Morin-Adeline V, Vogelnest L, Dhand NK, Shiels M, Angus W, and Šlapeta J. (2011). Afternoon shedding of a new species of *Isospora* (Apicomplexa) in the endangered Regent Honeyeater (*Xanthomyza phrygia*). Parasitology 138(6): 713-724 [http://dx.doi.org/10.1017/S0031182011000126]

Afternoon shedding of a new species of *Isospora* (Apicomplexa) in the endangered Regent Honeyeater (*Xanthomyza phrygia*)

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Running head: Afternoon shedding of a new species of *Isospora*

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SUMMARY

The Regent Honeyeater (*Xanthomyza phyrigia*) is an endangered Australian bird species. Breeding populations have been established at Australian zoos in support of re-introduction programs. This species is the host of a new species of *Isospora* (Apicomplexa). Oocysts are spherical, 25.8 (22.5-28.75) by 23.8 (20-26.25) µm with a colourless to pale yellow smooth wall undergoing rapid exogenous sporulation, 90% sporulated oocysts in 8 hours at 20 °C. Each oocyst contains one polar granule. Sporocysts are ovoid, 18.67 (17-19) by 9.49 (9-10) µm with a flat Stieda body and spherical substieda body devoid of a hyaline body. The asexual stages and sexual phase is within the enterocytes of the duodenum and jejunum. Faeces collected in the morning (AM, n=84) and in the afternoon (PM, n=90) revealed significant diurnal periodicity in oocyst shedding; 21% (18 of 84) of the AM were positive with the mean of 499 oocysts.g^{-1} compared to the PM with 91% (82 of 90) bird faeces positive with the mean of 129,723 oocysts.g^{-1}. Therefore, parasite checks for these birds should be carried out in the afternoon to obtain an accurate result. In conclusion, it is plausible that captive birds with high parasite burdens could be less likely selected by females for reproduction after release due to their duller plumage than their wild counterparts.

Key words: coccidia, *Isospora*, Regent Honeyeater, honeyeater, diurnal shedding, oocysts, recovery program.

INTRODUCTION

Establishing baseline data for potentially threatening infectious agents are necessary for recovery and reintroduction programs (Polley *et al.*, 2010; Thompson *et al.*, 2010). Identification and knowledge of the life history of infectious agents in wildlife is imperative for the implementation of satisfactory recovery programs. One of the most common infectious agents of birds are coccidian parasites (Grulet *et al.*, 1982; Levine, 1988). For example, poultry coccidiosis caused by *Eimeria* spp. is a highly contagious disease that is estimated to cost the broiler industry in excess of $1.5 billion per annum worldwide (Sharman et al., 2010). Intestinal coccidian parasites in the genus *Isospora* are ubiquitous intestinal parasites of birds, however clinical and ecological implications are yet to be fully understood (Levine, 1988). All coccidian parasites undergo asexual and sexual development leading to production of environmentally resistant oocysts (Belli et al., 2006). What distinguishes *Isospora* species in birds from other coccidian parasites is their diurnal periodicity of life cycle and oocyst release. Boughton (1933) published the seminal paper describing release of oocyst in the late afternoon. This was later confirmed for wide range of species in diverse passerine birds (Brawner & Hill, 1999; Brown *et al.*, 2001; Grulet *et al.*, 1982; López *et al.*, 2007; Misof, 2004; Stabler & Kitzmiller, 1972). It has been experimentally documented that it represents an adaptive trait against desiccation and ultraviolet radiation (Martinaud et al., 2009). Little information exists about the pathology caused by *Isospora* species in birds, despite significant impact of parasites on bird's fitness and reproductive success (Grulet *et al.*, 1986b; Hõrak *et al.*, 2004; McGraw *et al.*, 2002; Tung *et al.*, 2007). Avian *Isospora* prevalence surveys that do not take into account the diurnal periodicity of the oocyst shedding will lead to incorrect results (Filipiak et al., 2009).

The Regent Honeyeater, *Xanthomyza phrygia* (Shaw, 1794) (Aves: Passeriformes), is endemic to south-eastern Australia (Franklin et al., 1989)**.** Historically, this bird could be seen overhead in flocks of hundreds ranging from Queensland to South Australia. It is no longer found in much of its former range (Franklin et al., 1989; Thomas, 2009). Its population is fragmented, and

the only remaining breeding habitat is in north-eastern Victoria, Capertee valley and the central coast of New South Wales. The primary threatening process for this species is extensive loss of its box ironbark eucalyptus forest habitat throughout its range. The Regent Honeyeater feeds on nectar and insects within box-ironbark eucalypt forests. They are a highly mobile species, which roams widely in search of unpredictable food sources.

The Regent Honeyeater is classified as Endangered in the IUCN Red List of Threatened Species - Red List Category C2a (ii) & Criteria ver 3.1. (Bird-Life-International, 2008). The population of the Regent Honeyeater is estimated at between 800 and 2000 and is continuing to decline (Garnett & Crowley, 2000; Thomas, 2009). In Australia a National Recovery Program has been established and managed by the NSW National Parks and Wildlife Service and Parks Victoria to protect this endangered native species from possible extinction. In the past decade the Recovery Program has become a large-scale project involving habitat restoration, wild population monitoring and a zoo based breeding program operating at Taronga Zoo since 1995. A number of birds suitable for reintroduction were bred. In May 2008, 27 zoo bred Regent Honeyeaters were released to ironbark woodlands near Chiltern, Victoria. A further 44 zoo bred Regent Honeyeaters were released in the same area in May 2010.

The aim of this study was to undertake a parasitological survey of a cohort of the Regent Honeyeaters at Taronga Zoo, Australia which were part of a breeding and reintroduction program for the species. We describe a new *Isospora* species representing the first coccidian species described from Australian endemic passerine birds. We confirm diurnal periodicity of oocyst shedding in this species. This information is useful in establishing appropriate health screening protocols for this species, particularly pre-release protocols prior to reintroduction to the wild.

MATERIAL AND METHODS

Animals

The Regent Honeyeaters used in this study were housed in four aviaries at Taronga Zoo, Mosman, New South Wales, Australia. Aviaries I-III were pre-release quarantine aviaries housing young birds prior to release. Aviary IV housed juveniles and adult breeding birds that were not part of the release cohort. All birds had been bred either at Taronga Zoo or Adelaide Zoo, South Australia, Australia. There was no difference in temperature, water, food supplements or contact to other endemic birds between the aviaries.

Quarantine aviaries I and II

Aviaries I and II were situated adjacent to each other with a corrugated iron gate between them (Taronga Zoo reference numbers BHH001-4). The perimeter of the aviaries was constructed from squared steel mesh, 3.5-4 ×13×5 m and 3.5-4 × 7×5 m (height× width × depth). Roof-high tree branches were placed in both aviaries as perches. Flooring in both aviaries was concrete and approximately half of the roof was covered for shelter from rain. The birds in these two aviaries were in contact with each other and for the purpose of this study were treated as a single population. Together these aviaries housed 36 birds. These birds varied in age (<1 year to adults) being held in pre-release quarantine prior to their release to the wild.

Quarantine aviary III

This aviary was a single row of four consecutive adjacent aviaries, each with an approximate dimension of 3.5-3.8×1.5×5 m (Taronga Zoo reference numbers BOB011-014). The aviaries were

separated from each other and the external environment by steel mesh. All aviaries were covered by a common roof covering half the aviary space, which sheltered the birds from rain. Roof-high tree branches were used as perches. The floor in the aviary was concrete. In total, eight birds (two in each) were housed in the aviary and were treated as single population. These birds were yearlings recently relocated from Adelaide Zoo also destined for release.

