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Evaluation of the Mood-Stabilizing Agent Valproic Acid as a Preventative for Toxoplasmosis In Mice and Activity Against Tissue Cysts in Mice

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ABSTRACT: *Toxoplasma gondii* is a common intracellular protozoan infection of humans worldwide. Severe disease can occur in immunocompromised individuals and the in the fetuses of nonimmune pregnant women. Chronic infection is associated with vision and hearing problems, and functional mental alterations, including schizophrenia. The mood-stabilizing agent valproic acid has been shown to inhibit the development of *T. gondii* in vitro at dosages that are normally achieved in the serum and cerebral spinal fluid of human patients and to have positive effects on the behavior of rats chronically infected with *T. gondii*. The present study was done to examine the in vivo activity of valproic acid against acute toxoplasmosis in mice. Two studies were done with valproic acid given in the drinking water at concentrations of 1.5 mg/ml (Experiment 1) or 3.0 mg/ml (Experiment 2). In a third experiment (Experiment 3), valproic acid was injected intraperitoneally (i.p.) at doses of 200 or 300 mg/kg every 12 hr. Valproic acid was not effective in preventing acute toxoplasmosis. All mice treated with valproic acid died or were killed and did not ($P > 0.05$) live significantly longer than the controls. Tachyzoites were demonstrated in the tissues of infected valproic-acid–treated mice. A fourth study was done to determine if valproic acid has activity against *T. gondii* tissue cysts in chronically infected mice. Mice were chronically infected with the ME-49 strain of *T. gondii* for 8 wk and then treated orally with valproic acid at approximately 6.6 mg/ml (800 mg/kg/day) in the drinking water for 10 wk (amount was varied due to increasing mouse weights). No significant differences ($P > 0.05$) were present in tissue cyst numbers in valproic-acid–treated *T. gondii* chronically infected mice and in mice chronically infected with *T. gondii* but not given valproic acid. Our results indicate that valproic acid, although effective in vitro against *T. gondii* tachyzoites, is not effective as a preventative in mice inoculated with *T. gondii* tachyzoites. Additionally, no activity against tissue cysts was observed in chronically *T. gondii*–infected valproic-acid–treated mice.

Toxoplasma gondii is a protozoan parasite that infects humans and most other warm-blooded animals. Humans become infected by ingesting meat containing tissue cysts or by ingesting sporulated oocysts in the environment. Researchers indicate that there are 1,500,000 cases of toxoplasmosis in the United States each year, and about 15% of those infected have clinical toxoplasmosis (Jones, Kruszon-Moran et al., 2001). The importance of maternal infection and congenital toxoplasmosis has long been recognized (Jones, Lopez et al., 2001). The role of chronic *T. gondii* infection on human health was manifested in the AIDS epidemic, with the numerous cases of toxoplasmic encephalitis due to reactivated infection and clinical toxoplasmic encephalitis. The association of chronic *T. gondii* infection and behavioral changes has come to light over recent years and has been strengthened by many studies in humans, mice, and rats (Holliman, 1997; Webster, 2001). Most notably, the association of chronic *T. gondii* infection and schizophrenia has gained attention (Yolken et al., 2001; Ledgerwood et al., 2003; Torrey and Yolken, 2003; Bachmann et al., 2005; Brown et al., 2005; Wang et al., 2006).

Valproic acid is a mood stabilizer used in the treatment of mental illness, including bipolar disorder and schizophrenia (see Bowden, 2007). It has also been shown to inhibit *T. gondii* reproduction in human fibroblast cell cultures (Jones-Brando et al., 2003; Strobl et al., 2007). The IC_{50} of valproic acid (sodium salt) in a tachyzoite production assay was 266 μg/ml (Strobl et al., 2007), whereas in an ELISA-based assay the IC_{50} of valproic acid (free acid) was 4.7 μg/ml and the IC_{50} of valproic acid (sodium salt) was 4.1 μg/ml (Jones-Brando et al., 2003). These studies indicate that this drug is active against tachyzoites of *T. gondii*.

Rats chronically infected with *T. gondii* lose their innate fear of cat odor (Berdoy et al., 2000; Vyas et al., 2007). This makes them easier prey for cats and enhances the transmission of the parasite. The effects of valproic acid on the feline avoidance behavior of chronically infected rats was examined by Webster et al. (2006) and it was shown to help treated *T. gondii*–infected rats (40 mg/kg valproic acid/day orally) retain their innate avoidance of cat smell.

The present study was done to examine the anti–*T. gondii* activity of valproic acid in the prevention of acute toxoplasmosis in mice or activity against the tissue cyst stage in chronically infected mice. Female ICR mice were housed in groups of 5 mice per cage (Experiments 1–3) or 3–4 mice per cage (Experiment 4). Mice in Experiments 1–3 were inoculated subcutaneously with 5×10^3 tachyzoites of the RH strain of *T. gondii* on Day 0. Valproic acid was given in the drinking water 1 day prior to subcutaneous inoculation of mice in Experiments 1 and 2. Valproic-acid–containing water in lightproof water bottles was provided ad libitum for the remainder of the study. Fresh valproic-acid–containing water was provided every 2 days. For dosing considerations, we assumed that each mouse would drink 4 ml of water each day. Saccharin was added at 0.2% (w/v) to mask the flavor of the valproic-acid–treated water.

Experiment 1 contained 10 mice treated with 1.5 mg/ml valproic acid (Groups 1 and 2) in the drinking water supplemented with 0.2% (w/v) saccharin and 10 mice not treated with valproic acid (Groups 3 and 4) (Table I). Saccharin was added at 0.2% (w/v) to 1 group of 5 of these mice (Group 3) given water without valproic acid. Experiment 2 contained 5 mice treated with 3 mg/ml valproic acid in the drinking water (Group 5) and 5 mice not treated with valproic acid (Group 6) (Table II). The mean weight of valproic-acid–treated mice in Experiment 1 was 24 g and in Experiment 2 the mean weight was 22 g. Valproic-acid concentrations in the drinking water provided doses of 250 mg/kg/day (Experiment 1) and 545 mg/kg/day (Experiment 2). These dosages translate to 300 mg/kg and 600 mg/kg for a 20-g mouse, respectively.

Experiment 3 was done to evaluate valproic acid administered intraperitoneally (i.p.) at doses of 200 or 300 mg/kg every 12 hr (Table III). The mean weight of valproic-acid–treated mice was 23 g. Valproic acid was dissolved in sterile saline (0.14 M NaCl solution) and i.p. injections were started 2 days prior to RH strain *T. gondii* infection with 5×10^3 tachyzoites s.c. There were 2 groups (Group 7 and 8) of 5 mice each that received the 400-mg/kg/day total dose and 2 groups (Group 9 and 10) of 5 mice each that received the 600-mg/kg/day total dose. A group of 5 mice (Group 11) were infected controls and treated every 12 hr with i.p. sterile saline only.

Experiment 4 (Table IV) was conducted to determine if valproic acid has activity against *T. gondii* tissue cysts in vivo. Eight mice (4 mice in Group 12 and 4 mice in Group 13) were s.c. infected with 1×10^3 tachyzoites of the ME49 strain of *T. gondii* in 0.5 ml HBSS and left untreated for 8 wk. Three mice (Group 14) were s.c. inoculated with 0.5 ml HBSS and treated similarly. After 8 wk, mice in Groups 12 and 14 were provided drinking water containing 0.2% saccharin and approximately 6.6 mg/ml (amount varied because of mouse weights, which changed during the study) valproic acid for 10 wk to deliver a daily dose of 800 mg/kg. Mice in Groups 12–14 were killed 10 wk after valproic-acid treatment, and their brains were removed. The left half of the brain was homogenized in 2 ml HBSS for 2 min with the use of a stomacher machine (Seward Lab Blender Stomacher 80, London, England). The numbers of tissue cysts in a 50-μl sample of the homogenized brain was determined with the use of light microscopy.

