Validation of various parasite detection tests for use in the Australian marsupials quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*)

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Short running title: Validation of various parasite tests in quenda and possums

Abstract.

We aimed to validate the use of: 1) the modified agglutination test and a PCR protocol in detecting *Toxoplasma gondii* infection in quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*); 2) immunofluorescence microscopy of feces and a PCR and sequencing protocol in detecting *Giardia* spp. infection in quenda; and 3) a fecal flotation protocol in detecting gastrointestinal helminth infections of quenda. Quenda and brushtail possum carcasses, and samples from trapped quenda, were tested with 2 parasite detection tests per parasite, and results were modelled using Bayesian latent class analysis to estimate test sensitivity and specificity. The modified agglutination test and the PCR protocol were highly specific at detecting *T. gondii* infections in quenda and brushtail possums (≥93%), however data were insufficient to assess sensitivity with adequate precision. Immunofluorescence microscopy and the PCR and sequencing protocol were both highly specific at detecting *Giardia* spp. in quenda (≥96%), but the PCR and sequencing protocol was relatively insensitive (69%, 95% credible interval [CrI] 60 – 77%), compared to the highly sensitive immunofluorescence microscopy (98%, 95% CrI 93 – 99%). The fecal flotation protocol was generally highly specific in the detection of gastrointestinal helminth infections (≥94%, with the exception of *Trichuris* spp. (88%; 95% CrI 71 – 99%). The fecal flotation protocol was moderately to highly sensitive (≥74%) in the detection of strongyles, *Labiobulura* spp., *Linstowinema* spp., and *Trichuris* spp. Sensitivity was poor for detection of the cestode genus *Potorolepis* (36%; 95% CrI 14 – 67%).

Keywords

Giardia, helminths, marsupial, tests, *Toxoplasma gondii*, validation, wildlife

Introduction

In epidemiologic investigations of infections in wildlife populations, validation of the test(s) used to detect the infectious agent in the host population under study is required for scientific rigor. Estimates of detection test sensitivity and specificity are essential for data interpretation and are valuable at the study design stage. A lack of validated detection tests is a substantial hindrance to the investigation of infectious disease epidemiology in wildlife worldwide,[38] and this is particularly true of parasitic infection epidemiology in Australian marsupial species. The lack of validated tests corresponds to a lack of "gold standard" tests (tests of 100% sensitivity and specificity) against which other tests can be compared in marsupial hosts. This deficiency may be overcome, however, by using Bayesian latent class analysis, which allows for estimation of detection test sensitivity and specificity in the absence of a gold standard.[18]

The protozoan parasite *Toxoplasma gondii* is of particular epidemiologic interest in Australian marsupial species.[16] The marsupials quenda (syn. southern brown bandicoots, *Isoodon obesulus*) and brushtail possums *(Trichosurus vulpecula*) are susceptible to infection with *T. gondii*,[6, 30,31] and may be at particular risk of infection with this parasite given their common occurrence in urban environments.[15,39] However, there is not yet a validated test for use in antemortem identification of *T. gondii* infection in quenda or brushtail possums, nor in any member of their respective order (Peramelemorphia) or suborder (Phalangeriformes). Similarly, quenda are susceptible to infection with parasites of the genus *Giardia*,[1,40] which is of epidemiologic interest as a result of potential conservation and public health implications.[39,40] However, no studies have investigated the accuracy of screening tools for detection of *Giardia* spp. infection in this species, nor in any other species in the order Peramelemorphia. Finally,

61 although fecal flotations of helminth eggs are common methodologies used in identifying
62 gastrointestinal helminths present in marsupials sampled antemortem for epidemiologic
63 purposes,[9,33,42] no fecal flotation protocol has been validated in any Australian marsupial species.
64 We aimed to use Bayesian latent class analysis to validate the use of a number of parasite
65 detection tests in quenda and brushtail possums: 1) the modified agglutination test and a PCR
66 protocol in detecting *T. gondii* infection in both host species; 2) immunofluorescence microscopy
67 and a PCR and sequencing protocol in detecting *Giardia* spp. infection in quenda; and 3) a fecal
68 flotation protocol in detecting gastrointestinal helminth infections in quenda.
69

70 **Materials and methods**

71 Sixty-two quenda and 22 brushtail possum carcasses originating from the Statistical
72 Division of Perth (Western Australia) were obtained opportunistically across 2013 - 2014.
73 Sources included a wildlife hospital and animals found dead (e.g., killed by vehicle strike).
74 Seventeen quenda carcasses and 1 possum carcass were sampled within 72 h of being found,
75 after storage at 4˚C. Forty-five quenda carcasses and 21 possum carcasses were frozen prior to
76 collection of samples. Frozen carcasses were left to defrost at 4˚C for 12 - 36 h prior to sample
77 collection (time dependent on body size). Likely cause of death was determined based on clinical
78 signs prior to death (where carcasses were obtained from the wildlife hospital), circumstances
79 surrounding the death, and gross findings on postmortem examination.
80 For each animal, sex and whether the animal was an adult or subadult were recorded.
81 Male possums were classified as adult if at least 1 testis was greater than 1 cm wide, or subadult
82 if each testis was ≤1 cm wide. Male quenda were classified as adult if their scrotal width was ≥2
83 cm, or subadult if their scrotal width was <2 cm. Female possums and quenda were classified as

adult if they had a parous pouch, or as subadult if they had a non-parous pouch. In adult females, pouch activity was noted - adult females were classified as having an active pouch if lactating, or an inactive pouch if not lactating.