Aviary IV

This aviary consisted of three separate aviaries immediately adjacent to each other and separated by steel mesh (Taronga Zoo reference numbers BHH035-037). The aviaries shared a common roof across the back that covers a portion of the space from rain. The walls of the aviary were steel mesh. The three aviaries were of unequal size and shape, approximately 4×3×5 m. Each aviary contained roof-high perches constructed from tree branches. In total, nine birds (three in each) were housed in the aviary and were treated as single population due to the close contact between the birds. These birds were part of the permanent collection at the Zoo and were not destined for release at this time.

Faecal collection

Faecal samples were collected on two consecutive days in April, 2010 (Sydney GMT+11; daytime: 11h 35 min; sunrise at 6:10 am, sunset 5:45 pm). Sampling was carried out over a three hour period twice a day, with samples designated as 'AM' and 'PM'. The collection involved placing clean plastic white bin-liners in each corner of the aviaries between 8:00 to 11:00 am for AM samples and between 2:30 to 5:30 pm for PM samples. At the end of each three hour sampling interval, individual faeces on the bin-liners were transferred into 2 ml sterile Eppendorf tubes. The sample tubes were labelled according to the day, time and the aviary from which they were

collected. The morning samples were kept at room temperature until the afternoon samples were collected. All samples were then preserved with 500 µl of 2.5% potassium dichromate ($K_2Cr_2O_7$) added to each tube and stored at 4 ºC until parasitological examination. Since the birds were housed in grouped aviaries, individual bird identification was not possible. We have collected faecal samples from Aviary I+II (27 on day 1, 48 on day 2), Aviary III (14 on day 1, 25 on day 2) and Aviary IV (18 on day 1, 42 on day 2).

Parasitological examination

Samples were examined at the University of Sydney, NSW, Australia. Sample vials containing faeces and 500µl of 2.5% $K_2Cr_2O_7$ were centrifuged for 2 minutes at 1000 g. The potassium dichromate was pipetted out and the faecal pellet weighed to the nearest 0.001 g using an electronic balance. The pellet was then gently homogenised with 500 µl saturated salt flotation solution. For each sample, the McMaster chamber was used for counting oocysts. Each preparation was rested for at least a minute before counting to allow oocysts to float to the top. An oocyst average was taken from three grids to obtain oocyst number per sample. Coccidian oocyst counts per total volume representing the oocyst faecal content was converted to oocysts.g^{-1} of faeces (OPG).

Coccidian oocysts were examined and measured with a calibrated ocular micrometer using bright field microscopy using 100x oil objective on an Olympus BX60 microscope equipped for Nomarski interference (DIC) contrast microscopy and photographed using an Olympus DP70 camera. Images were recorded as TIFF and adjusted in Adobe Photoshop CS3.

Statistical analysis

Proportions of faecal samples positive were calculated, overall as well as by time of the day, sampling day, and by the aviary. Unconditional association of these three explanatory variables

with the outcome variable (presence or absence of oocysts in a sample) was evaluated using univariable logistic regression. Stratified analyses were conducted to investigate if the odds ratio between the morning and afternoon samples was confounded/ modified by the day collected or by the aviary. This included calculation of stratified odds ratios for each stratum (each day and each enclosure, respectively), testing them for heterogeneity using the Breslow-Day test, and combining them to calculate adjusted or Mantel-Haenszel odds ratios if there was no evidence of heterogeneity. Significance of adjusted odds ratios were tested by a Cochran-Mantel-Haenszel chi-square test. Finally, a multivariable logistic regression model was fitted to evaluate the combined effect of all three variables by using a backward stepwise approach.

To compare parasite burden OPG between times of the day, sampling dates and the cages, summary statistics were calculated by each of the categorical explanatory variables, box-and-whiskers plots (GraphPad Prism 4 Software, Inc., La Jolla, CA). All negative samples were excluded for this analysis and OPG was log transformed to satisfy the assumption of normality and equal variance. An outlier with 1,535,439 oocysts.g^{-1} count (PM sample) was removed before conducting analyses. Two sample *t*-tests were used to compare the mean log OPG between time of the day and sampling day, and ANOVA was used to compare the means between aviaries. All three variables and their first order interactions were tested in multiple linear regression models to test their association with log OPG by backward stepwise approach and retained if significant ($P<0.05$). The assumptions of linear regression were evaluated using residual diagnosis.

Analyses were conducted using SAS statistical software (release 9.1, 2002-03, SAS Institute Inc., Cary, NC, USA) and UniLogistic macro (Dhand, 2010); all P-values were two sided, and odds ratios are reported with 95% confidence intervals (CI), unless indicated to be otherwise.

Histological examination

Regent Honeyeater material held within the Australian Registry of Wildlife Health (Taronga Conservation Society Australia, Mosman, NSW, Australia) was obtained. In total, tissues from six birds were retrieved (1999-2010) that were catalogued with "coccidiosis". Due to autolysis we excluded ARWH 2340.1. For the remaining five, ARWH 1881.1, ARWH 2204.1, ARWH 7298.1, ARWH 7341.1 and ARWH 7457.1, we retrieved paraffin blocks and cut 2 µm sections and stained with H&E and Giemsa for histopathological examination and identification of coccidian life cycle stages.

Two birds from the cohort examined in this study were found dead after release; ARWH 7598.1, was processed as above, however ARWH 7585.1 was too autolysed to examine coccidian development.

Molecular characterisation

Nucleic acid was extracted from 10^6 oocysts purified from a single faecal sample using the FastDNA Soil Kit Protocol with a Fast Prep-24 Homogenisation System equipped with QuickPrep Adapter (MP Biomedicals, Australia); the speed setting used was 6.0 for 40 s as described previously (King et al., 2010). A nested PCR amplification of a fragment of the subunit I of the cytochrome c oxidase gene (COI) from the parasite mitochondrial genome was applied according to Dolnik et al. (2009). Each reaction of 25µl contained 12.5 µl of 2x SAHARA Mix (BioLine), 0.5 µl of each 10 mM primer, and 100 ng of extracted DNA; deionised sterile water was used as a negative control. A touch down temperature profile was utilised for the first PCR according to Dolnik et al. (2009). PCR was performed in an Eppendorf Mastercycler Personel. Resulting products were resolved in 2% (w/v) agarose gel. PCR product of approximately 250 bp was considered as positive and cloned using the TA-TOPO Cloning Kit (Invitrogen, Australia) according to the manufacturer's instructions. Four randomly selected plasmids with target inserts were sequenced bidirectionally using primers targeting sequences located within the vector by

Macrogen Inc. (Seoul, South Korea). Sequences were assembled, aligned with related sequences and analysed using the CLC Main Workbench 5.5 (CLC bio, Denmark). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

RESULTS

Parasite description and identification

Parasitological examination of the Regent Honeyeaters housed at Taronga Zoo revealed oocyst and parasite development of the genus *Isospora* Schneider, 1875. The sporulated oocyst is the stage that new coccidian species are predominantly defined by, because the oocyst is the most readily available stage in the life cycle. Besides specific guidelines for oocysts circumscription, it was emphasised that endogenous development and ecological parameters should be included whenever possible with the species description (Duszynski & Wilber, 1997). Morphological and ecological investigations showed that this parasite represents a new species, the description of which follows.