TABLE I. Protocol and results of Experiment 1 on 1.5-mg/ml dose of valproic acid given in the drinking water on acute toxoplasmosis in mice.

Group	Mouse number	Dose of valproic acid*	Day post inoculation died/killed
1	1	1.5 mg/ml	Died 9
1	2	1.5 mg/ml	Died 10
1	3	1.5 mg/ml	Died 11
1	4	1.5 mg/ml	Died 11
1	5	1.5 mg/ml	Died 12
2	6	1.5 mg/ml	Died 10
2	7	1.5 mg/ml	Died 11
2	8	1.5 mg/ml	Died 12
2	9	1.5 mg/ml	Died 12
2	10	1.5 mg/ml	Died 13
3	11	None	Died 10
3	12	None	Died 10
3	13	None	Died 11
3	14	None	Died 11
3	15	None	Died 11
4	16	None	Died 10
4	17	None	Died 11
4	18	None	Died 11
4	19	None	Died 11
4	20	None	Died 12

* Provided continuously in water in lightproof drinking bottle to provide an estimated total dose of 250 mg/kg/day.

The right half of the brain was fixed in 10% neutral buffered formalin solution and processed routinely for histological examination following staining with hematoxylin and eosin.

Impression smears were made from the livers or lungs of mice that died or were killed during the study. They were examined unstained for tachyzoites with the use of an Olympus BH60 microscope equipped with interference contrast optics.

Kaplan–Meier survival analysis was performed with the use of PrismGraphpad version 4.0 on mice in Experiments 1–3. The data were analyzed for statistical significance with the use of the chi-square and log-rank tests with a P value of 0.05. Tissue cyst counts from mice in Experiment 4 were examined with the use of a 1-way ANOVA and an unpaired t-test with a P value of 0.05.

Acute toxoplasmosis occurred in all mice given the RH strain (Tables I–III). Neither oral (Experiments 1 and 2) nor i.p. (Experiment 3) treatment with valproic acid was effective. Deaths occurred in treated mice from 8 to 13 days after inoculation with tachyzoites and in untreated mice 9–12 days after tachyzoite inoculation (Tables I–III). There was no significant positive effect of valproic-acid treatment on mouse survival ($P > 0.05$). Tachyzoites were seen in tissues of all mice given the RH strain of *T. gondii*.

None of the mice inoculated with tachyzoites of the ME49 strain of *T. gondii* died during the study (Groups 12 and 13). None of the mice

TABLE II. Protocol and results of Experiment 2 on 3-mg/ml dose of valproic acid given in the drinking water on acute toxoplasmosis in mice.

Group	Mouse number	Dose of valproic acid*	Day postinoculation died/killed
5	21	3.0 mg/ml	Died 11
5	22	3.0 mg/ml	Died 11
5	23	3.0 mg/ml	Died 12
5	24	3.0 mg/ml	Died 12
5	25	3.0 mg/ml	Died 12
6	26	None	Killed 10
6	27	None	Killed 10
6	28	None	Died 10
6	29	None	Killed 11
6	30	None	Killed 11

* Provided continuously in water in lightproof drinking bottle to provide an estimated total dose of 545 mg/kg/day.

TABLE III. Protocol and results of Experiment 3 examining 2 doses of valproic acid given intraperitoneally every 12 hr on acute toxoplasmosis in mice.

Group	Mouse number	Total dose of valproic acid*	Day post inoculation died/killed
7	31	400 mg/kg	Died 9
7	32	400 mg/kg	Killed 9
7	33	400 mg/kg	Died 10
7	34	400 mg/kg	Killed 10
7	35	400 mg/kg	Killed 10
8	36	400 mg/kg	Died 8
8	37	400 mg/kg	Killed 9
8	38	400 mg/kg	Killed 10
8	39	400 mg/kg	Died 10
8	40	400 mg/kg	Killed 10
9	41	600 mg/kg	Died 9
9	42	600 mg/kg	Killed 10
9	43	600 mg/kg	Died 10
9	44	600 mg/kg	Killed 10
9	45	600 mg/kg	Killed 10
10	46	600 mg/kg	Killed 9
10	47	600 mg/kg	Killed 10
10	48	600 mg/kg	Killed 10
10	49	600 mg/kg	Killed 10
10	50	600 mg/kg	Killed 10
11	51	None†	Killed 9
11	52	None	Killed 10
11	53	None	Died 10
11	54	None	Died 10
11	55	None	Killed 11

* Given intraperitoneally as 200 or 300 mg/kg every 12 hr.
† Sterile saline given intraperitoneally every 12 hr.

TABLE IV. Protocol and results of Experiment 4 on 6.6-mg/ml dose of valproic acid given in the drinking water* for 10 wk on chronic toxoplasmosis in mice.

Group	Mouse number	Dose of valproic acid	Inoculated with *Toxoplasma gondii*	Number of tissue cysts
12	56	3.0 mg/ml	Yes	0
12	57	3.0 mg/ml	Yes	1
12	58	3.0 mg/ml	Yes	1
12	59	3.0 mg/ml	Yes	3
13	60	None	Yes	0
13	61	None	Yes	1
13	62	None	Yes	3
13	63	None	Yes	4
14	64	3.0 mg/ml	No	0
14	65	3.0 mg/ml	No	0
14	66	3.0 mg/ml	No	0

* Provided continuously in water in lightproof drinking bottle to provide an estimated total dose of 800 mg/kg/day.

given only valproic acid (Group 14) died during the study. Tissue cysts were structurally normal when viewed as fresh preparations with light microscopy. They were also normal when viewed in stained histological sections. The mean number of tissue cysts in 50 μl was 1.3 ± 1.3 (range, 0–3) for mice in Group 12 and 2.0 ± 1.8 (range, 0–4) for mice in Group 13. No tissue cysts were seen in the brains of mice in Group 14. There were no significant differences ($P > 0.05$) in tissue cyst counts in mice infected with the ME49 strain of *T. gondii* and treated with valproic acid (Group 12) and those infected but not treated with valproic acid (Group 13).

Valproic acid is active against tachyzoites of *T. gondii* in 2 different cell-culture–based assays (Jones-Brando et al., 2003; Strobl et al., 2007). The studies of Jones-Brando et al. (2003) indicate that valproic acid is active at lower concentrations than reported by Strobl et al. (2007), but both indicate that it is active and the differences are probably due to different test systems used to determine activity.

The results of the present study indicate that valproic acid is not active in preventing acute toxoplasmosis in mice. The highest oral daily dose of 800 mg/kg/day we tested is approaching the oral LD_{50} dose of this agent in mice (1,098 mg/kg); the highest i.p. dose of valproic acid tested, 600 mg/kg/day, is greater than the LD_{50} in mice for this route of administration (470 mg/kg) (American Pharmaceutical Partners, Inc., Bedford Labs, Bedford, Ohio). Therefore, further increasing the dose of valproic acid is impractical. In contrast, Webster et al. (2006) demonstrated that oral treatment of chronically *T. gondii*–infected rats with 40 mg/kg/day of valproic acid was beneficial in their study system. Treated *T. gondii*–infected rats retained their innate avoidance of feline smell (cat urine). The mode of action of valproic acid, including which stage of *T. gondii* is affected by valproic acid in the rats, is not known. Because the rats were chronically infected, it is possible that valproic acid acted on the tissue cyst/bradyzoite stages of *T. gondii*. Valproic acid might also influence the bradyzoite-to-tachyzoite or tachyzoite-to-bradyzoite stage conversion. We did not demonstrate a significant ($P > 0.05$) effect of valproic acid on the numbers of tissue cysts in treated mice versus controls. Additional study is needed to examine the activity of valproic acid and similar agents against the tissue cysts/bradyzoites of *T. gondii*.