Where available, the following samples were collected from each carcass:

1) Hemorrhagic or serohemorrhagic chest fluid was obtained from the chest cavity, centrifuged at 314 x *g* for 10 min, then the serous top layer of the sample (if available) or the top half of the sample (if a serous layer was not available) was transferred to a storage tube and frozen at -20˚C until analysis.

2) Organ samples: brain tissue was removed via the foramen magnum and stored in 70% ethanol (at a ratio of at most 1:4), and 2 mL of heart, liver, tongue and diaphragm tissue (or the whole organ, if total volume was lower) was cut to ~1 cm width and added to separate tubes of 8 mL 70% ethanol. All organ samples were stored at 4˚C until analysis.

3) Feces from the large intestine (quenda only): 2 mL feces were added to 8 mL 10% buffered formalin and mixed thoroughly. One mL feces were added to 8 mL 70% ethanol and mixed thoroughly. Samples were stored at 4˚C until analysis. Similarly, fecal samples were obtained from quenda trapped as part of a parasite survey in the Statistical Division of Perth. Trapped quenda had age, sex, and pouch activity recorded, as described for the quenda carcasses.

4) Gastrointestinal helminths (quenda only): the entire gastrointestinal tract and its contents were screened for helminths under a dissection microscope. After the fecal flotations were completed, helminths were also recovered from the fecal matter used for the flotation. All observed nematodes were removed and stored in 70% ethanol. Cestodes were removed and stored in 10% buffered formalin. Helminths were stored at 4˚C until analysis.

All samples were obtained under Murdoch University Animal Ethics Permit R2530/12, and Department of Parks and Wildlife Regulation 17 (SF009640) and Regulation 4 (CE004287) permits.

Chest fluid samples from quenda and possum carcasses were tested using a commercial modified agglutination test kit,[a] according to manufacturer's instructions. The animal was considered to have tested positive on the modified agglutination test if it tested positive at titers of 1:40 and / or 1:4000. The same observer performed all these tests.

For the *T. gondii* PCR, DNA was extracted from tissue samples using a blood and tissue kit[b] with some variations. A freeze/thaw step was conducted prior to addition of proteinase K, to facilitate disruption of *T. gondii* tissue cysts. Tissue was macerated in 50 µL PBS in a screw-cap tube, 180 µL buffer ATL was added, and tubes were subjected to 8 cycles of alternating between 1 min in liquid nitrogen and 1 min in a 95°C waterbath. Proteinase K was added, samples were incubated overnight at 56°C, and the protocol was then continued according to the manufacturer's instructions, with a final elution volume of 50 µL. Eluted DNA was stored at -20°C until required for PCR. A positive control (*T. gondii* infected quenda liver) and negative control (phosphate-buffered saline) were included in each batch of DNA extractions.

The target for PCR amplification of *T. gondii* DNA was the B1 gene, using a nested PCR with primers: external forward 5'-TGTTCTGTCCTATCGCAACG; external reverse 5'-ACGGATGCAGTTCCTTTCTG; internal forward 5'-TCTTCCCAGACGTGGATTTC; internal reverse 5'-CTCGACAATACGCTGCTTGA.[11] Each reaction of the nested PCR was performed in a 25 µL volume consisting of 1-2 µL extracted DNA, 1.5 mM $MgCl_2$, 1x reaction buffer,[c] 200 µM of each dNTP, 0.4 µM of each appropriate forward and reverse primer, and 0.2 units of Taq DNA polymerase[c] in a cresol loading dye solution. PCR was performed in a thermocycler[d] with

the following conditions: 95°C for 1 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension of 72°C for 5 min. Each PCR plate contained a positive control (purified DNA from cultured *T. gondii* tachyzoites) and negative controls. PCR products were either used immediately, or stored at 4°C until required for either the internal PCR or visualization. After the internal PCR amplification, PCR products were separated on a 2% agarose gel containing DNA stain[e] by electrophoresis, and gels were photographed under ultraviolet light. Carcasses were considered to have tested positive by PCR if specific DNA bands of the correct product size for *T. gondii* were amplified from any or all of the brain, heart, diaphragm, liver, or tongue. The same observer performed all PCR tests.

Of the non-specific DNA bands that were sometimes amplified by PCR (faints bands that were not the correct product size for *T. gondii*), the 10 non-specific bands that were closest to the size of *T. gondii* were sequenced to ensure that *T. gondii* had not been amplified. These DNA samples were purified using a purification system[f], following the manufacturer's instructions. Samples were sequenced in both directions using a sequencing kit[g] on a DNA analyzer.[h] Sequences were analyzed using sequencing software.[i]

Immunofluorescence microscopy was used to identify *Giardia* spp. cysts in fecal samples obtained from trapped quenda and quenda carcasses, using a commercial kit[l] according to the manufacturer's instructions for a non-concentrated sample. Samples were considered positive on immunofluorescence microscopy if at least one cyst of appropriate fluorescence and *Giardia* spp. morphology was identified. The same observer performed all immunofluorescence microscopy tests.

PCR and sequencing was undertaken on all immunofluorescence microscopy positive samples and a random sample of 10 immunofluorescence microscopy negative samples. Fecal

samples preserved in 70% ethanol had DNA extracted for amplification of *Giardia* spp. at 3 loci - 18S rRNA, ITS1-5.8s-ITS2, and *gdh*, and all amplified products on PCR were subject to sequencing, as described previously.[17] Quenda were considered positive for *Giardia* spp. via this PCR and sequencing protocol if genetic material was amplified, and amplified product sequenced as *Giardia* spp., at 1 or more loci. The same observer performed all PCR work.