Alveolata Cavalier-Smith, 1991

Apicomplexa Levine, 1970

Eimeriidae Minchin, 1903

***Isospora lesouefi* sp. n.**

(Fig. 1, 2, 3, 4)

Oocyst. Oocysts broadly spherical, 25.8 (22.5-28.75) µm by 23.8 (20-26.25) µm; shape index (length/width) 1.07 (1-1.17) (n=50). Oocyst wall smooth, colourless to pale yellow. Oocyst wall bilayered, 1 µm thick (outer layer 0.7 µm, inner layer 0.3 µm). One polar granule 1.83 (1.5-2) µm by 1.67(1-3) µm, grain shaped or rounded. Oocyst residuum absent. Sporocysts ovoid, 18.67 (17-19) µm by 9.49 (9-10) µm, with thin, smooth, well defined unilayered sporocyst wall 0.5 µm thick. Sporocyst shape index 1.97 (1.81-2.11). Stieda body flat, 1.75 (1.5-2) µm by 1 µm. Substieda body spherical, 2.67 (2-3) by 2 µm. Absence of hyaline body protruding from the Stieda into substieda body. Sporocyst residuum present, composed of numerous granules of approx. 0.3 µm each,

condensed into oval cluster 8-5 µm in diameter. Sporozoites elongate, arranged head to tail within sporocyst, in some oocysts overlapping with the substieda body. Each sporocyst contains four sporozoites. Sporozoites with two refractile bodies, one bean-shaped refractile body (3.5 by 2.5 µm) and a smaller more spherical (2 by 2.5 µm) body. Sporozoite nucleus oval situated between refractile bodies. In between sporozoite refractile bodies and nucleus conspicuous transverse ridges. Sporozoites and sporozoite residuum float free within the sporocyst, not enclosed in a membrane. Oocysts were unsporulated when voided. Sporulation exogenous, up to 50% sporulated in 4 hours at 20 °C and up to 90% sporulated in 8 hours at 20 °C.

Nucleotide signature sequence. The haplotype fragment of the subunit I of the cytochrome c oxidase gene (COI) from the mitochondrial DNA of *I. lesouefi* sp. n. was identical across all four clones sequenced and submitted to GenBank™ (HQ221885). When comparing the sequence of *I. lesouefi* sp. n. to available sequences of *Isospora hypoleucae* (from Pied Flycatcher, *Ficedula hypoleuca*; haplotype iFICEHYP1: FJ269363) and *Isospora* spp. (from Blackcap, *Sylvia atricapilla*; haplotypes iSAT1-iSAT6: FJ269357- FJ269362) we found sequence divergences between 2.8 and 4.8%. On a phylogenetic tree (Fig. 1), the *I. lesouefi* sp. n. haplotype clustered outside the Blackcap's iSAT1, iSAT3 and iSAT4 possessing extraintestinal stages (Dolnik et al., 2009).

Endogenous development. The parasite development was detected in the columnar epithelium of the duodenum and jejunum. Parasites were found intracellularly in enterocytes. Asexual development was detected in ARWH 7598.1 (Fig. 3). The asexual stages were detected in low numbers in Lieberkühn's crypts surrounded by minimal host response (Fig. 3 A). We could detect two distinct types of meronts (Fig. 3 B, C) - according to by Grulet and colleagues (1986b).

Sexual development was detected in ARWH 7457.1 (Fig. 4). The sexual stages were associated with loss of epithelial structure due to necrosis in the duodenum (Fig. 4 A, B) and jejunum (Fig. 4 C, D). The sexual stages were localised along the whole microvillus epithelium,

younger forms were at base of the cells (bellow the host cell nucleus), while more mature larges stages were progressively displacing the host nucleus to the side and moving towards the lumen. The enlarged epithelial cells were parasitised by one or more parasitic stages (dominantly by developing marcogametes and early oocysts). Similar endogenous development associated with moderate to marked intestinal coccidiosis of duodenum and jejunum were detected in ARWH 1881.1, ARWH 2204.1, ARWH 7298.1, and ARWH 7341.1.

Diurnal periodicity of *Isospora lesouefi* sp. n. oocyst shedding

The proportions of positive faeces were based on freshly voided faeces collected from 53 Regent Honeyeaters (Table 1, Supplementary Table S2). The proportions of ***I. lesouefi*** sp. n. oocysts positive samples were significantly different between morning (AM, 91% positive) and afternoon (PM, 21% positive) samples (Table 1, Fig. 5 A). The crude odds ratios indicate that PM samples were 37.6 times more likely to be positive compared to AM samples (Table 1). Breslow-Day test for homogeneity of odds ratios between days was non-significant (P =0.48) indicating that it was appropriate to combine stratified odds ratios to calculate an adjusted odds ratio. Mantel-Haenszel odds ratio adjusted for sampling day was 35.9 (95% CI: 14.68, 87.66) and was statistically significant (Cochran-Mantel-Haenszel chi-square test statistic 84.0; P <0.001). The confounding by sampling day was negligible (4.6%). Stratified odds ratios calculated for each aviary were not significantly different (Breslow-Day test for homogeneity of odds ratios P = 0.13). Therefore, after adjusting for aviaries, Mantel-Haenszel odds ratio was 39.2 (95% CI: 13.87, 110.75) and was statistically significant (Cochran-Mantel-Haenszel chi-square test statistic 78.2; P <0.001). This suggests that it was significantly more likely for oocysts to be detected in PM samples than in AM samples even after adjusting for sampling day or aviary.

To control for both the variables and their interactions simultaneously, multivariable logistic regression analyses were conducted. Neither sampling day nor its interaction with sampling time

was significant and therefore both were removed from the model. The final model suggest that after adjusting for the variation due to enclosures, PM samples had 42 times greater odds to be positive compared to AM samples (Supplementary Table S3 A). However, samples from aviaries I/II and III were 5.6 and 3.4 times more likely to be positive compared to from aviary IV (Supplementary Table S3 A). Similar results were obtained when aviaries were controlled as a random effect rather than a fixed effect (odds ratio - PM versus AM = 38.35; 95% CI: 14.68, 100.16).

We analysed data for *I. lesouefi* sp. n. burden in positive samples (Fig. 5 B, Supplementary Table S2). The means we significantly different, 499 (95% CI: 124-523) oocysts.g^{-1} (n=18) and 129,723 (95% CI: 83,846-175,601) oocysts.g^{-1} (n=82) in the AM and PM samples, respectively (Fig. 6). The geometric mean oocyst count in PM samples was 200 times greater than in AM samples (95% CI: 86.26 to 462.48 times). There was no significant difference in the mean oocyst count between sampling days (t_{97}= 0.91; *P* = 0.37) or between aviaries ($F_{2,96}$ = 0.95; *P*= 0.39).