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Molecular Characterization of *Babesia kiwiensis* From the Brown Kiwi (*Apteryx mantelli*)

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ABSTRACT: To further investigate the recently described avian piroplasm, *Babesia kiwiensis*, blood samples were collected from 13 wild-caught and 8 zoo-captive brown kiwi (*Apteryx mantelli*) and screened for the presence of piroplasm DNA using a nested-polymerase chain reaction (PCR) targeting the 18S rRNA gene of most members of Piroplasmida. All captive birds gave a negative PCR result, while 12 wild-caught birds were PCR positive. The nearly full-length 18S rRNA gene for *B. kiwiensis* was sequenced. Upon phylogenetic analysis, it was found to belong to the babesid group of piroplasms and was ancestral, yet genetically similar, to the *Babesia canis*-related species. An insight into the current taxonomy of the avian piroplasms is also given. An *Ixodes anatis* tick collected from 1 of the North Island brown kiwi was also screened using PCR and was found to be positive for *B. kiwiensis* DNA.

The avian piroplasms (Piroplasmida) remain an understudied group of protozoans. In comparison to many of the mammalian species of

Babesia and *Theileria*, very little is known about the life cycle or biology of any of the avian species (Peirce, 1975, 2000). To date, approximately 18 species of *Babesia* have been described from birds, although much confusion exists over the correct taxonomic classification of each of these (Peirce, 2000; Peirce et al., 2003; Peirce, 2005). The introduction of molecular-based analysis is likely to provide greater insights into the biology of these piroplasms, in addition to offering improved methods of detection, differentiation, and solutions to current taxonomic idiosyncrasies.

An avian species of piroplasm, *Babesia kiwiensis*, was recently described on the basis of morphology alone in the brown kiwi (*Apteryx mantelli*) of the North Island of New Zealand (Peirce et al., 2003). The kiwi is the smallest member of the ratites (Struthioniformes) and is one of the most primitive birds alive today (Heather and Robertson, 2005). All kiwi species are currently are currently threatened with extinction (Stattersfield et al., 2000), with the key factor contributing to their decline being predation by introduced mammals (McLennan et al., 1996). The pathogenicity of *B. kiwiensis* is currently unknown, as is much of the life cycle and biology of this species.

The present study aimed to identify kiwi infected with *Babesia* sp. by using PCR, to characterize *B. kiwiensis* on the basis of the 18S rRNA gene sequence, and phylogenetically compare this species to other members of *Babesia*.

Blood samples used in this study were collected by venipuncture from 13 wild-caught juvenile North Island brown kiwi at 3 locations (Rarewarewa, Riponui, and Purua) in Northland, New Zealand, during a field trip in September and October 2003. For a description of the study sites, see Robertson et al. (1999). Additional blood samples were collected from 8 captive North Island brown kiwi in the Auckland Zoo. Thin blood films were made immediately; blood was mixed into EDTA anticoagulant and stored at −20 C until PCR analysis. Ticks were also collected from the wild-caught birds and preserved in 70% ethanol. Both blood and tick samples were transported on ice to Australia for analysis.

Thin blood films were stained with Wright-Giemsa stain (Hematek Stain Pak, Bayer Diagnostics Europe, Ltd., Dublin, Ireland) and examined using an Olympus BX50 microscope (Olympus, Tokyo, Japan) using ×100 magnification and oil immersion. A relatively uniform region of the blood film was viewed and approximately 2,000 erythrocytes were examined for the presence of parasites.

Individual ticks were morphologically identified to the species level, then macerated with a sterile scalpel blade before DNA was extracted using a QIAamp tissue kit (QIAGEN, Berlin, Germany). DNA was purified from the blood samples using the QIAamp blood mini kit (QIAGEN) following the manufacturer's instructions.

All samples were initially screened for piroplasm species using a nested PCR capable of detecting most members of the Piroplasmida (Jefferies et al., 2007) that amplified an approximately 800 bp region of the 18S rRNA gene. Amplification of the near-complete 18S ribosomal RNA gene was performed using primers previously described by Criado-Fornelio et al. (2003a). PCR assays were performed in a final reaction volume of 25 μl that consisted of the following: 10× polymerase buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 μM of each primer (12.5 ng/ml), 0.5 U Tth DNA polymerase and 2 μl extracted template DNA. PCR products were electrophoresed on a 1% agarose gel and visualised using ethidium bromide and UV illumination. Amplified products were purified using an UltraClean Gelspin DNA Purification Kit (MO Bio Laboratories Inc., Sohlana Beach, California). Sequencing reactions were performed using an ABI Prism DyeTerminator Cycle Sequencing Core kit (Applied Biosystems, Foster City, California) and sequence data was analysed using 4peaks analysing software (v 1.7.1 for Mac OS X, A. Griekspoor and Tom Groothuis, mekentosj.com).

Using ClustalW, a 1,472 bp region of the 18S rRNA gene of *B. kiwiensis* was aligned with sequences from 55 other *Babesia* and selected other piroplasm species (GenBank accession numbers are given in Fig. 1) obtained from the GenBank database. *Hepatozoon canis* (GenBank AY789075) was also included in analyses as an outgroup species. Phylogenetic relationships were determined using TREECON v1.3b (using the Gaultier and Gouy distance method and neighbor-joining method algorithms) (Van de Peer and De Wachter, 1993) and MEGA v2.1 softwares (minimum evolution) (Kumar et al., 2001). Statistical support was provided by bootstrapping 1,000 replicates. Bootstrap values greater than 70% were considered statistically significant.

In total, 8 blood films, corresponding to 8 of the 13 wild-caught birds, were available for microscopic examination. Intra-erythrocytic piroplasms similar to those described by Peirce et al. (2003), including a tetrad ('Maltese cross') form, were observed in 6 of the 8 blood films. An approximately 800 bp region of the 18S rRNA gene was amplified by nested PCR from blood in 12 of 13 wild-caught birds. No DNA amplification was observed for any of the captive kiwi. A tick, identified as *Ixodes anatis*, was removed from 1 of the infected birds and subjected to DNA extraction; an approximately 700 bp product was also amplified by nested-PCR. All PCR products were sequenced and found to be identical.

The near-complete 18S rRNA gene was then amplified and 1,510 bp were sequenced from 3 of the infected kiwi, then BLAST searched and found to be most homologous to *Babesia canis vogeli* (GenBank AB083374), sharing 94% sequence identity. All 3 kiwi sequences were identical and submitted to the GenBank database (GenBank EF551335). Distance-based and neighbor-joining phylogenetic analysis on the basis of the 18S rRNA gene (1,472 of the total 1,510 bp; 531 informative sites) revealed 3 distinct groups within *Babesia* (Fig. 1). Each cluster was classified in accordance to groups proposed by Criado-Fornelio et al. (2003b). Strong statistical support was provided for the separation of the archaeopiroplasmids, combined prototheilerid-theilerids, and babesid species into 3 major clades. Similar groups were also observed for minimum evolution analysis with the exception of the prototheilerids and theilerids being separated into 2 distinct clades (data not shown). The sequence for *B. kiwiensis* was shown to be genetically distinct and ancestral to the *B. canis* subspecies, *Babesia gibsoni*, *Babesia odocoilei*, *Babesia capreoli*, and *Babesia divergens* within the babesid group, forming a branch divergent from all other known piroplasm species. The other avian species included in the analysis were ancestral from *B. kiwiensis*, with *Babesia poelea* forming a clade with *Babesia conradae* and *Babesia duncani* within the prototheilerid/theilerid group and *Babesia bennetti* clustering with *Babesia ovis* and *Babesia bovis*.