As an addendum to the *Giardia* spp. validation, during the fecal flotations for identification of gastrointestinal helminth eggs (described below), the microscopist assessed the sample for the presence of *Giardia* spp. cysts.

For the fecal flotation protocol, each formalin-preserved quenda fecal sample originating from a carcass was centrifuged at 850 x *g* for 2 min, with the formalin supernatant discarded. The sample was then divided between 2 10-mL centrifuge tubes. The samples were re-homogenized in 9 mL distilled water, and centrifuged at 850 x *g* for 2 min, with the supernatant discarded.

One tube was analyzed using zinc sulfate flotation. The fecal matter was thoroughly mixed with 9 mL zinc sulfate solution (SG 1.20), then centrifuged at 850 x *g* for 2 min. A flamed wire loop was used to transfer material from the surface of the flotation to a slide, and a coverslip was added. Slides were systematically scanned at 200x magnification via a light microscope.[m] The second tube was analyzed using sodium nitrate flotation. The fecal matter was mixed thoroughly with 9 mL sodium nitrate solution (SG 1.37) and centrifuged at 850 x *g* for 2 min. The tube was then topped up with sodium nitrate solution to create a meniscus. A coverslip was placed on top of the tube and left for 10 min, then the coverslip was placed on a slide and systematically scanned at 200x magnification via a light microscope.[m] Before the first sodium nitrate slide was examined, the sodium nitrate tube was topped up with sodium nitrate solution

again to create another meniscus, and a second coverslip was placed on top. This was transferred to a slide and examined at 100x magnification via a light microscope,[m] directly after the first sodium nitrate flotation slide had been examined (a pilot study indicated that examining a second slide from the sodium nitrate tube substantially increased the likelihood of detecting *Trichuris* spp. eggs in quenda feces (A. Hillman- unpublished data)).

Helminth eggs were identified morphologically, using features and size ranges described in the literature[22, 36, 43] and in microscopic photography records collated from prior quenda fecal analyses (A. Elliot, unpublished data). Quenda were considered positive for the respective helminth taxon by the fecal flotation protocol if at least 1 egg of appropriate morphological features was identified on any or all of the 3 fecal flotations performed as part of the fecal flotation protocol. All fecal flotations were undertaken by the same observer. To aid observers using this fecal flotation protocol in the future, photographic records of eggs of the respective helminth taxa, and ranges of egg length and width, were obtained. Of all helminth taxa eggs observed by the fecal flotation protocol, at least one egg per taxon per host was measured microscopically at 400x magnification to include in reported ranges of egg length and width. However, the reported ranges of *Trichuris* spp. egg length and width were restricted to dimensions of eggs obtained from the feces of quenda from which *Trichuris* spp. worms were obtained from the gastrointestinal tract. Photographs and measurements were undertaken at 400x magnification.

The gastrointestinal helminths obtained from the quenda carcasses via the microscopic gastrointestinal tract helminth screen were differentiated to genus, family, or infraorder level by light microscopy[m] (at 4x to 200x magnification, depending on the worm genus / family / infraorder), using published quenda helminth descriptions.[22,36,43] Where cestodes were too

198 degraded to have retained identifying features (particularly rostellar hooks), the morphologic
199 features of eggs obtained from gravid proglottids were used to identify the genus of cestode. If
200 gravid proglottids were not present, and thus the cestode was not identifiable morphologically,
201 the sample was excluded from cestode sensitivity and specificity calculations. Quenda were
202 considered to have tested positive for the respective nematode taxon on microscopic
203 gastrointestinal tract examination if at least one nematode of the taxon was obtained on
204 microscopic gastrointestinal tract examination and differentiated as such morphologically.
205 Quenda were considered to have tested positive for a cestode genus on microscopic
206 gastrointestinal tract examination if at least one cestode of that genus was obtained and
207 differentiated as such morphologically, or if eggs of that cestode genus were obtained and
208 identified from gravid proglottids of a cestode that was not otherwise morphologically
209 identifiable. All gastrointestinal helminth identifications were undertaken by the same observer.
210 The sensitivity and specificity of the parasite detection tests were calculated via Bayesian
211 latent class analysis, using a software package[j] with a software interface.[k] Briefly, in the
212 Bayesian latent class analyses used in this study (where 2 detection test results are available per
213 animal, with tested animals obtained from 1 population), 5 parameters require consideration -
214 infection prevalence, and the sensitivity and specificity of each test. Parameters are entered as
215 either informative or non-informative distributions. As the number of parameters exceeds the 3
216 degrees of freedom provided by the data, informative prior distribution inputs are required for at
217 least 2 of the 5 model parameters for model identifiability. Published data (where available) and /
218 or expert knowledge can be used to guide informative (beta) prior distribution inputs. Non-
219 informative (uniform) prior distributions can be used where no information is available to guide
220 prior distribution estimations. The prior distribution inputs are then modelled with the detection

test data obtained from the study and the unknown latent variable (representing the true infection status of the host) using the iterative Gibbs sampler Markov-chain Monte Carlo method, to obtain posterior distribution estimates of the sensitivity and specificity of each detection test and of the infection prevalence[18] (although in this study, prevalence is not of interest as an output).