The multiple linear regression analyses conducted to investigate if the parasite burdens in AM and PM samples (=time of day) are influenced by sampling day or by the aviary revealed that the effect of time of sampling did not vary by sampling day or by the aviary (Supplementary Table S3 2 B). However, after adjusting for variation due to time of sampling, there were significant differences in mean oocyst counts between aviaries, with samples from enclosure IV having significantly higher mean log oocyst counts than enclosure I/II (*P* = 0.02) but not enclosure III (*P*= 0.65). There was no significant difference in the mean log counts between aviary I/II and aviary III (*P* = 0.36). After adjusting for this variation in aviaries, the geometric mean oocyst counts in the afternoon samples were 233.4 times greater than in the morning samples (95% CI: 101.28, 537.60). Similar results were obtained using linear mixed model and considering enclosures as random effects. The ratio of geometric mean between afternoon and morning samples was determined to be 199.7 (95% CI: 86.26, 462.48) and aviaries accounted for about 8% of the variance in the model (intra cluster correlation = 8.11%, *P* = 0.069).

DISCUSSION

Coccidia belonging to the genus *Isospora* in birds are a taxonomically difficult group due to (i) ambiguities in the morphology and (ii) unknown host specificity (Grulet et al., 1982; Levine, 1982). The name *Isospora lacazei* (Labbé, 1893) has been used loosely for many years for *Isospora* species of many different birds. Levine (1982) reviewed the historical literature and proposed to restrict the name *I. lacazei* to the species from European goldfinch (*Carduelis carduelis*) in Spain. To stabilise the taxonomy of the genus *Isospora* in birds, Levine (1982) assumed "that a coccidian species may be transmissible from one species to another in the same genus, but not from one genus to another in the same family until otherwise demonstrated". The same conclusion has been adopted by Grulet and colleagues (1982; 1986a) to describe new bird *Isospora* species in House sparrows and to revise existing bird *Isospora* species (Grulet et al., 1982; Grulet et al., 1986a). Our newly described *I. lesouefi* sp. n. is the first *Isospora* species in the host genus *Xanthomyza* that is monotypic within the family Meliphagidae. Molecular phylogeny has demonstrated that the Regent Honeyeater is nested within Wattlebirds of the genus *Anthochaera* (Driskell & Christidis, 2004). No coccidian parasites have previously been named from the genera *Xanthomyza* or *Anthochaera*.

In Australia, the House sparrow (*Passer domesticus*) is an introduced urban bird. They are known to be infected all year round with multiple *Isospora* species. In a study from France, wild House sparrows were infected with up to 12 distinct species based of freshly sporulated oocysts (Grulet et al., 1982; Grulet et al., 1986a). Characters of the Stieda apparatus (Stieda body, substieda body and their inclusions) together with the shape and number of polar granules were used to review and distinguish these species from each other and previously named species (Grulet *et al.*, 1982; Grulet *et al.*, 1986a; Grulet *et al.*, 1986b). Eight of these *Isospora* species were identified in House sparrow specimens from Adelaide, Australia (Grulet et al., 1986b). While size and shape of our species overlaps with majority of *Isospora* species from the House sparrow, the combination of the single grain shaped or rounded polar granule together with the simple symmetric Stieda

apparatus distinguishes our species from all known species in the House sparrow and the majority of described bird *Isospora* species. The shape and size of the oocyst resembles *Isospora passeri* Levine, 1982 that was described from House sparrows in Illinois, US (Levine, 1982; Levine & Mohan, 1960). In our species we neither observed endostideal bodies according to Levine and Mohan (1960) "sometimes [oocyst of *I. passeri*] contains a cylindrical core extending part way down from the Stieda body" nor are sporozoites and sporocyst residuums "enclosed in a membrane, forming more or less of a ball within the sporocyst". These differences distinguish *I. passeri* from our species. Oocysts, Stieda apparatus and shape of polar granule resemble *Isospora petrochelidon* Stabler and Kitzmiller, 1972 from Cliff swallows from US (Stabler & Kitzmiller, 1972). Our species is distinguished by the presence of single polar granule and absence of a sporocyst membrane enclosing the sporozoites and residuum. Compared to the rapid sporulation of *I. lesouefi* sp. n., the average time for completion of *I. petrochelidon* sporulation was 24 hr at 21-28 ˚C (Stabler & Kitzmiller, 1972). Sporulation of *Isospora* spp. in passerine birds take 24 hours to 7 days (e.g., Anwar, 1966; Berto *et al.*, 2009; Perrucci *et al.*, 1998; Rossi *et al.*, 1996; Upton *et al.*, 1995). We are not aware of any other coccidian species with exogenous sporulation that would match sporulation time (8 hours) together with 90% efficiency of sporulation as demonstrated for *I. lesouefi* sp. n.

The diurnal periodicity of the *I. lesouefi* sp. n. oocyst release in the afternoon faeces is homologous to other *Isospora* species in birds (Brawner & Hill, 1999; Brown *et al.*, 2001; Grulet *et al.*, 1982; López *et al.*, 2007; Misof, 2004; Stabler & Kitzmiller, 1972). Our results confirm that shedding of *I. lesouefi* sp. n. was diurnal and that faeces collected in the afternoon reflect the true parasite prevalence. For example, by pooling all morning and afternoon faecal samples we would end up with only 57% (100/174) positive compared to 91% (82/90) positive faeces in the afternoon. Sampling before noon even indicated absence of the parasite (0/5 in Aviary 3 on Day 1) despite 100% (9/9 in Aviary 3 on Day 1) positive faeces in the afternoon, thus suggesting that all birds in this aviary were infected with *I. lesouefi* sp. n. Therefore, parasite surveys that do not take into

account the diurnal periodicity of the oocyst shedding will lead to incorrect results (Filipiak et al., 2009).

There was ~200 times more oocysts *I. lesouefi* sp. n. in the afternoon faeces that contained tens of thousands of oocyst per gram compared to the morning samples with only few hundreds of oocysts per gram of faces. Similar to *Isospora* in Blackcaps (*Sylvia atricapilla*) (Dolnik, 2006), our results show that the production of oocysts is comparable from day to day, but is in contrary to *Isospora* in Blackbirds (*Turdus merula*) whose oocyst output strongly varied between successive days (Filipiak et al., 2009). It implies that a single faecal sample from the Regent Honeyeater collected in the afternoon processed using the McMaster chamber will produces an accurate measure of the parasitic load. This is important when health screening captive and wild birds and should also be taken into consideration when health screening other passerine species. Moreover, investigation whether oocyst shedding is diurnal should be a compulsory part of any new *Isospora* species in passerine birds.

Histological examination of tissues from Regent Honeyeaters revealed endogenous *Isospora* development in the duodenum associated with marked necrosis of the intestinal villi. Whether these histopathological changes are reflected in clinical sings is unlikely because similar extent of *Isospora* development was reported in clinically healthy house sparrows (Grulet et al., 1986b). Some *Isospora* species in birds are known to undergo extraintestinal and possibly devastating disease - atoxoplasmosis, formally thought to be caused by a distinct parasite of the genus *Atoxoplasma* (Barta et al., 2005; Schrenzel et al., 2005). Molecular techniques have now provided direct evidence that these extraintestinal stages belong to the same *Isospora* species in the intestine (Schrenzel et al., 2005). Histopathological investigation has not provided evidence of such extraintestinal *I. lesouefi* sp. n. development in Regent Honeyeaters and the obtained sequence did not cluster with those with extraintestinal stages. Nevertheless, molecular probes based on the sequenced markers of *I. lesouefi* sp. n. will be instrumental in resolving this phenomenon, because histopathological investigation may have missed the presence of these stages.