The present study has successfully characterized *B. kiwiensis* on the basis of the 18S rRNA gene, adding to only 2 previously genetically-described avian species. Interestingly, this species is not closely related to *B. poelea* from the brown booby, *Sula leucogaster* (Work and Rameyer, 1997; Yabsley et al., 2006) or *B. bennetti* from the yellow-legged gull, *Larus cachinnans* (Merino, 1998; Criado et al., 2006). It is more genetically similar, yet ancestral, to the *Babesia canis*-related species within the babesid group described by Criado-Fornelio et al. (2003b). Indeed, the unique branch separation of *B. kiwiensis* suggests that this species may be representative of an as yet uncharacterized group of piroplasms that may have co-evolved with the palaeognathes (ratites and tinamous), which are themselves taxonomically separated from all other living birds (neognathes) (Cooper et al., 2001). Identification of possible piroplasm species within other members of the Struthioniformes, such as the emu and ostrich, may also give further insight into the evolutionary origins of *B. kiwiensis*. Alternatively, *B. kiwiensis* may have originated in a mammalian host, introduced to New Zealand with the animals accompanying Polynesians (dogs and Pacific rats) or European settlers (including dogs, cats, rats, mustelids, pigs, and brushtailed possums), none of which has been screened for *B. kiwiensis* to the best of the authors' knowledge. However, with the exception of *B. kiwiensis*, New Zealand is considered free of *Babesia* spp. (Beban, 2003).

The kiwi itself, being both flightless and ground dwelling, is also susceptible to tick infestation, principally by *Ixodes anatis*, which is found in considerable numbers in kiwi nests and engorged on kiwi chicks (Peirce et al., 2003). The identification of *B. kiwiensis* DNA in a semi-engorged *I. anatis* tick taken from an infected kiwi chick raises the possibility, originally suggested by Peirce et al. (2003), that this tick species is a vector candidate. It is important to note, however, that detection of DNA in the tick does not imply a life cycle contribution, as all hematophagous arthropods can potentially contain the DNA of blood pathogens. Further research is, therefore, necessary before a definitive vector can be elucidated.

From a taxonomic viewpoint, the classification of *B. kiwiensis* within *Babesia* by Peirce et al. (2003) is in agreement with the 18S rRNA gene phylogenetic analysis provided by the present study. The discovery of tetrad schizonts stages in the *B. kiwiensis* lifecycle (Peirce et al., 2003) is, however, anomalous with other members of the babesid group, but similar to the morphology observed in most other avian piroplasms

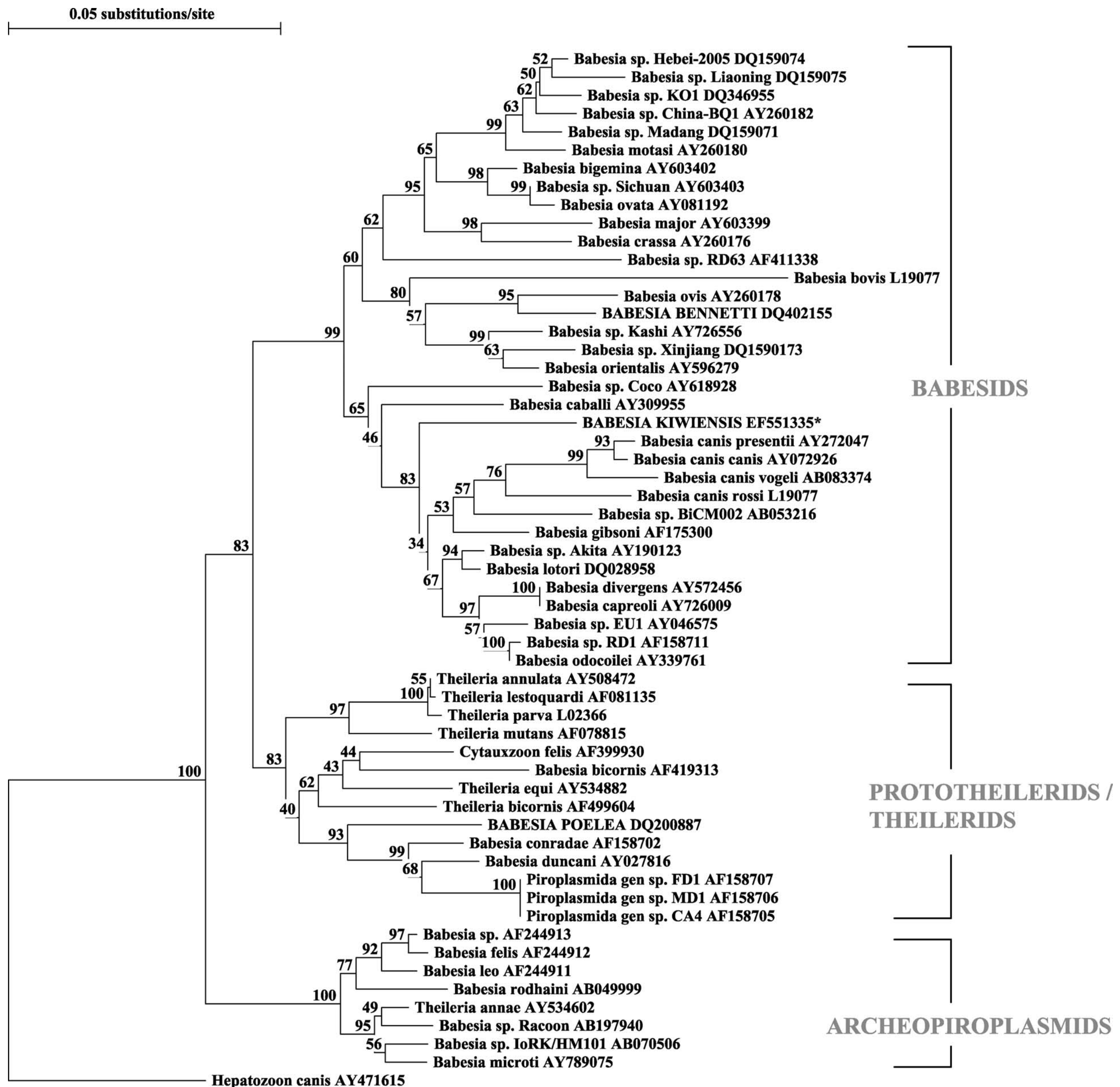

FIGURE 1. Phylogenetic tree constructed using partial 18S rRNA gene sequences based on distance (Gaultier and Gouy) and neighbor-joining analyses. Numbers above branches represent bootstrap percentages of 1,000 replicates. Avian species are shown in capitals and the novel sequence is indicated with an asterisk.

(Peirce, 2000). One of the greatest points of contention surrounding the classification of the avian piroplasms within *Babesia* is that a majority of avian species develop cruciform tetrad schizonts in typical 'Maltese cross' formations and some may develop exoerythrocytic schizont life cycle stages, although this has been disputed (Peirce, 2000). The latter of these life cycle stages has previously been considered to be characteristic of *Theileria* (Zahler et al., 2000). Analyses carried out by Yabsley et al. (2006) and the present study found *B. poelea* to be phylogenetically similar to *B. duncani* and *B. conradae* (the Western clade piroplasms-prototheilerids), which also exhibit tetrad schizonts (Conrad et al., 2006; Kjemtrup et al., 2006) as do many of the species within the archaeopiroplasmid group (Zahler et al., 2000; Yokoyama et al., 2003) and could, therefore, be an ancestral characteristic among the piroplasms. It is possible that *B. kiwiensis* has simply retained this ancestral trait not observed in the other babesid group species and this warrants further study.