For the Bayesian latent class analysis of the *T. gondii* tests, tests were modelled as conditionally independent. The prior distribution inputs were identical for the quenda and brushtail possum models (Table 1). The relatively conservative prior distribution for modified agglutination test specificity (0.50 - 0.99) was based on findings from several macropod species (taxonomic family Macropodidae). An ELISA was validated as highly specific, and this ELISA was shown to have excellent agreement with the modified agglutination test[28] (see also Parameswaran N. *Toxoplasma gondii* in Australian marsupials [PhD thesis]. Perth, Australia: Murdoch University; 2008. p 52). The more precise prior distribution used for PCR specificity was based on previous research findings at the B1 locus (Table 1).

For the Bayesian latent class analysis of the *Giardia* spp. tests, tests were modelled as conditionally independent, as the widely reported high sensitivity and specificity of *Giardia* spp. detection via immunofluorescence microscopy of feces in other species indicated that conditional dependence between tests was unlikely to impact these models (Table 1). The relatively conservative informative prior distribution used for PCR and sequencing sensitivity was chosen in view of the variable results observed via PCR in different studies of *Giardia* spp. that used varying protocols on a variety of host species.[41] The high and narrow prior distribution used for PCR and sequencing specificity was selected as the use of sequencing in our case definition made false positive results highly unlikely.

For the Bayesian latent class analysis of the gastrointestinal helminth tests, tests were modelled as conditionally independent (Table 1). All informative priors were based on expert opinion, obtained in consulting expert personnel involved in past and current marsupial parasitology at Murdoch University. The informative prior distributions used for the specificity of the fecal flotation protocol in detection of *Labiobulura* spp., *Linstowinema* spp., *Physaloptera* spp., and *Potorolepis* spp. were recommended based on the distinctive morphologic features of these helminth eggs rendering false positives unlikely. The prior distribution for fecal flotation specificity for the detection of strongyles and strongyloids was recommended in consideration of the possibility that parasite eggs of quenda prey items may be present in the quenda feces, and there are strongyle and strongyloid helminths with similar eggs parasitic in a wide range of species. The prior distribution for fecal flotation specificity for the detection of *Trichuris* spp. infection was recommended in consideration of the risk of eggs of other capillarids (superfamily Trichinelloidea, e.g., *Capillaria* spp.) being mistaken for those of *Trichuris* spp. The prior distributions for the sensitivities of the observer finding *Labiobulura* spp., *Linstowinema* spp., *Physaloptera* spp. and *Potorolepis* spp. helminths in the gastrointestinal tract of infected quenda and correctly differentiating them to genus level were recommended based on the relatively large size of adult helminths, and the reasonably uncomplicated taxonomic differentiation required, rendering false negatives relatively unlikely. Uninformative prior distributions were used for sensitivity of detection and differentiation of strongyles (infraorder Rhabditomorphia), strongyloids (family Strongyloididae) and *Trichuris* spp., based on the tiny size (strongyles and strongyloids) and the tiny (coiled) size and apparent fragility of *Trichuris* spp. worms after recovery from frozen carcasses, causing uncertainty about the potential for false negatives. The informative prior distribution inputs for the specificity of the observer finding helminths in the

quenda gastrointestinal tract and correctly differentiating to a genus / family / infraorder level were recommended based on the distinctiveness of the helminth morphology at the taxonomic level of differentiation required, rendering false positives unlikely.

The number of Gibbs sampler iterations for each model started at 10,500 iterations, with the first 500 "burn in" iterations dropped from the results. The number of iterations was doubled if the model did not adequately converge using the lower number of iterations (Table 1).

Model validity for each infection type was established in a number of ways. First, Gibbs sampler trace plots were evaluated to ensure convergence, and models were rerun with different starting values 5 times to ensure consistency of output. Second, the observed vs. the median predicted test profiles were compared to ensure they were similar. Finally, the observed and expected probabilities of agreement between the tests were compared to ensure values were similar, and the associated probability statistic was between 0.05 and 0.95.

The median value of the modelled test sensitivities and specificities were reported as the point estimates, with 95% credible intervals (CrI) (credible intervals are Bayesian analogues of frequentist confidence intervals). Where fewer than 6 animals tested positive or negative for a parasite taxon by one or both tests, data were considered insufficient for sensitivity or specificity calculations (respectively), and were not reported in results. The prevalence outputs, and the output of the sensitivity and specificity of the observer finding and correctly identifying helminths to a genus / family / infraorder level were also not reported. This was because this study was not designed to measure infection prevalences, and although the data provided by the observer obtaining and differentiating helminths were valuable components of the latent class analyses, the sensitivity and specificity of this test is not of practical value of itself to future research.

Sensitivity analyses of all models were undertaken, by varying each informative prior distribution separately, while all other model inputs were held constant. The credible limits of the informative priors were decreased by 10% and 20% (gross), respectively. A change in the sensitivity and / or specificity point estimate by 5% (gross) or more was considered 'substantial', and reported in the results.

Results

Of quenda carcasses used in this study, 54/62 (87%) were judged to have been killed by vehicle strike, attack by another animal, household poisons (such as rat and snail baits), or drowning in backyard pools or ponds. Of possum carcasses used in this study, 15/22 (68%) were judged to have been killed by traumatic injuries (particularly vehicle strike or attack by another animal), electrocution, or household poisons (such as rat and snail baits).