In the Regent Honeyeater, males are characterised by a black upper body, decorated by bright yellow ornamentation especially around at the tip of the wings and tail and the belly area while females are duller and smaller in size (Higgins *et al.*, 2001; Oliver, 1988). In wild bird populations, the brightness of male birds' plumage reflects a character for mate selection (Hamilton & Zuk, 1982). Females will select a mate according to the extent of development of such characteristics within a population to ensure that they have chosen the best available genotype to reproduce (Hamilton & Zuk, 1982; Zahavi, 1975). Plumage colour can originate either from melanin pigments or carotenoid pigments producing either black or brown colours or hue ranging from red to yellow respectively (McGraw & Hill, 2000; McGraw *et al.*, 2002). In captive male Greenfinches (*Carduelis chloris*) tail feathers of birds infected with *I. lacazei* parasites contained 52% less carotenoids and also had smaller values of chroma and hue compared to tail feathers of Greenfinches medicated with coccidiostats (Hõrak *et al.*, 2004). The colour deposition is compromised during parasitic infection, because a trade-off between the use of carotenoids for ornamental displays and the immune function in response to infection (Baeta *et al.*, 2008; Lozano, 2001). This could induce conflict between the social signal intended by the individual bird and that conferred by its appearance (Hill & Brawner, 1998). Whether *I. lesouefi* n. sp. infected birds are disadvantage over their wild counterparts, who are subjected to a different parasitic burden and carotenoid deposits in their plumage, remains to be investigated.

We do not know yet what the ecological significance of an ongoing *I. lesouefi* n. sp. infection in the wild is. Nevertheless, after release the captive bred Regent Honeyeaters interact with each other and with wild Regent Honeyeaters in exactly the same way that wild Regent Honeyeaters interact and over both releases (2008 and 2010) captive birds demonstrated both courtship and nest building behaviour with wild birds (Ingwersen, personal observations). An investigation towards the reproduction and survival success of released birds in the wild correlated with the parasite burden is a logical step in our investigation and recovery of the Regent Honeyeater population in Australia. This information will not only be critical in the recovery of the Regent Honeyeater, but

also other remnant communities in the threatened box-ironbark forests of Victoria and New South Wales including the Painted Honeyeater or Swift Parrot and Superb Parrot.

Taxonomic summary

Isospora lesouefi sp. n. (Apicomplexa: Eimeriidae)

Type host: Regent Honeyeater, *Xanthomyza phrygia* (Shaw, 1794) (Aves: Passeriformes: Meliphagidae); syn. *Anthochaera phrygia* (Shaw, 1794).

Type locality: Zoo breading flock at Taronga Zoo, Mosman, Sydney, New South Wales, Australia. Animals are alive at the Taronga Zoo or were released.

Site of infection: Enterocytes. Duodenum and jejunum. Unsporulated oocysts recovered from faeces.

Prevalence: Oocyst found in 21% (18/84) of morning faces and 91% (82/90) of afternoon faeces from enclosures with 53 captive birds.

Type material / hapantotype: ARWH 7598.1. Formalin fixed paraffin embedded blocks at the Australian Registry of Wildlife Health, Mosman, NSW, Australia. Nucleotide sequence of the cytochrom oxidase I (COI) is available in GenBank™ under Accession No. HQ221885.

Etymology: The specific epithet "*lesouefi*" is given in honour from the surname of Albert Sherbourne Le Souëf (1877-1951), the first director of Taronga Zoo who insisted that all walls and fences were camouflaged. As a Bachelor of Veterinary Science, he was a dedicated leader of the zoological community and passionate supporter of faunal and floral reservations and sanctuaries.

Remarks: This is the first *Isospora* species described from a passerine bird in the genus *Xanthomyza*. The invasive House sparrow (*Passer domesticus*) from Adelaide are known to be infected with several *Isospora* spp. identical to those in Europe (Grulet et al., 1986b). While size and shape of our species overlaps with majority of *Isospora* species from House sparrow, the oocysts' polar granule together with the Stieda apparatus distinguishes our species from all known species in the House sparrow.

ACKNOWLEDGEMENTS

The Taronga Conservation Society Australia and Taronga Zoo is acknowledged for assistance with conducting this study. Dean Ingwersen (Regent Honeyeater recovery team co-ordinator) for insight on released birds. We thank Denise McDonell for expert technical assistance, Derek Spielman for insight on pathology (both University of Sydney), and Karrie Rose, Sheryl Sangster and Jane Hall (Australian Registry of Wildlife Health) for access to the registry records. We thank anonymous reviewers for their comments that improved the manuscript.

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Legends to figures

Fig 1. Composite line drawing of sporulated *Isospora lesouefi* sp. n. oocyst in the Regent Honeyeater (*Xanthomyza phrygia*) at the Taronga Zoo and its phylogenetic relationship with related cytochrom oxidase I (COI) sequences. The Minimum Evolution tree was reconstructed using Kimura 2-parameter distances and bootstraped (1000 replicates). Tree rooted using COI of *Eimeria tenella* (EF174188).

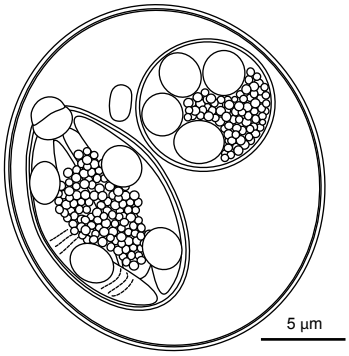
Fig 2. Photomicrographs of sporulated *Isospora lesouefi* sp. n. oocysts in the Regent Honeyeater (*Xanthomyza phrygia*) at the Taronga Zoo. Arrowhead, polar granule. DIC and blue autofluorescence; scale bar represents 5 μm.

Fig 3. Intestinal coccidiosis caused by *Isospora lesoueffi* n. sp. Coccidian asexual development, merogony, in the jejunum of the Regent Honeyeater (*Xanthomyza phrygia*, ARWH 7598.1C). Intracellular developing meronts (arrows) with merozoites (arrowheads) are within the columnar epithelium (c) of the Lieberkühn's crypts (A). Two types of meronts are recognized, meronts with delineated circular outline (large arrowhead, C) and meronts with undefined outline (arrow, B). Host inflammatory response is minimal (A-C). 2μm section, H&E. Scale bars represent 10 μm.

Fig. 4. Intestinal coccidiosis caused by *Isospora lesoueffi* n. sp. Coccidian sexual development, gamogony (arrows), in the duodenum (A, B) and the jejunum (C, D) of the Regent Honeyeater (*Xanthomyza phrygia*, ARWH 7457.1B). Intracellular developing macrogametes, mature macrogametes and early oocyst are within the enterocytes of the columnar epithelium (c). Developmental stages are surrounded by necrosis (apoptotic nuclei, arrowhead) and the columnar epithelium (c) is enlarged (D). Maturating oocysts destroy the columnar epithelium. Host inflammatory response is minimal. 2μm section, H&E. Scale bars represent 20 μm.

Fig. 5. Presence of oocysts of *Isospora lesouefi* sp. n. in the Regent Honeyeater (*Xanthomyza phrygia*) at the Taronga Zoo aviaries. A. Quantitative representation of proportions of ***I. lesouefi*** sp. n. positive faeces in all (n=174) faecal samples collected across two different days (Day 1, n=59; Day 2, n=115). The pie charts size is proportional to the number of faecal samples (see Supplementary Table S1). Morning samples (AM) and afternoon (PM) samples are side by side. B. Qualitative representation of all faecal samples sorted into negative and five positive categories according to ***I. lesouefi*** sp. n. and time of the day, morning samples (AM, n=84) and afternoon (PM, n=90).