The current taxonomic paraphyly within the Piroplasmida (Reichard et al., 2005; Allsopp and Allsopp, 2006) is also exposed by the present study, with members of *Babesia* represented in all 3 phylogenetically distinct clades. While molecular-based analysis of these species will hopefully allow for a greater understanding of piroplasm biology, the taxonomic confusion that exists within *Babesia* and the Piroplasmida needs to be addressed urgently.

The current study has also shown that PCR offers an effective method of rapidly and accurately identifying kiwi infected with *B. kiwiensis* and is more sensitive than microscopic-based diagnosis in accordance with numerous other studies (Almeria et al., 2001; Ano et al., 2001). This detection method can, therefore, potentially be used to identify infected kiwi and to assess the pathogenicity of this infection in future studies aiming to conserve these threatened bird species.

The authors are grateful to Ngati Hine for supporting the collection and export of the samples from their traditional lands for this study. The samples were obtained from kiwi being studied as part of a Department of Conservation and Bank of New Zealand Save the Kiwi

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Ticks, *Amblyomma rotundatum* (Acari: Ixodidae), on Toads, *Chaunus schneideri* and *Chaunus granulosus* (Anura: Bufonidae), in Northern Argentina

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ABSTRACT: This communication provides notes on 2 species of toads, *Chaunus schneideri* and *Chaunus granulosus*, infested with ixodid ticks, *Amblyomma rotundatum*, from the provinces of Corrientes and Formosa in northern Argentina. *Chaunus schneideri* is a new amphibian host record for *A. rotundatum*, a species previously reported to parasitize other anurans and also reptiles. We examined 74 ticks on 5 toads. All ticks were *A. rotundatum*; all adults were females, and all developmental stages were randomly attached to host body parts. Ticks remained

attached to one of the toads for from 7 to 17 days after the host was captured. One toad, encumbered with 33 ticks, was moribund when found and died shortly thereafter.

On the evening of 9 November 2006, we captured 4 male bufonid toads, *Chaunus schneideri* (Werner 1894) (formerly *Bufo paracnemis* Lutz 1925), on the 8-ha compound of the Centro de Ecología Aplicada del Litoral (CECOAL), a field station in the suburbs of the city of Corrientes, in Corrientes Province (27°30′S, 58°45′W). CECOAL is operated by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina. *Chaunus schneideri* is a giant toad, the largest species in Argentina. Weights and snout vent lengths (SVL) of the 4 toads were as follows: 654 g, 185.5 mm; 474 g, 154.4 mm; 546 g, 186.6 mm; and 516 g, 160.7 mm. We noticed that the first of these toads that we encountered feeding on insects (mostly beetles) at an insect light trap was conspicuously infested with hard ticks (Ixodidae). This prompted us to search the CECOAL compound for additional toads. We found 3 more males, and all 3 males carried ticks. We encountered no female *C. schneideri* on the compound. Ticks on male toads represented larvae (seed ticks) and engorged nymphs or adults. Five, 6, 9, and 18 were the tick counts for the 4 toads.

On 11 November 2006, we noted the locations of ticks attached to the 4 toads, and then we used ice to relax the ticks, which were removed from 3 of the toads with watchmakers' forceps. We observed that each site of tick attachment to the toads' skin showed a small, 2–4 mm reddened spot of inflammation. After we removed the ticks, we released the toads back into the CECOAL compound. The toads were robust, active, and none seemed negatively affected by their tick loads or by having had the ticks removed. Ticks were attached to the toads' legs, feet, throat, abdomen (venter), back (dorsum), posterior quarter, and on the head. The points of attachment seemed random and evidenced no pattern to suggest that they had preferred feeding locations on the toads. However, we observed that no ticks were attached to these bufonids' large paratoid glands, which may contain high concentrations of the toxins bufonin/bufotalin. We removed ticks from the toads, and we killed the ticks in 100% ethanol; alcohol was changed after approximately 1 hr. We used a computer based video microscope system (ProScope®; Bodelin Technologies, Lake Oswego, Oregon) with 10 and 50× lenses to produce scaled images of each tick, and we used the NIH public domain ImageJ software to measure these images. We removed 29 ticks from 3 toads. The ticks ranged in size (length × width) from 1.03 × 0.78 mm to 9.28 × 5.33 mm. The range in ratios of length to width (a possible index of degree of engorgement) was 1.17 to 1.55. We held one of the infested toads in a laboratory basin, and we observed it once a day to determine when its ticks would voluntarily abandon their host. Abandonment for 4 ticks occurred at 7, 11, 12, and 17 days from the day of host capture. All of the ticks were *Amblyomma rotundatum* (Koch 1844) (Keirans and Oliver, 1993; and independently determined by Dr. Lorenza Beati).

On 6 December 2006, 1 of us (E.F.S.) captured an adult male *Chaunus granulosus* (Spix, 1824) at La Maravilla, 60 km southwest of Ingeniero G. Juárez, Formosa Province (23°54′S, 61°51′W). This specimen of a very small species was just 60 mm SVL and weighed only 16.2 g. It was infested with 3 *A. rotundatum* (confirmed by Dr. Lorenza Beati), each attached to a different leg. *Chaunus granulosus* has previously been recognized as a host of *A. rotundatum* (Burridge and Simmons, 2003, and references therein). It would seem that this relatively tiny toad species might be especially vulnerable to a critical loss of blood taken by feeding ticks.

Years earlier, on 26 December 1998, 1 of us (A.I.K.) found a heavily tick-infested male *C. schneideri* on the CECOAL compound. This individual contained 33 ticks on its legs, feet, and body. The toad was 137.8 mm long (SVL) and emaciated, weighing a slight 365 g. Remarkably, this toad was found during the day walking very slowly and in a labored manner. The air temperature at the time was >30 C. *Chaunus schneideri* is rarely seen moving during the daylight hours and especially not when the air temperature is high. Each time the toad had advanced approximately 40–50 cm, the animal arched its back with nose and posterior pointed high creating a contorted and aberrant concave posture that it held for several seconds. This pattern of behavior was observed repeatedly before the toad was taken to the laboratory, where it flattened itself against the floor and remained motionless until it died <30 min later. We attribute the morbidity and mortality of this toad to exsanguination (see Keirans and Durden, 1998) by its extraordinary load of ticks. The toad with its attached ticks was preserved in formaldehyde shortly after it died. Recently, we removed 9 ticks from the preserved specimen, and we measured them with calipers. These ticks ranged in size from 5.6 × 4.4 to 15.8 × 11.4 mm (length × width). The 9 randomly removed ticks were all *A. rotundatum* (determined by Dr. Lorenza Beati).

This toad's morbidity and death reinforces previous assertions that ticks may play an important role in regulating natural toad populations in tropical and subtropical regions of the New World (Oba and Schumaker, 1983; Lampo and Bayless, 1996). If this is true, ticks could have value as a biological control agent for exotic ''giant toads'' that have been introduced into Australia and the United States from Central and South America. Unregulated populations of these enormous exotic toads have expanded dramatically, poisoning pets and predators, and they are threatening native species by both indirect and direct competition. *Amblyomma rotundatum* has been reported on exotic giant toads, *Chaunus marinus* (Linnaeus, 1758) (formerly *Bufo marinus*), from the greater Miami, Florida area (Oliver et al., 1993), but the impact of the ticks on the introduced U.S. *C. marinus* population has not been evaluated.