Chest fluid and body tissues were available from 56 quenda carcasses and 22 brushtail possum carcasses. The quenda carcasses comprised 6 subadult females, 12 adult females, 21 subadult males, 16 adult males, and an additional subadult quenda of undetermined sex. Of the adult female quenda, 9 had an active pouch and 3 had an inactive pouch. The possum carcasses comprised 2 subadult females, 9 adult females, 4 subadult males, and 7 adult males. Of the adult female possums, 2 had an active pouch and 7 had an inactive pouch.

All 5 targeted body tissues (brain, heart, liver, diaphragm, and tongue) were available from 51 quenda carcasses and all brushtail possum carcasses. Because of traumatic injuries, diaphragmatic tissue was not recoverable from 3 quenda, heart tissue was not recoverable from 1 quenda, and sampling of brain tissue was severely compromised in 1 quenda. All carcasses positive on the modified agglutination test had all 5 tissues available for testing.

The *Toxoplasma gondii* tests in brushtail possums were 96% concordant (21/22 possums). One possum was positive on the modified agglutination test, whilst bands specific for *T. gondii* were not amplified from any brushtail possum tissues. In quenda, the *T. gondii* tests were 93% concordant (52/56 quenda). Four quenda were positive on the modified agglutination test; bands specific for *T. gondii* were not amplified from any quenda tissues (Table 2). On sensitivity analysis, no substantial changes occurred to the point estimates of the specificity of either *T. gondii* test in either species (Table 3).

The 10 faint, non-specific bands amplified by *T. gondii* PCR that were closest to the expected size for *T. gondii* (from 10 tissues obtained from 6 quenda) did not sequence as *T. gondii*. One of the 22 possums and 1/56 quenda were known to have non-specific neurologic signs prior to death. Both animals were negative on both the modified agglutination test and PCR.

Fecal samples available for the *Giardia* spp. test validation were from 110 immunofluorescence microscopy-positive quenda and 10 immunofluorescence microscopy-negative quenda. The samples were obtained from 6 subadult females, 42 adult females, 11 subadult males, and 61 adult males. Of the adult females, 33 had an active pouch and 9 had an inactive pouch. Eleven samples were from quenda carcasses; 109 samples were obtained from trapped quenda.

The *Giardia* spp. infection detection test results were concordant in 83/120 (69%) quenda (Table 4). Thirty-six quenda were positive for *Giardia* spp. infection on immunofluorescence microscopy, but negative by PCR and sequencing. *Giardia* spp. cysts were identified in 22 (61%) of these quenda, by light microscopy during the fecal flotations.

On sensitivity analysis, no substantial changes occurred to the point estimates of sensitivity and specificity of both *Giardia* spp. tests (Table 3).

Gastrointestinal tract helminth screens and fecal flotation analyses were available from 49 quenda carcasses. This comprised 4 subadult females, 11 adult females, 19 subadult males, and 15 adult males. Of the adult female quenda, 2 had an inactive pouch and 9 had an active pouch.

Six nematode taxa, consisting of strongyles (infraorder Rhabditomorphia), *Labiobulura* spp., *Linstowinema* spp., *Physaloptera* spp., strongyloids (family Strongyloididae), and *Trichuris* spp., were detected by the fecal flotation protocol (Figs. 1 – 7) and / or the microscopic gastrointestinal helminth screen. The cestode genus *Potorolepis* was detected by the fecal flotation protocol (Figs. 8, 9) and the microscopic gastrointestinal helminth screen. Three quenda carcasses contained unidentifiable cestode segments and were thus excluded from *Potorolepis* spp. results, and from the *Potorolepis* spp. sensitivity and specificity calculations. Cestode eggs were not identified in the feces of any of these 3 quenda.

The concordance of the gastrointestinal helminth detection tests were: 34/49 (69%) for strongyles; 40/49 (82%) for *Labiobulura* spp.; 41/49 (84%) for *Linstowinema* spp.; 46/49 (94%) for *Physaloptera* spp.; 44/49 (90%) for strongyloids; 40/49 (82%) for *Trichuris* spp.; and 38/46 (83%) for *Potorolepis* spp. (Table 5).

On sensitivity analysis, no substantial changes occurred to the point estimates of: the sensitivity and specificity of the fecal flotation protocol in detecting *Labiobulura* spp., *Linstowinema* spp. and *Trichuris* spp. infections; the sensitivity of the fecal flotation protocol in detecting strongyle infections; and the specificity of the fecal flotation protocol in detecting *Physaloptera* spp., strongyloids, and *Potorolepis* spp. infections (Table 3). When the lower

credible limit of the informative prior distribution for the specificity of the fecal flotation protocol in identifying strongyle infections was extended to 0.6, the point estimate of specificity of the fecal flotation protocol in detecting strongyle infections decreased to 88% (95% CrI 57 – 99%). When the lower credible limit of the informative prior distribution of the specificity of the microscopic gastrointestinal tract exam in identifying *Potorolepis* spp. infections in quenda was widened to 0.75, the point estimate for the sensitivity of detecting *Potorolepis* spp. via the fecal flotation protocol increased to 45% (95% CrI 16 – 92%).

Discussion

This study presents the first validation data regarding parasite tests for use in the detection of *Giardia* spp. and gastrointestinal helminth infections in any Australian marsupial species (superorder Australidelphia), and the first validation data for tests to detect *T. gondii* infection in any species of the order Peramelemorphia and suborder Phalangeriformes.

