye terminator technology at the Micromon DNA Sequencing Facility, Monash University, Clayton, Victoria, Australia. All amplicons were sequenced in both forward and reverse directions with the same primers as were used for the secondary PCR (EIMR and 989).[18 4] Sequence analysis was performed using Geneious v11.1.5 [19] and sequence identity was compared with the GenBank database using the basic local alignment search tool (BLAST).[20]

Transitional Electron Microscopy

Formalin fixed upper intestinal tissues from one of the goldfinches that died in 2018 (accession number W509-18) were subject to examination by electron microscopy.

Results

Grossly thickened upper intestinal walls were detected in the affected finches.

Gross examination of the greenfinches from 2017 (accession W599-17 and W600-17) found significant atrophy of pectoral muscles, distention of the coelomic cavities, and enlargement and multi-focal tan-cream discolouration of livers. Upper intestines appeared distended and, on cross section, the intestinal wall appeared grossly thickened (table 1).

At necropsy the body weights of the ten euthanised goldfinches (W509-18), ranged from 13 to 18 g. Upper intestine appeared moderately dilated and pale. Cross section of the intestinal wall looked grossly thickened in all finches. Also, the liver of some of the finches appeared enlarged. One finch also had an enlarged spleen (table 1).

The six sparrows (W508-18) ranged in body weight from 28 to 31 g. No obvious thickening of the upper intestine was detected but mildly subjectively enlarged livers were detected in most birds. One sparrow also had an enlarged spleen (table 1).

Microscopic evidence of Apicomplexa-like parasites detected in the intestines.

Histopathological examinations of the tissues from all finches found a severe thickening of the upper intestinal wall (Figure 1a), chronic enteritis associated with mononuclear and lymphocytic cells infiltrated into the lamina propria (Figure 1b). Large protozoal organisms were found in the epithelial cells consistent with *Isospora* oocytes (Figure 1c). Smaller protozoal organisms containing meroziotes

were found in the lymphocytes infiltrating the lamina propria of the upper intestine (Figure 1c). There was also an apparent lymphocytosis in the blood vessels associated with increased number of myeloid cells (not shown). Large numbers of lymphocytes were also found in the capillaries of the liver some containing merozoites (Figure 1d). Finches of accessions W599-17 and W600-17 also had evidence of *Macrorabdus ornithogaster* detected in the interventricular (isthmus) region. Liver, kidney and spleen of the affected birds also had lymphoplasmocytic cell infiltration with some cells containing small protozoal organisms.

Tissues from some of the dead sparrows (W508-18) had advanced autolysis making them unsuitable for complete microscopic examination. Apicomplexa parasite oocyts were detected in the epithelium of the lower intestinal samples with the absence of changes in the lamina propria.

The thickness of the lamina propria directly correlated with the weight loss of the goldfinches.

The average thickness of the intestinal lamina propria was measured at six points around the sectioned tissue for each of the 10 goldfinches and average figures for each bird was analysed against body weights. A relatively strong inverse correlation coefficient of 0.63 was detected between the thickness of the intestine and the weight of the birds.

Ultrastructural examination found protozoal organisms with features consistent with isospora spp.

Electron microscopy of the lamina propria of the upper intestine of finches W509-18 found organisms within the cytoplasm of the lymphocytes. The organisms had an apical complex, with conoid, a nucleus, and multiple micronemes (Figures 2 a, b and c). These structural features were consistent with apicomplexia organisms including *Isospora* spp.[2, 21, 22]

A combination of PCR and DNA sequencing revealed that intestinal and systemic isosporosis caused by at least 2 species of Isospora.