All of the ticks we recovered from the 6 toads we examined were females. Populations of *A. rotundatum* from within its historic range in Central and South America have long been known to be parthenogenetic (Robinson, 1926), but 1 laboratory-reared male (Keirans and Oliver, 1993) and 1 male from a field-collected host (Labruna et al., 2005) have been found. Parthenogenesis may have evolved in these ticks of reptiles and amphibians because of the difficulty of movement on reptilian and amphibian skin (compared with feathered or furred skin) by adult males to find and mate with adult females. If the parent species to *A. rotundatum* was a sexually reproducing parasite of mammals, birds, or both, and in the course of its speciation it underwent a host shift to amphibians and reptiles, we can imagine that a proportion of females of the new species might have gone unmated owing to the relative difficulty of male movement to access females on the slippery amphibian and reptilian skin. Failure to be mated could have created intense selection on females to produce asexual offspring. Molecular phylogenetics data indicate that the hypothesized host shift from mammals or birds to amphibians and reptiles within species of *Amblyomma* is probable, suggesting that the taste for amphibian and reptilian blood is derived (Klompen et al., 1996).

Dr. Lorenza Beati, U.S. National Tick Collection, Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, Georgia, kindly examined and identified all of tick specimens and helped enlighten R.L.S. about tick evolution. All of the tick specimens have been contributed to the U.S. National Tick Collection and received accession numbers 124001–124010. R.L.S. and J.A.S. are grateful to Drs. Juan José Neiff and Alicia Poi de Neiff for providing living quarters, assistance, congenial company, and generous hospitality during their stay at the CECOAL in Corrientes.

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A Cautionary Note on the Use of Nested PCR for Parasite Screening—An Example From Avian Blood Parasites

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ABSTRACT: The use of new powerful nested polymerase chain reaction (PCR) techniques to identify and screen for prevalence of parasites has a huge potential. It allows for the detection and identification of low-intensity infections, but its high sensitivity and technical setup may also induce problems. Here, we report a cautionary note regarding misleading amplification of avian malaria species (*Haemoproteus* and *Plasmodium*) during *Leucocytozoon* spp. detection. We used a previously described nested PCR method for the molecular detection of avian malaria and *Leucocytozoon* spp. In the first step of the PCR protocol, these parasites are detected simultaneously; in the second PCR, *Haemoproteus* and *Plasmodium* spp. are separated from *Leucocytozoon* spp. However, in certain cases when a bird was infected with avian malaria, we obtained a slightly longer PCR product during the detection of *Leucocytozoon* spp. Our data imply that these ''false'' *Leucocytozoon* fragments are the consequences of strong amplification of certain malaria lineages in the first PCR, which can also be detected after the second PCR amplification that is specific to *Leucocytozoon* spp. parasites. Because these ''false'' *Leucocytozoon* fragments are slightly longer than the normal *Leucocytozoon* fragments, we suggest the use of well-separating agarose gels, several positive controls, and molecular standards to facilitate their separation. If one obtains a fragment that differs in length from the one expected for *Leucocytozoon* spp., sequencing is essential. More generally, in order to limit this type of problem with nested PCR protocols, we suggest that the first and the second primer pair be chosen so that they have different annealing temperatures.

Using molecular methods for detecting microorganisms requires high sensitivity because these organisms often occur in low numbers (intensities) in their hosts or the environment. One way of increasing the sensitivity of a polymerase chain reaction (PCR) is to apply a nested approach, where the screening is conducted with the use of 2 PCRs that are performed sequentially. However, this approach is more costly and takes additional time. In addition, along with the increase in sensitivity comes the risk of contaminations and amplification of ''nonspecific'' genes, i.e., genes for which the primers were not designed (reviewed in Burkardt, 2000; Freed and Cann, 2006). To ensure that the correct target gene has been amplified, most studies also sequence the PCR product. However, as the sample sizes in data sets used for molecular, biological, and ecological studies steadily increase, combined with a decrease in the cost of running PCRs, large-scale ecological and biological studies may use nested PCR protocols just to screen samples for positive or negative amplifications for a group of parasites or microorganisms. To ensure the validity of such studies, it is therefore of importance to investigate and note any shortcomings or pitfalls that occur when nested PCR methods are used to screen for microorganisms.

The study of avian haemosporidian parasites, i.e., *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* spp., is one area that has greatly benefited from the use of different PCR methods in the detection of parasites from blood samples (Li et al., 1995; Bensch et al., 2000; Fallon, Ricklefs et al., 2003). Thus, several studies have demonstrated that PCR-based methods have higher sensitivity at low levels of parasitemia compared to the traditional microscopic examination of blood smears (Richard et al., 2002; Waldenström et al., 2004), though they are not flawless (Cosgrove et al., 2006; Valkiūnas et al., 2006). Comparing different molecular methods further showed that nested PCRs have the ability of detecting lower degrees of parasitemia compared to single PCRs (Waldenström et al., 2004). Thanks to these new methods for detecting blood parasites, there has been a recent boom in studies on the distribution and prevalence of avian haemosporidian parasites all over the world (Perkins and Schall, 2002; Waldenström et al., 2002; Fallon, Bermingham, and Ricklefs, 2003; Jarvi et al., 2003; Beadell et al., 2004; Kimura et al., 2006; Hellgren et al., 2007).

Here, we report a cautionary note regarding misleading amplifications due to a carryover effect of the first PCR round primers in the second reaction, when a nested PCR protocol is being used. The procedure was designed by Hellgren et al. (2004) for the simultaneous detection of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* spp. We applied it to 495 blood samples collected from different bird species, including both European migrant and African resident species. In short, the blood samples were collected in SET buffer (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0), kept at −20 C until analysis, and extracted using the standard ammonium–acetate protocol (Nicholls et al., 2000). The concentration of genomic DNA was set to 25 ng/μl; a nested PCR was performed. In the first PCR, 2 μl of the host DNA was used. The first primer pair, targeted at the mtDNA of the parasites, amplifies a 570-bp-long fragment (617-bp fragment including primers) of the cytochrome *b* gene from species of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*. In the second PCR, the detection of avian malaria parasites (*Haemoproteus* and *Plasmodium*, sensu Pérez-Tris et al., 2005) is separated from the detection of *Leucocytozoon* spp. by the use of different and more specific primers. In these reactions, 2 μl or 1 μl PCR product from the first reaction was used (for *Leucocytozoon* spp. and avian malaria, respectively). The second primer pairs bind to the fragment amplified in the first reaction, producing smaller, 478- and 480-bp-long fragments (526 and 527 bp with primers), for *Leucocytozoon* spp. and avian malaria, respectively (Hellgren et al., 2004). All reactions were performed in 25-μl volumes and both negative (ddH_2O) and positive controls (samples from birds that were previously confirmed to be infected) were applied to control for possible contamination or failures during PCRs. Amplified PCR products were sequenced with the use of the Amplicycle® sequence kit on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, California), in accordance with the manufacturer's recommendations. Sequences were edited and aligned with the use of the program BioEdit (Hall, 1999) and identified to genus level based on their position in the phylogenetic tree of previously identified parasites (Hellgren et al., 2007) with the use of the software MEGA2 (Kumar et al., 2001).