The robustness of the Bayesian models on sensitivity analysis suggests that the results were primarily driven by the data, rather than unduly influenced by the choice of credible limits for the informative prior distributions. On sensitivity analysis, substantial changes to the point estimates of the specificity of the fecal flotation protocol in detecting strongyle infections, and the sensitivity of the fecal flotation protocol in detecting *Potorolepis* spp. infections only occurred when informative prior distribution limits that were considered highly implausible were used. Therefore, it is not considered that these sensitivity analysis findings invalidate the results presented here.

The apparently accidental cause of death of the majority of carcasses used in the study may have avoided substantial bias of results towards that of "sick" animals, therefore

maintaining the applicability of the results to epidemiologic studies in the broader host populations.

Our findings suggest that both the modified agglutination test (at the titers used in this study) and the PCR protocol are highly specific in the detection of *T. gondii* infection in quenda and brushtail possums. This study used chest fluid for the modified agglutination test, rather than serum, which may bias findings if extrapolating these results to the use of serum. However, findings of a study comparing *T. gondii* ELISA results in tissue fluid vs. serum did not suggest ELISA specificity was substantially affected when using tissue fluid.[14] The finding of high modified agglutination test specificity in quenda and brushtail possums concurs with findings in other host species.[7,13,21]

The data were insufficient to assess the sensitivity of these tests in an adequately precise manner, as the apparent prevalence (using both tests) of *T. gondii* was low in both the quenda and possum study samples. The non-specific bands amplified from some quenda tissues on *T. gondii* PCR were faint and not the correct product size for *T. gondii*. The 10 bands that were closest in size to *T. gondii* did not sequence as *T. gondii,* and this supports our interpretation of such bands as negative results.

This study found both immunofluorescence microscopy and the PCR and sequencing protocol to be highly specific in the detection of *Giardia* spp. infections in quenda, which concurs with high specificities observed in other host species.[8,10,25]

Immunofluorescence microscopy was highly sensitive in detecting *Giardia* spp. infection in quenda feces, and substantially more so than the PCR and sequencing protocol. This reflects other research findings, in which amplification by PCR was not successful in all *Giardia* positive fecal samples tested.[23, 27] The identification of *Giardia* spp. cysts by fecal flotations in the

majority of the immunofluorescence microscopy-positive, PCR and sequencing-negative fecal samples provided reassurance that the discrepancy between the number of samples positive by immunofluorescence microscopy vs. those positive by the PCR and sequencing protocol was not because of lack of specificity by immunofluorescence microscopy. As *Giardia* spp. detection by fecal flotations has been shown to lack sensitivity in other species,[10,32] the fact that *Giardia* spp. cysts were not identified in all fecal flotations is not unexpected. The sensitivity of both immunofluorescence microscopy and PCR and sequencing in detecting *Giardia* spp. infection in quenda may have been overestimated if there are commonly periods in which infected quenda do not shed any cysts in their feces.

As only 2 fecal samples tested positive for *Giardia* spp. by PCR and sequencing at the *gdh* locus, and these samples also tested positive for *Giardia* spp. at the 2 other loci tested, the *gdh* locus could be excluded from the PCR and sequencing protocol with no change in protocol sensitivity or specificity (further detail of the PCR and sequencing results at the individual loci are described previously).[17]

The specificities of detection of various gastrointestinal helminth infections by the fecal flotation protocol were generally high. The fecal flotation protocol specificities for *Labiobulura* spp., *Linstowinema* spp., *Physaloptera* spp., and *Potorolepis* spp. may have been biased downwards as we chose to be relatively conservative in inputting prior distributions, despite the reasonable expectation of a very high degree of specificity in the protocol for these helminths (distinctive helminth egg morphology, with all flotations performed by an experienced observer). However, we cannot exclude the possibility that these specificities reflect a small degree of misclassification. The relatively lower specificity for *Trichuris* spp. may have been attributable to eggs of other capillarids being mistaken for those of *Trichuris* spp. If this is the case, our

observations on microscopy suggest that there is very little difference in the morphology or size range of eggs between *Trichuris* spp. and other capillarid genera in quenda. The published description of smooth-shelled eggs of a size range of 65 x 35µm for *T. peramelis*[22] was not commonly reflected in the *Trichuris* spp. eggs frequently observed in the feces of quenda in which *Trichuris* spp. worms were obtained from their gastrointestinal tract, and in the *Trichuris* spp. worms themselves. Eggs observed in this study did not have smooth shells and were typically 55-57.5 µm x 22.5-25 µm - only occasionally larger or smaller (A. Hillman - unpublished data).

The fecal flotation protocol detected the helminths *Labiobulura* spp., *Linstowinema* spp., *Trichuris* spp. and strongyles in quenda with a moderate to high degree of sensitivity, although results are limited by a lack of precision. The imperfect sensitivity of the fecal flotation protocol in detecting gastrointestinal helminth infections may be influenced by factors such as prepatent infections (particularly given the relatively large number of subadult quenda in the study sample) and intermittent shedding of eggs. Although also limited by a lack of precision, the relatively low sensitivity of the fecal flotation protocol in detecting the cestode genus *Potorolepis* spp. reflects a lack of sensitivity in the detection of other intestinal cestodes via similar methods in other host species.[19, 20, 26] Our point estimate of 36% sensitivity may have been an overestimate if any or all of the 3 unidentifiable cestode-positive quenda were infected with *Potorolepis* spp., which is considered highly likely. *Physaloptera* spp. and strongyloids were not sufficiently prevalent in our study to provide an adequately precise estimate of fecal flotation protocol sensitivity.