Fig. 6. Statistical comparison of log transformed *Isospora lesouefi* sp. n. positive quantitative (oocysts.g^{-1}) data in the Regent Honeyeater (*Xanthomyza phrygia*) at the Taronga Zoo aviaries. A. All positive samples for morning (AM, n=18) and afternoon (PM, n=82). Data represented as a scatter dot plot of individual samples (circles) and a box-and-whisker plot (whiskers: min. & max, mean: +). B. Positive samples split according to aviaries they were collected in and morning (AM: Aviary I+II, n=7; Aviary III, n=6; Aviary IV, n=5) and afternoon (PM: Aviary I+II, n=51; Aviary III, n=12; Aviary IV, n=19). Data represented as a box-and-whisker plot (box: whiskers: min. & max, mean: +). Unpaired t-test values are shown above AM and PM plots, means are significantly different if $P<0.05$.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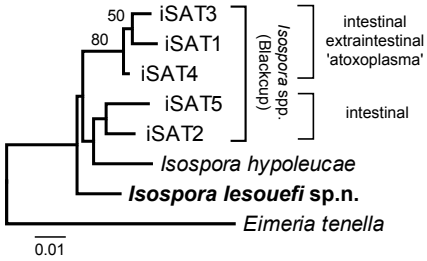
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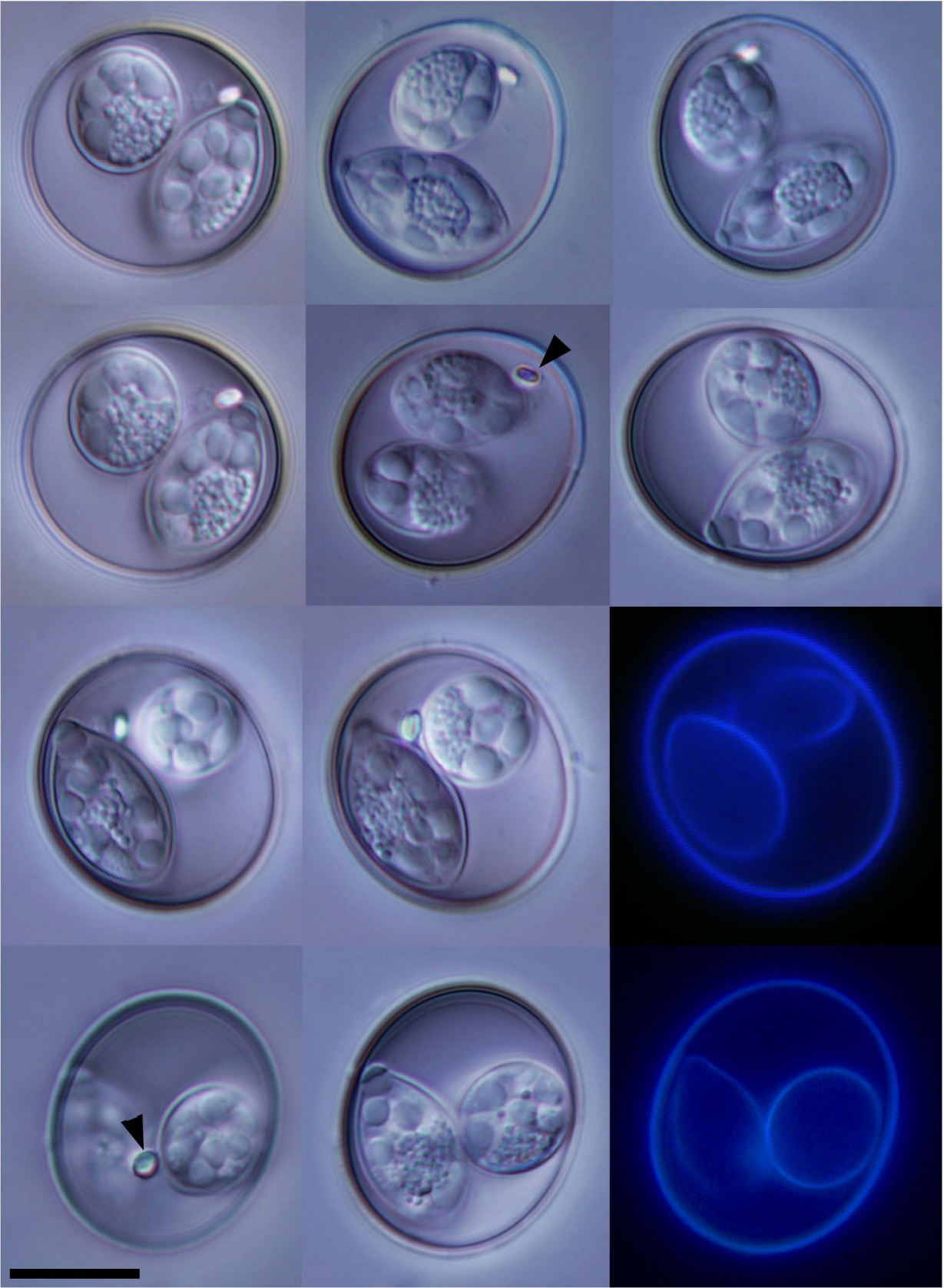
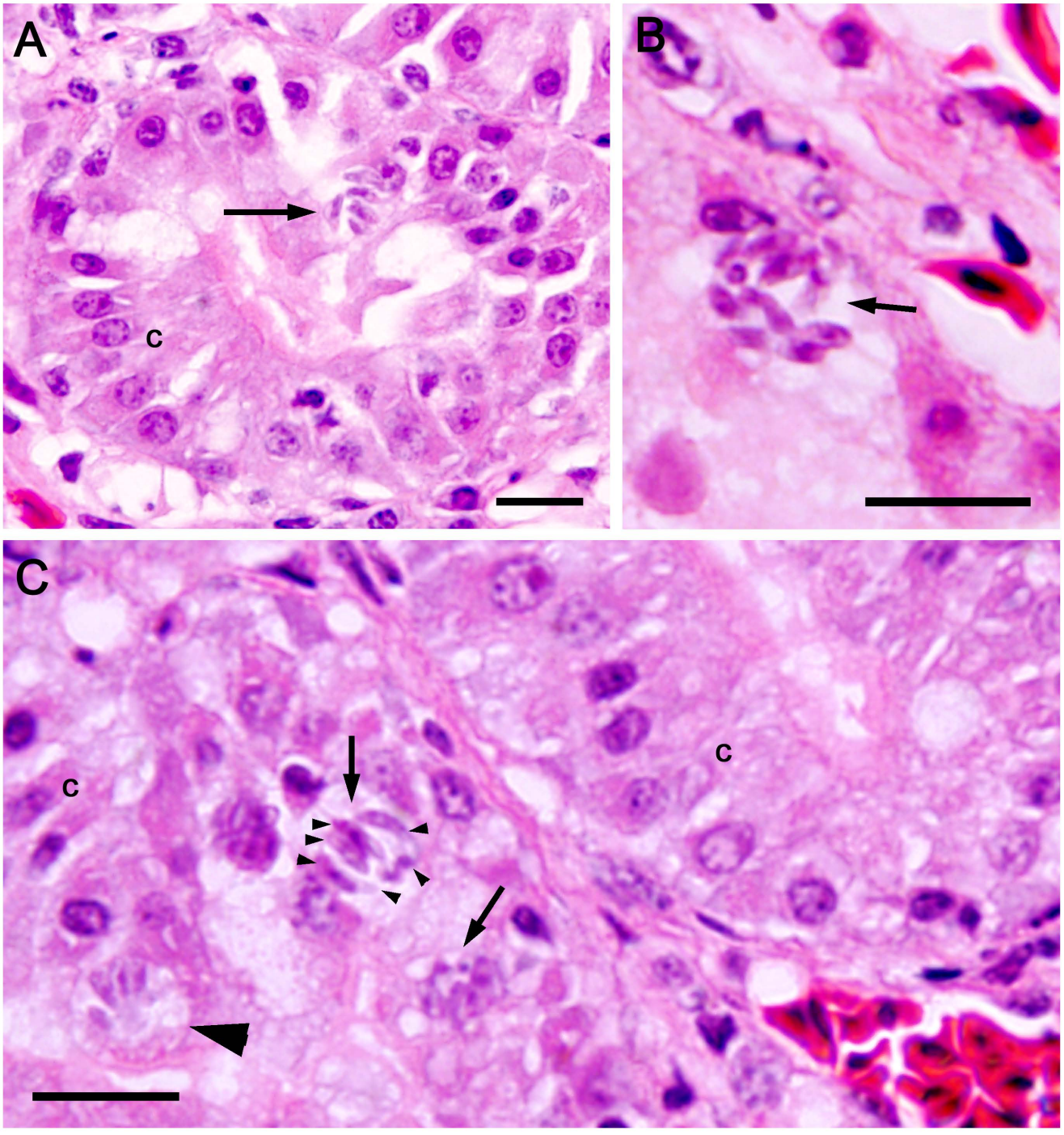
Isospora lesouefi sp.n.
Eimeria tenella
0.01