In the second PCR, specific for *Leucocytozoon* spp., 123 of the 495 samples produced a PCR product. However, in 23 cases, the fragments seen on agarose gels were slightly longer than the usual 526-bp-long *Leucocytozoon* species–specific fragment, including primers (Hellgren et al., 2004) (Fig. 1). Despite several trials, we were not able to se-

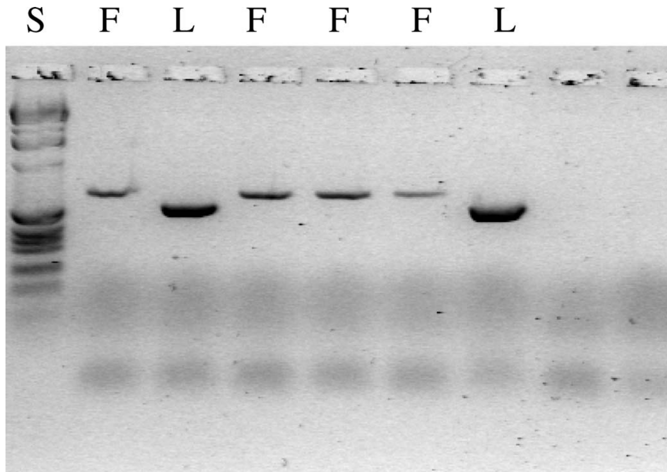

FIGURE 1. Products from the nested PCR specific to *Leucocytozoon* spp. after running in 2% agarose gel (for 30 min with 5 V/cm). S: 1-kb molecular standard, L: normal *Leucocytozoon* spp. bands, F: false *Leucocytozoon* spp. bands.

quence these fragments with the primers that are designed for the direct sequencing of *Leucocytozoon* spp. New extractions and rerunning of the PCRs under sterile conditions gave the same result, excluding the possibility of contamination during the reactions. Interestingly, these longer fragments were amplified only if the birds were infected with either *Haemoproteus* or *Plasmodium* spp. Sequencing with the first primer pair (which is designed for the simultaneous detection of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* spp.) showed that this 617-bp-long fragment (including primers) was a *Haemoproteus* or *Plasmodium* spp. sequence, indicating ''false'' amplification during the reactions specific for *Leucocytozoon* spp. parasites. Therefore, we then investigated how these *Haemoproteus* or *Plasmodium* spp. parasite sequences can be amplified in a reaction, which was designed to detect only *Leucocytozoon* spp.

We supposed that the reason for this ''false'' amplification was not that the *Leucocytozoon* species–specific primers in some cases amplify avian malaria parasites, but instead that certain malaria lineages were amplified somehow better in the first PCR, and the result of this amplification was seen also after the second PCR. To test this hypothesis, we diluted the PCR products from the first reaction to the same concentration as we used in the second reaction and visualized the samples on 2% agarose gel. These tests never resulted in any PCR product visible on the gel, suggesting that the first reaction in itself is not enough to result in a visible 617-bp-long band on the agarose gel after the second PCR.

We then performed a special nested PCR. In the first step, we used the same conditions as described by Hellgren et al. (2004). However, in the second PCR specific to *Leucocytozoon* spp., all reagents except the second primer pair were added to the samples and the reaction was performed. After visualizing the products from the second PCR on an agarose gel, we obtained a 617-bp-long band that contained the same 570-bp-long *Haemoproteus* or *Plasmodium* spp. sequence generated when running the nested PCR with the *Leucocytozoon* species–specific primers. This means that the primers from the first PCR continued the amplification of the 570-bp fragment from the first reaction also in the second reaction, though no additional primers were added and a 12.5 times dilution was applied.

The fact that we did not always obtain these longer fragments when the birds were infected with avian malaria species suggests that there are a few lineages for which the amplification by the first primer pair is stronger. Indeed, of the 63 avian malaria lineages that were found in our 181 malaria-positive samples, only 9 produced the 617-bp-long ''false'' *Leucocytozoon* spp. bands. The 9 lineages that caused these amplifications were not particularly closely related (mean Jukes-Cantor distance: *Haemoproteus* spp.: 0.062 ± 0.008 SE; *Plasmodium* spp.: 0.059 ± 0.006 SE) compared to the mean genetic distance between all lineages in our database (mean Jukes-Cantor distance: *Haemoproteus* spp.: 0.055 ± 0.006 SE; *Plasmodium* spp.: 0.058 ± 0.006 SE). This indicates that the 9 lineages do not group into a closely related clade of avian haemosporidians and the strong amplification might be a result of high parasite intensity, or analogous mutations in the primer binding sites that increase the amplification success. The latter notion is supported by the study of Valkiūnas et al. (2006), who found that in case of mixed infections of avian malaria parasites, some lineages were detected preferentially, but that this was not related to the level of parasitemia of different lineages in the blood. In addition, Sowmya et al. (2006) showed that differences in the primer-binding sites can affect the amplification success.

Based on our results, we suggest the necessity to apply molecular standards and positive controls in each gel during the detection of *Leucocytozoon* spp. parasites and run PCR products in a well-separating agarose gel for a period long enough to be able to detect differences between fragment lengths. If deviations from the standard *Leucocytozoon* species fragment length are detected, then sequencing is essential to avoid the risk of considering ''false'' detections as normal *Leucocytozoon* spp. infection. More generally, to avoid the problems that primer pairs from the first reaction continue to amplify also in the second PCR when a nested approach is used, we suggest the following. First, primer pairs should be designed so that the optimal annealing temperatures for the first and second primer pairs, if possible, are different. Second, the amount of primers used in the first reaction should be optimized so that the amount of leftover is reduced without affecting the outcome of the results.

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Helminth Fauna of the Nine-Banded Armadillo (*Dasypus novemcinctus*) in North-Central Florida

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ABSTRACT: The gastrointestinal tracts of 32 free-ranging, 9-banded armadillos (*Dasypus novemcinctus*) from north-central Florida were examined for the presence of helminths during July 1991 to November 1993. *Aspidodera* sp. (Nematoda: Aspidoderidae), most closely resembling *Aspidodera sogandaresi*, were recovered from 20 of 32 armadillos examined. Total numbers of *A. sogandaresi* ranged from 1 to 1,021 per infected animal, and followed an inverse correlation to body condition index for those animals. The cystacanth stage of 1 acanthocephalan, *Macracanthorhynchus ingens*, was present in 1 armadillo, and is the first report of *M. ingens* in the 9-banded armadillo. The present study contributes to the known natural history of the 9-banded armadillo, an important animal research model.

Armadillos (*Dasypus novemcinctus*) are an important animal model with unique research potential that predominantly reflects their tendency to produce monozygous quadruplets and an unusual susceptibility to *Mycobacterium leprae* and other human disease agents (Storrs, 1971). Attempts to breed these animals in captivity have been largely unsuccessful thus far, prompting most research programs to utilize wild-caught armadillos (Storrs, 1987). However, limited epidemiological studies have shown that 5–10% of wild-caught armadillos from Texas and Louisiana have leprosy (Walsh et al., 1975; Meyers et al., 1978; Walsh et al., 1986; Storrs, 1987). Because wild armadillos in peninsular Florida are free of leprosy, these are the preferred choice for use in research (Storrs, 1987). Consequently, it is desirable to gather data concerning factors that might impact the general health of wild armadillos in peninsular Florida. In the present study, the prevalence and abundance of helminths in free-ranging armadillos from north-central Florida are described.

In 1991–1993, 32 9-banded armadillos were collected in Alachua County, Florida. Armadillos were either the victims of automobile impact or had been shot as pests. Upon collection, the gastrointestinal (GI) tracts were dissected; the stomach, small intestine, and large intestine were examined separately for the presence of helminths. The mucosa of each section of the GI tract was scraped and combined with gut contents from that section, then processed by sedimentation. The resultant sediment was examined in a backlit box. Helminths were identified from each section of GI and either a total count or estimate was performed for all armadillos. Fecal samples from 21 specimens were examined for parasite ova by flotation concentration with the use of sodium nitrate. A body condition index (BCI) was calculated for 25 animals with the use of the following equation:

$$\text{BCI} = \text{actual body mass (kg)}/\text{expected body mass (kg)},$$

where

$$\text{expected body mass} = [\text{pectoral length (cm)} + \text{pelvic length (cm)}]^3(0.22725) + 1.4893.$$

The equation used to calculate expected body weight was determined by regressing body weight against the cubed sum of pelvic length and pectoral length for a large number of adult armadillos (L. H. Herbst, pers. comm.). Thus, a BCI of 1.0 indicates an animal of ''average'' condition, and deviations above and below reflect those animals that weigh more or less than expected, respectively.