Compared to our findings, both the sensitivity and specificity of fecal flotations may be influenced through the use of different observers, or if the particular fecal flotation protocol varies to that utilized in this study. However, as quenda helminth eggs are relatively large and

449 have distinct morphological features, we believe that observers with prior experience
450 undertaking fecal flotation microscopy (in any species) would achieve similar sensitivity and
451 specificity of parasite detection using this described fecal flotation protocol.
452

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463

464 **Sources and manufacturers**

465 [a] Toxo-Screen DA, bioMérieux, France.

466 [b] QIAGEN GmbH, Hilden, Germany.

467 [c] Fisher Biotec, Perth, Australia.

468 [d] BioRad thermocycler, Gladesville Australia.

469 [e] SYBR safe DNA stain, Molecular Probes Inc., Eugene, OR.

470 [f] Agencourt AMPure XP, Beckman Coulter, Beverley, CA.

471 [g] ABI Prism^{TM} Terminator Cycle Sequencing kit, Applied Bio-systems, CA.

[h] Applied Biosystems 3730 DNA Analyzer, Thermo Fisher Scientific, Waltham, MA.

[i] Geneious 7.2. (Kearse M, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 2012:28;1647-1649)

[j] R v3.1.1 (R Development Core Team. R: A language and environment for statistical computing (Version 3.1.1). R Foundation for Statistical Computing, Vienna, Austria. December 2015. URL http://www.R-project.org).

[k] BayesLatentClassModels v1.13 (Dendukuri N, et al. BayesLatentClassModels: A program for estimating diagnostic test properties and disease prevalence (Version 1.13). December 2015. URL: http://www.nandinidendukuri.com/blcm).

[l] Merifluor Cryptosporidium/ Giardia, Meridian Bioscience, Inc., Cincinnati, OH.

[m] BX50 microscope, Olympus, Tokyo, Japan.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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595 **Tables**

596

597 **Table 1.** Prior distribution inputs for Bayesian latent class analysis of parasite detection tests in

598 quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*)

Test validation: parasite (host)	Prior distributions	Prior distribution inputs	Informative prior distribution references/ expert opinion*	No. Gibbs sampler iterations
Toxoplasma gondii (quenda and possums)	Prevalence	Non-informative	-	21,000†
	Modified agglutination test (MAT) sensitivity	Non-informative	-	
	MAT specificity	0.50 – 0.99	28‡	
	PCR sensitivity	Non-informative	-	
	PCR specificity	0.95-0.99	5, 12, 29, 34, 35.	
Giardia spp. (quenda)	Prevalence	Non-informative	-	10,500§
	Immunofluorescence microscopy (IMF) sensitivity	0.8 – 0.99	2,8, 10, 32, 37.	
	IMF specificity	0.9 – 0.99		
	PCR & sequencing sensitivity	0.6 – 0.99	3,4, 23, 24, 37.	
	PCR & sequencing specificity	0.95 – 0.99		
Strongyles[ı], strongyloids[#] (quenda)	Prevalence	Non-informative	-	21,000†
	Fecal flotation protocol (FFP) sensitivity	Non-informative	-	
	FFP specificity	0.8 – 0.99	Expert opinion	
	GI¶ helminth screen sensitivity	Non-informative	-	
	GI¶ helminth screen specificity	0.95 – 0.99	Expert opinion	
Labiobulura spp., *Linstowinema* spp., *Physaloptera* spp., *Potorolepis* spp. (quenda)	Prevalence	Non-informative	-	21,000† (*Physaloptera* spp. only) 10,500§ (all others)
	FFP sensitivity	Non-informative	-	
	FFP specificity	0.9 – 0.99	Expert opinion	
	GI¶ helminth screen sensitivity	0.8 – 0.99	Expert opinion	
	GI¶ helminth screen specificity	0.95 – 0.99	Expert opinion	
Trichuris spp. (quenda)	Prevalence	Non-informative	-	10,500§
	FFP sensitivity	Non-informative	-	
	FFP specificity	0.5 – 0.99	Expert opinion	

	GI¶ helminth screen sensitivity	Non-informative	-
	GI¶ helminth screen specificity	0.95 – 0.99	Expert opinion

599 * Expert opinion was obtained by consulting experts involved in past and current marsupial parasitology at Murdoch
600 University, Australia
601 † First 1,000 "burn in" iterations dropped from results
602 ‡ See also Parameswaran N. *Toxoplasma gondii* in Australian marsupials [PhD thesis]. Perth, Australia: Murdoch
603 University; 2008. p 52
604 § First 500 "burn in" iterations dropped from results
605 ¦ Infraorder Rhabditomorphia
606 # Family Strongyloididae
607 ¶ GI= gastrointestinal

608 **Table 2.** Results of the modified agglutination test (MAT) and PCR protocol in detecting
609 *Toxoplasma gondii* infections in quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus*
610 *vulpecula*)

Host			PCR	
			Positive	Negative
Brushtail possums	Modified agglutination test	Positive	0	1
		Negative	0	21
Quenda	Modified agglutination test	Positive	0	4
		Negative	0	52

611

612 **Table 3:** Sensitivity and specificity of various parasite detection tests in quenda (*Isoodon*
613 *obesulus*) and brushtail possums (*Trichosurus vulpecula*)