Amplicons of 457 bp were generated from 18S ribosomal RNA (18S rRNA) gene using a nested PCR primer pair (EIMR and 989) binding to all species of Eimeria and Isospora. DNA extracted from

intestinal and liver tissues as well as faecal samples from the greenfinches (W599-17 and W600-17), goldfinches (W509-18) and the sparrows (W508-18) were used as template in PCR and generated amplicaons of an expected size. To confirm the identity of the organism, PCR amplicons were subjected to DNA sequencing. Comparison of the sequences with those available in the GenBank database revealed that the products from liver tissues (W599-17, W508-18 and W509-18) had closest identity (99%) to *Atoxoplasma* spp. MS-2003 (Systemic *Isospora* spp.) (GenBank AY331571.1), *Isospora* spp. Strain MEO-xd24 (GenBank KT184357), *Isospora gryphoni* strain UWO_2010 (GenBank KF854254), *Isospora streperae* (GenBank KJ634021), and *Isospora* spp. ex Myodes glareolus isolate 57MG (GenBank MH698576.1). The sequence from the faeces (W600-17, and W509-18) were 99% identical to sequences of *Isopora serinuse* RY-2015a (GenBank KR477877.1). Alignment of the nucleotide sequences revealed that sequences of amplicons from liver samples were almost 100% identical to each other but differed from sequences from the faecal sample by at least 18 nucleotides. Alignment of the nucleotide sequences from the liver samples of W509-18 (greenfinch) and W508-18 (sparrow) had 100% and 99.9% identity to sample W599-17 (goldfinch), respecticvely. These sequences did not have identical matches in Genbank and have been submitted for future reference. The liver samples W508-18 (sparrow) GenBank MT032502, W509-18 (greenfinch) GenBank MT032503 and W599-17 (goldfinch) GenBank MT032505. The faecal sample W600-17 (greenfinch) GenBank MT032504.

Discussion

Through sequence analysis of the 18s rRNA gene, the *Isospora* DNA extracted from the faecal material didn't match that found in the tissue samples, indicating the presences of at least two different species of *Isospora* parasitising the birds in this study. Neither sequences has been previously reported in GenBank. For complete species identification of *Isospora* spp. morphological assessment of sporulated oocysts as well as DNA sequencing is required.[23] Few oocysts were recovered in faecal samples such that a complete morphological analysis was not performed. Poor oocyte recovery is likely the result

of coccidial treatment which controls the intestinal stages of *Isospora* spp.[18] The gross and histological lesions detected in the greenfinches and the goldfinches were comparable, consisting of hepatic and intestinal changes (especially in the lamina propria) leading to gross weight loss. This, in addition to the molecular examination of the parasite, is highly suggestive that the outbreaks of the captive birds were likely to have been caused by the same species of *Isospora* despite the lack of oocyst morphological data. This is supported by other studies on *Isospora* in passerines that have identified *Isospora* spp. cross infecting inter-familial passerine species.[23, 24] The infection found in the sparrows was much more subtle, histological lesions were found in the lower part of the intestines and no thickening of the lamina propria was detected. This may suggest that the sparrows were infected by a different species of *Isospora*, or that they were affected differently by the same species. Previous studies have shown that infection of *Isospora* spp in sparrows is highly specific.[11] it has been largely accepted that *Isospora* spp. are specific to species and genus of passerine such that infection across families has not been shown.[23] Recently this theory has been challenged as *Isospora bioccai* has been identified in *Serinus canaria* which was previously described in *Carduelis chloris*.[25] However, this discovery was based on morphology without any DNA sequencing. In our study the sparrow sequencing of the 18s RNA matched that of the greenfinches and goldfinches but intra-familial parasitism between the greenfinch, goldfinch and the sparrow could not be confirmed due to the lack of morphological identification. Next generation sequencing of whole genomes may be another option to consider for identify *Isospora* spp. in the future. Therefore, although we were concerned that the sparrows may have been the source of the infection in the greenfinches and goldfinches, we were unable to confirm this.

A separate investigation directed towards the cause of death in sparrows in this study found that organophosphate toxicity was most likely involved (results not shown). Isosporosis was not considered the cause of death for these birds. This along with the lack of histological changes detected in the intestines and the liver correlates with findings by Grulet et al suggesting that systemic isosporosis acts as a latent component of the life cycle for some species of isospora.[3] Observations by Gill et al

suggest that free ranging wild birds can coexist with systemic isosporosis only to succumb under stress such as capture.[15] A separate investigation has also reported that young birds and stressed adults were most likely to develop systemic isosporosis.[4] These studies identify stress as a trigger for a latent systemic Isoporosis to develop. The greenfinches and goldfinches in this study originated from the same source but didn't develop disease until some months later. Although we speculated that the source of infection may have been the sparrows, it is also possible that the infection was latent and came from the breeding aviaries. We were unable to investigate the aviaries from which these birds came to confirm this.