Aspidodera species were recovered from the large intestines from 20 of the 32 armadillos, and were also present in the small intestines of 10 of these 20 infected animals. Total numbers were counted in 12 of the 20 armadillos, and ranged from 1 to 1,021 per infected host. In the remaining 8 armadillos, the number of *Aspidodera* sp. present was estimated to be either greater than or less than 100 nematodes. For purposes of this study, intensities were defined as heavy (greater than 100 nematodes) or light (0–100 nematodes); 14 and 18 animals had heavy and light infections, respectively.

The expected body weight and condition index were calculated for 25 animals. When the relationship between body condition and the total number of *Aspidodera* sp. was examined for 12 animals, a significant inverse correlation between body condition and infection status was observed (Fig. 1). We used a Student's *t*-test to compare average body condition for 10 animals with greater than 100 *Aspidodera* sp. nematodes (high intensity) to animals with fewer than 100 *Aspidodera* sp.

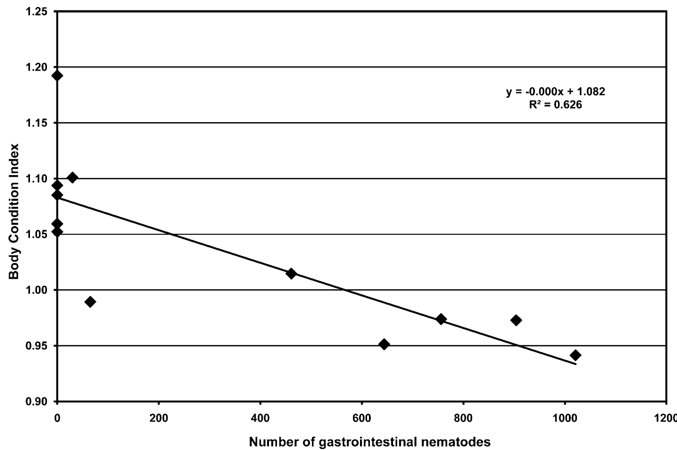

FIGURE 1. Correlation between body condition and infection status of armadillos with *Aspidodera sogandaresi*.

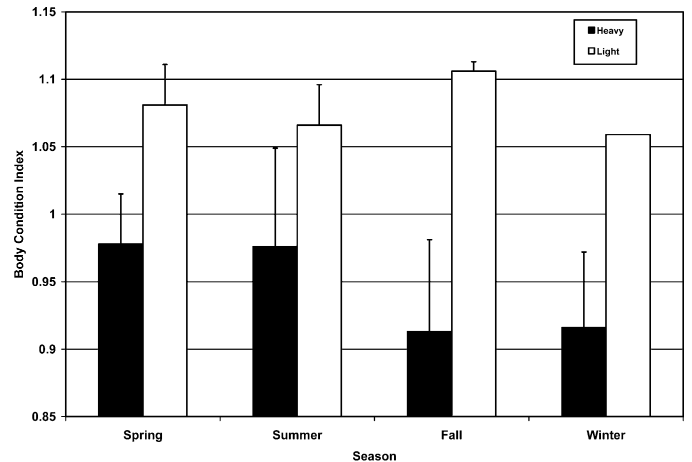

FIGURE 2. Seasonal variation in body condition index of armadillos with light and heavy infections.

(light load). Animals with light intensities had a significantly higher ($P < 0.05$) BCI than animals with heavy intensities. This inverse relationship was also significant when analyzed by season in which animals were collected (Fig. 2).

Of the 10 species of *Aspidodera* that have been described, 7 are found in armadillos. All 7 species occur in the Neotropical region; only 2, *Aspidodera fasciata* and *A. sogandaresi*, occur both in the Neotropical and Nearctic regions. A characteristic feature of species of *Aspidodera* is the presence of cephalic cordons that form usually 6–9 longitudinal loops. In the present study, a total of 10 females and 13 males of *Aspidodera* sp. were examined under a compound microscope after being cleared in lactophenol. In these specimens, 6 cordons were observed, the same number as both *A. fasciata* and *A. sogandaresi*. However, the average length of all specimens was 5.97 mm (5.39–6.44 mm) for females and 6.50 mm (4.78–7.31 mm) for males, lengths that are more consistent with *A. sogandaresi*. In addition, egg dimensions from ova examined in utero in specimens from this study were more oval (mean 49.7 × 72.6 μm) compared to *A. fasciata* (40 × 57 μm) and dimensions were more similar to *A. sogandaresi* (50 × 71 μm) (Santos et al., 1990; Jiminez-Ruiz et al., 2006). Some measurements, however, were not consistent with *A. sogandaresi* or *A. fasciata*. For example, mean distance from vulva to cephalic end for these *Aspidodera* sp. was 3,528 μm, whereas mean distances for *A. sogandaresi* and *A. fasciata* were 2,265 and 2,900 μm, respectively, although values were not significantly different statistically. Mean esophagus lengths were also somewhat different, but fell within the range of both *A. sogandaresi* and *A. fasciata*. Based on the most morphological similarities, the *Aspidodera* sp. recovered from armadillos in the present study most closely resemble *A. sogandaresi*.

In addition to *A. sogandaresi*, a cystacanth stage of the acanthocephalan *M. ingens* was recovered from mesenteries of the small intestine of an armadillo heavily infected with the *Aspidodera* species. Other unidentified nematodes were also collected. An unidentified nematode and 3 unidentified pinworms were found in the stomachs of 2 individual armadillos, and an animal heavily infected with *A. sogandaresi* was coinfected with 1 pinworm in the small intestine and another unidentified nematode in the large intestine. No helminth ova were detected in the feces of the 21 fecal samples examined, although 10 of the 21 armadillos were heavily infected with *A. sogandaresi*.

The range of the 9-banded armadillo encompasses much of the Neotropical region; of the 20 species of armadillos, it is the only one to have extended its range into the United States. Its rapid expansion into the United States began in the mid-1800s as a result of European colonization in Texas, which effectively removed previous ecological barriers. The population in Florida was actually initiated by the release of several individual animals from a personal zoo in 1924, followed by the additional accidental release of armadillos from a circus truck in 1936 (Talmage and Buchanan, 1954; Taulman and Robbins, 1996). By 1972, armadillos expanding their range from Texas began entering into the western border of Florida (Humphrey, 1974; Storrs, 1987). Prior to this study, 10 helminths had been reported from 9-banded armadillos in the United States. These include the trematode *Brachylaemus virginianus*; a cestode, *Oochoristica* sp.; 2 acanthocephalans, *Hammanniella* sp. and *Onicola canis*; and 7 nematodes, *Dipetalonema averyi*, *Aspidodera fasciata*, *A. sogandaresi*, *Physocephalus* sp., *Ascarops* sp., *Physalotera* sp., and *Gnathostoma* sp., with the latter 3 using armadillos as a paratenic host (Chandler, 1946, 1954; Storrs, 1971; Eberhard, 1982; Cockman-Thomas et al., 1993; Jiminez-Ruiz et al., 2006). The present study reports the presence of an *Aspidodera* species that is most similar to *A. sogandaresi* and reports, for the first time, *M. ingens* in the 9-banded armadillo. Because there was a negative correlation between *Aspidodera* sp. numbers and BCI, these nematodes appear to have an adverse influence on the armadillo. Consequently, determining the helminth fauna and prevalence in 9-banded armadillos is critical for a comprehensive understanding of the animal's natural history, particularly when wild populations are to be used as models for human research.

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