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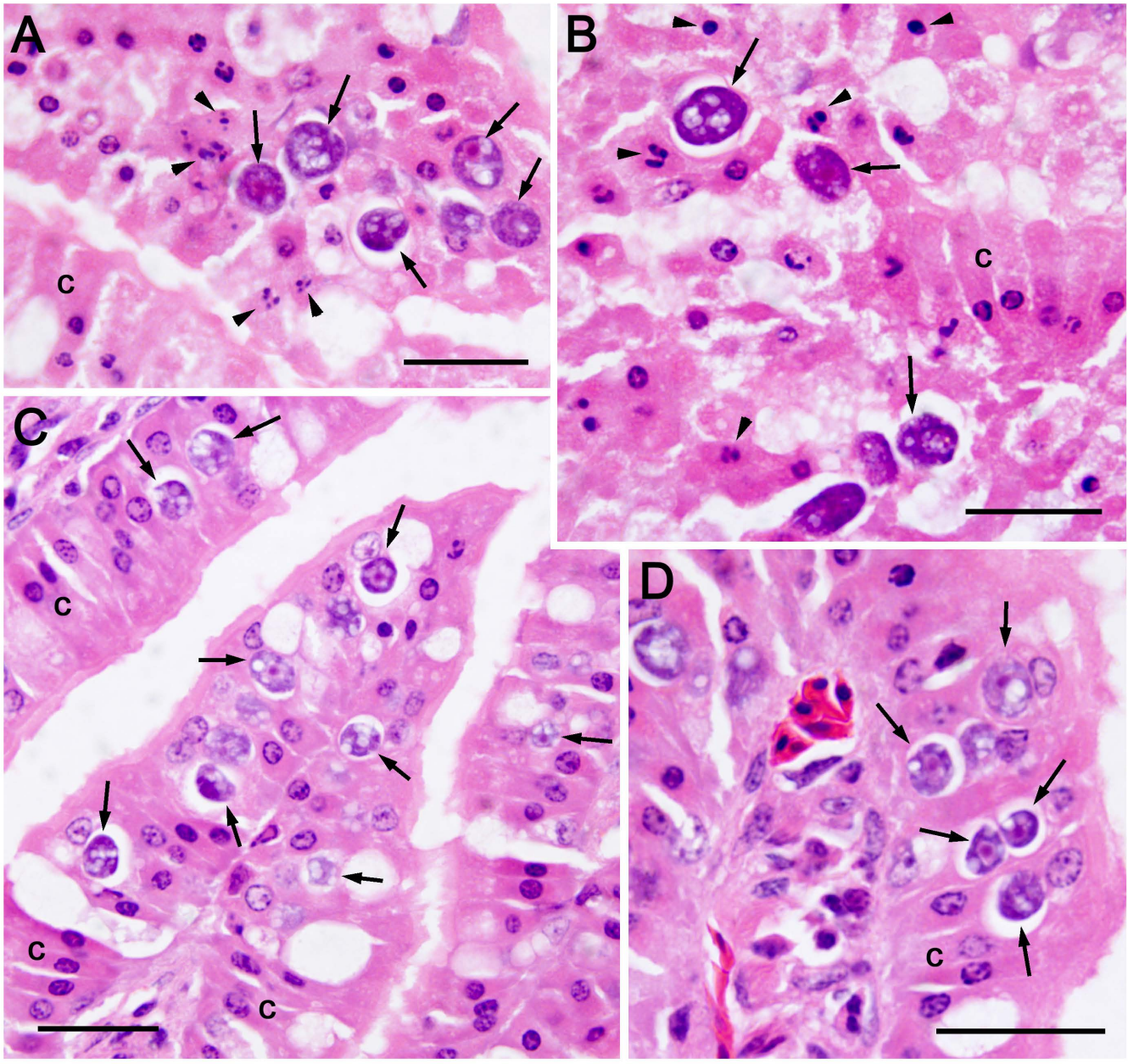
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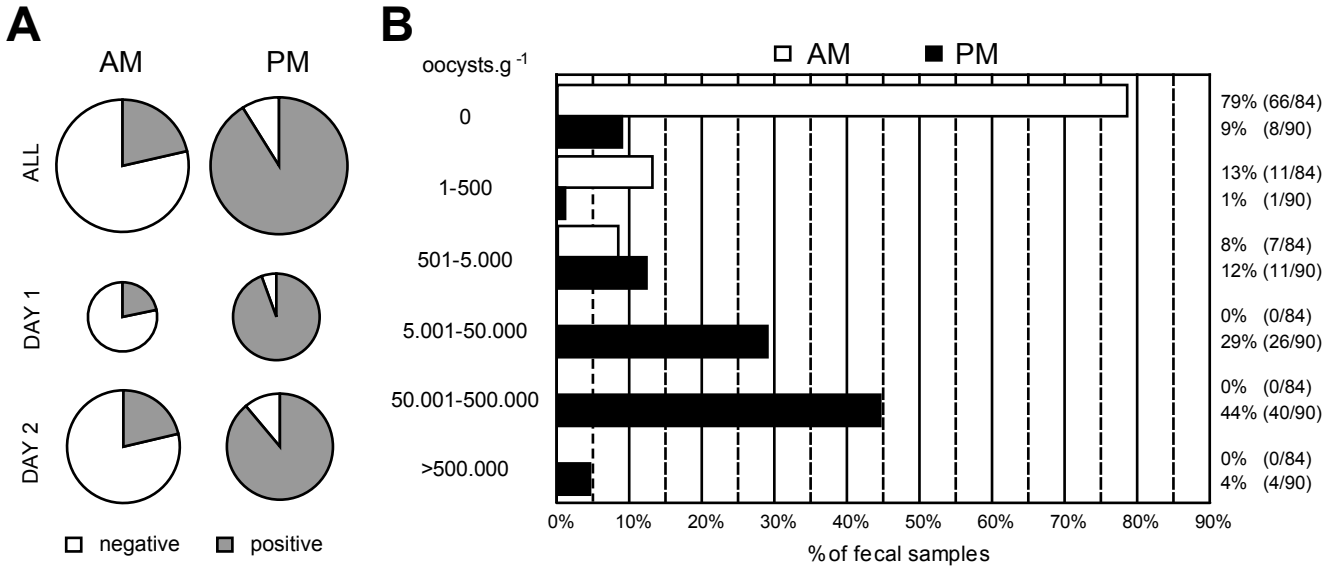
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>500.000
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9% (8/90)
13% (11/84)
1% (1/90)
8% (7/84)
12% (11/90)
0% (0/84)
29% (26/90)
0% (0/84)
44% (40/90)
0% (0/84)
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0%
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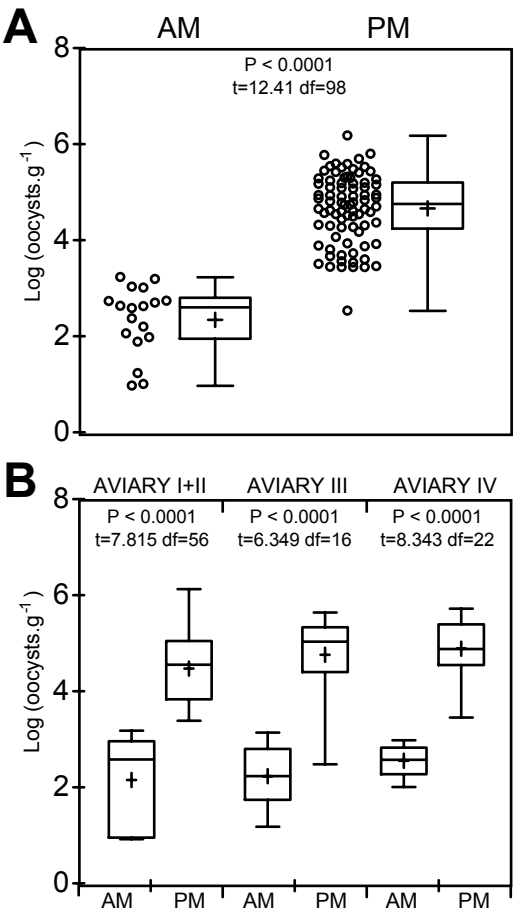
30%
40%
50%
60%
70%
80%
90%
% of fecal samples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P < 0.0001
t=12.41 df=98
Log (oocysts.g-1)
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AVIARY I+II
AVIARY III
AVIARY IV
P < 0.0001
t=7.815 df=56
P < 0.0001
t=6.349 df=16
P < 0.0001
t=8.343 df=22
Log (oocysts.g-1)
0
2
4
6
8
AM
PM
AM
PM
AM
PM