Host species	**Parasite**	**Parasite detection test**	**Sensitivity** median (95% CrI*)	**Specificity** median (95% CrI*)
Brushtail possums	*Toxoplasma gondii*	Modified agglutination test	Data inadequate for calculation	94% (78 – 99%)
		PCR protocol	Data inadequate for calculation	98% (95 – 99%)
Quenda	*T. gondii*	Modified agglutination test	Data inadequate for calculation	93% (79 – 99%)
		PCR protocol	Data inadequate for calculation	98% (96 – 99%)
Quenda	*Giardia* spp.	Immunofluorescence microscopy	98% (93 – 99%)	96% (90 – 99%)
		PCR and sequencing protocol	69% (60 - 77%)	98% (95- 99%)
Quenda	Strongyles†	Fecal flotation protocol	97% (83 – 100%)	94% (80 - 99%)
	Labiobulura spp.		74% (58 – 87%)	97% (92 – 99%)
	Linstowinema spp.		81% (66 – 92%)	96% (90 – 99%)
	Physaloptera spp.		Data inadequate for calculation	96% (91 – 98%)
	Strongyloids‡		Data inadequate for calculation	96% (88 – 99%)
	Trichuris spp.		91% (64 – 100%)	88% (71 – 99%)
	Potorolepis spp.		36% (14 – 67%)	98% (93 – 99%)

614 * CrI= credible interval

615 † Infraorder Rhabditomorphia

616 ‡ Family Strongyloididae

617 **Table 4.** Results of immunofluorescence microscopy and a PCR and sequencing protocol in

618 detecting *Giardia* spp. infections in quenda (*Isoodon obesulus*)

		Immunofluorescence microscopy	
		Positive	Negative
PCR and sequencing protocol	Positive	74	1
	Negative	36	9

619

620 **Table 5.** Results of a fecal flotation protocol (FFP) and microscopic examination of the
621 gastrointestinal (GI) tract in detecting gastrointestinal helminth infections in quenda (*Isoodon*
622 *obesulus*)

Helminth type			**Microscopic GI tract exam:**	
			Positive	Negative
Strongyles*	**FFP:**	Positive	20	15
		Negative	0	14
Labiobulura spp.	**FFP:**	Positive	27	0
		Negative	9	13
Linstowinema spp.	**FFP:**	Positive	27	2
		Negative	6	14
Physaloptera spp.	**FFP:**	Positive	0	3
		Negative	0	46
Strongyloids†	**FFP:**	Positive	0	2
		Negative	3	44
Trichuris spp.	**FFP:**	Positive	8	9
		Negative	0	32
Potorolepis spp.	**FFP:**	Positive	4	0
		Negative	8	34

623 * Infraorder Rhabditomorphia

624 †Family Strongyloididae

625

626

627 **Figure legends**

628 **Figure 1.** Unembryonated strongyle (infraorder Rhabditomorphia) egg observed in quenda feces
629 by fecal flotation microscopy at 400x magnification. Approximate dimensions of strongyle eggs
630 from quenda feces (range): length: 52.5µm – 72.5µm; width: 30µm – 45µm.
631 **Figure 2.** Embryonated strongyle (infraorder Rhabditomorphia) egg observed in quenda feces by
632 fecal flotation microscopy at 400x magnification. Approximate dimensions of strongyle eggs
633 from quenda feces (range): length: 52.5µm – 72.5µm; width: 30µm – 45µm.
634 **Figure 3.** *Labiobulura* spp. egg observed in quenda feces by fecal flotation microscopy at 400x
635 magnification. Approximate dimensions of *Labiobulura* spp. eggs from quenda feces (range):
636 length: 52.5µm – 65µm; width: 45µm – 60µm.
637 **Figure 4.** *Linstowinema* spp. egg observed in quenda feces by fecal flotation microscopy at 400x
638 magnification. Approximate dimensions of *Linstowinema* spp. eggs from quenda feces (range):
639 length: 35µm – 45µm; width: 32.5µm – 45 µm.
640 **Figure 5.** *Physaloptera* spp. egg observed in quenda feces by fecal flotation microscopy at 400x
641 magnification. Approximate dimensions of *Physaloptera* spp. eggs from quenda feces (range):
642 length: 45 µm – 55 µm; width: 27 µm – 32.5 µm.
643 **Figure 6.** Strongyloid (family Strongyloididae) egg observed in quenda feces by fecal flotation
644 microscopy at 400x magnification. Approximate dimensions of strongyloid eggs from quenda
645 feces (range): length: 40 µm – 45 µm; width: 15µm – 20 µm.
646 **Figure 7.** *Trichuris* spp. egg observed in quenda feces by fecal flotation microscopy at 400x
647 magnification. Approximate dimensions of *Trichuris* spp. eggs from quenda feces (range):
648 length: 47.5 µm – 60 µm; width: 22.5 µm – 27.5 µm.
649 **Figure 8.** *Potorolepis* spp. egg observed in quenda feces by fecal flotation microscopy at 400x
650 magnification. Image is focused on the egg wall. Approximate dimensions of *Potorolepis* spp.
651 eggs from quenda feces (range): length: 65 µm – 102.5 µm; width: 65µm – 80 µm.
652 **Figure 9.** *Potorolepis* spp. egg observed in quenda feces by fecal flotation microscopy at 400x
653 magnification. Image is focused on the hexacanth embryo. Approximate dimensions of
654 *Potorolepis* spp. eggs from quenda feces (range): length: 65 µm – 102.5 µm; width: 65µm – 80
655 µm.