Birds with heavy extraintestinal merogonic infection and accompanied intestinal isosporosis have been observed as losing weight in a syndrome termed as "going light.[26] In this study we demonstrated a statistically significant link between the increased thickness of the lamina propria of the upper intestine caused by lymphocytic infiltration and the weight of goldfinches infected with this species of *Isosopora*. The weight of the goldfinches inversely correlated with the thickness of the lamina propria of their upper intestines. A reduction in pectoral muscle mass was observed clinically and at necropsy. The thickened bowel wall resulting from the infiltration of lymphocytes caused by the *Isospora* infection is consistent with findings in American goldfinches (*Spinus tristis*) and house sparrows (*Passer domesticus*) documented by Cushing *et al* (2011) in which lymphocytic infiltration were found to have features consistent with lymphosarcoma.[5]

Studies on the prevalence of *Isospora* from Estonia,[13] Germany[14] Israel[15] and New Zealand[16] have suggested that *isospora* is wide spread in these countries. Findings from our study and the many reports identifying *Isospora* species in Australian birds is consistent with a suspected high prevalence. However, there appears to be few studies specifically documenting the prevalence and the impact of this parasite in captive and wild birds in Australia.

In this study we were able to confirm and characterise lesions caused by outbreaks of a systemic isosporosis in greenfinches and goldfinches. Histopathology, electron microscopy and molecular

analysis was able to detect at least two species of *Isospora*. One species had invaded the laminia propria of the upper intestine and caused systemic disease of the captive birds. As a result of the changes in the upper intestine we were able to show an inverse correlation between the thickness of the lamina propria caused by the *Isospora* and the weight of the goldfinches.

Acknowledgements

Funding to conduct this project was provided by Association of Avian Veterinarians Australasian committee (AAVAC) and the Asia Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, Melbourne University. The authors like to thank Professor Jan Slapeta, Sydney School of Veterinary Science, the University of Sydney, for commenting on the EM images.

Conflict of Interest

The author declares no conflict of interest for the work presented here. This work was support with a grant from the AAVAC.

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Figure 1a. A microscopic image of the cross section of the duodenum of a gold finch (W509-18), showing the thickening of the lamina propria due to infiltrating lymphocytes. The scale on the bottom of the image represents one of the six measurements used to calculate average thickness of the intestinal wall.

Figure 1b. A microscopic image of the duodenum of a gold finch (W509-19) demonstrating a severe infiltration of lympho-monocytic cells into the lamina propria. Large black arrow points to Isospora merozoites in the lymphocytes. The smaller arrow points to Isospora oocyst in the cytoplasm of the epithelial cells.

Figure 1c. A microscopic image of the duodenum. The smaller arrow showing merozoites in the mononuclear cells, the larger arrow indicating a gamont in an epithelial cell.

Figure 1d. A microscopic image of the liver of a gold finch (W509-18), showing infiltration of lymphocytes into the liver. Arrow points to a lymphocyte with cytoplasmic Isospora.

Figure 2a. An electron microscopic image of the isospora organism showing the apical complex including the Conoid (Co), and Micronemes (Mn).

Figure 2b. An electron microscopic image of isospora showing a cross section through the Conoid.

Figure 2c. Section through the Isospora organism showing the nucleus (N) and dense granules (arrow).

Table 1 Summary of the gross pathological changes detected across the three different species of bird

Accession number	**Bird species**	**Gross changes detected**
W599-17 and W600-17	Green finch (2 birds)	Pectoral muscle atrophy
		Coelomic distention
		Enlarged liver with focal discolouration
		Distention and thickening of upper intestinal wall
W509-18	Gold finch (10 birds)	Pectoral muscle atrophy (on some birds)
		Coelomic distention
		Enlarged liver with focal discolouration
		Distention and thickening of upper intestinal wall (all birds)
		Enlarged spleen (1 bird)
W508-18	Sparrows (6 birds)	Enlarged liver (2 birds)
		Enlarged spleen (1 bird)
		No obvious thickening of the intestinal wall