Table 1. Comparison between presence and absence of *Isospora lesouefi* n. sp. oocysts in faeces of the Regent Honeyeater, *Xanthomyza phrygia* collected in the morning (AM) and in the afternoon (PM) on two consecutive days at four Taronga Zoo aviaries.

Variables	Categories	Total	Positive (%)	Negative (%)	Odds-ratios	(95% CI)	*P*
Time	AM	84	18 (21.4%)	66 (78.6%)	1.00		<0.001
	PM	90	82 (91.1%)	8 (8.9%)	37.58	(16.17, 97.96)	
Day	1	59	39 (66.1%)	20 (33.9%)	1.00		0.097
	2	115	61 (53.0%)	54 (47.0%)	0.58	(0.30 1.10)	
Aviary	I and II	75	58 (77.3%)	17 (22.7%)	5.12	(2.42, 10.81)	<0.001
	III	39	18 (46.2%)	21 (53.8%)	1.29	(0.57, 2.90)	
	IV	60	24 (40.0%)	36 (60.0%)	1.00		

Appendix A: Supplementary data

Supplementary Table S1. Stratified analyses of the presence of *Isospora lesouefi* n. sp. oocysts in the morning (AM) and in the afternoon (PM) samples by sampling day and by aviary at which the birds were housed.

Variables	Categories	AM			PM		
		Total	Positive (%)	Negative (%)	Total	Positive (%)	Negative (%)
Day	1	23	5 (22%)	18 (78%)	36	34 (94%)	2 (6%)
	2	61	13 (21%)	48 (79%)	54	48 (89%)	6 (11%)
Aviary	I and II	23	7 (30%)	16 (70%)	52	51 (98%)	1 (2%)
	III	27	6 (22%)	21 (78%)	12	12 (100%)	0 (0%)
	IV	34	5 (15%)	29 (85%)	26	19 (73%)	7 (27%)

Supplementary Table S2. Burden of *Isospora lesouefi* n. sp. oocysts.g^{-1} in the morning (AM) and in the afternoon (PM) samples.

Categories	Total	AM (oocysts.g^{-1})	Total	PM (oocysts.g^{-1})
		Median (25-75% perc.) Mean (95% CI)		Median (25-75% perc.) Mean (95% CI)
ALL	18	406 (91-676) 499 (124-523)	82	58,009 (17,793-162,294) 129,723 (83,846-175,601)
Aviary I+II	7	432 (10-1,039) 540 (-46-1,127)	52	39,723 (7,621-126,606) 108,626 (44023-173228)
Aviary III	6	199 (76-809) 439 (-173-1,052)	12	123,518 (28,649-250,282) 155,362 (54,486-256,238)
Aviary IV	5	424 (252-817) 513 (66-959)	19	85,972 (39,882-281,600) 164,444 (86,513-242,375)
Day 1	5	424 (44-467) 289 (7-572)	34	62,763 (17,494-16,2294) 114,534 (65,308-163,759)
Day 2	13	388 (106-1,065) 580 (224-935)	48	55,011 (15,931-184,755) 140,483 (68,843-212,122)

Supplementary Table S3. Multivariable logistic regression analyses to compare the qualitative presence of *Isospora lesouefi* n. sp. oocysts in faeces of the Regent Honeyeater, *Xanthomyza phrygia*, collected in the morning (AM) and in the afternoon (PM) on two consecutive days at three Taronga Zoo aviaries. B. Multivariable linear regression analyses to compare the quantitative log counts of *Isospora lesouefi* n. sp. oocysts in faeces.

A

Variables	Categories	b	SE(b)	Odds-ratios	(95% CI for OR)	*P*
Intercept		-2.32	0.49			
Time	AM	0		1.00		<0.001
	PM	3.74	0.51	42.07	(16.47, 126.7)	
Aviary	I and II	1.72	0.56	5.57	(1.95, 17.74)	0.01
	III	1.23	0.61	3.41	(1.06, 12.05)	
	IV	0	0	1.00		

B

Variables	Categories	b	SE(b)	(95% CI)	*P*
Intercept		5.95	0.46	(5.03, 6.87)	
Time	AM	0			<0.001
	PM	5.45	0.42	(4.62, 6.29)	
Aviary	I and II	-1.03	0.39	(-1.80, -0.27)	0.030
	III	-0.43	0.49	(-1.42, 0.55)	
	VI	0			