Trust project. We also wish to acknowledge Russ Hobbs, Parasitology Department at Murdoch University, for identification of the tick specimens. Mr. Down's Honours Project was supported by a scholarship provided by Murdoch University.

LITERATURE CITED

ALLSOPP, M. T., AND B. A. ALLSOPP. 2006. Molecular sequence evidence for the reclassification of some *Babesia* species. Annals of the New York Academy of Sciences **1081:** 509–517.

ALMERIA, S., J. CASTELLA, D. FERRER, A. ORTUNO, A. ESTRADA-PENA, AND J. F. GUTIERREZ. 2001. Bovine piroplasms in Minorca (Balearic Islands, Spain): A comparison of PCR-based and light microscopy detection. Veterinary Parasitology **99:** 249–259.

ANO, H., S. MAKIMURA, AND R. HARASAWA. 2001. Detection of *Babesia* species from infected dog blood by polymerase chain reaction. Journal of Veterinary Medical Science **63:** 111–113.

BEBAN, H. 2003. Absence of *Babesia gibsoni* in New Zealand dogs. Surveillance **30:** 9–11.

COOPER, A., C. LALUEZA-FOX, S. ANDERSON, A. RAMBAUT, J. AUSTIN, AND R. WARD. 2001. Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution. Nature **409:** 704–707.

CONRAD, P. A., A. M. KJEMTRUP, R. A. CARRENO, J. THOMFORD, K. WAINWRIGHT, M. EBERHARD, R. QUICK, S. R. TELFORD III, AND B. L. HERWALDT. 2006. Description of *Babesia duncani* n. sp. (Apicomplexa: Babesiidae) from humans and its differentiation from other piroplasms. International Journal for Parasitology **36:** 779–789.

CRIADO-FORNELIO A., A. MARTINEZ-MARCOS, A. BULING-SARANA, AND J. C. BARBA-CARRETERO. 2003a. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part I. Epizootiological aspects. Veterinary Parasitology **113:** 189–201.

———, ———, AND ———. 2003b. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part II. Phylogenetic analysis and evolutionary history. Veterinary Parasitology **114:** 173–194.

CRIADO-FORNELIO A., J. MARTINEZ, A. BULING, J. C. BARBA, S. MERINO, R. JEFFERIES, AND P. J. IRWIN. 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. Veterinary Parasitology **142:** 238–247.

HEATHER, B. D., AND H. A. ROBERTSON. 2005. The field guide to the birds of New Zealand. Penguin, Auckland, New Zealand, 432 p.

JEFFERIES, R., U. M. RYAN, AND P. J. IRWIN. 2007. PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. Veterinary Parasitology **144:** 20–27.

KJEMTRUP, A. M., K. WAINWRIGHT, M. MILLER, B. L., PENZHORN, AND R. A. CARRENO. 2006. *Babesia conradae*, sp. nov., a small canine *Babesia* identified in California. Veterinary Parasitology **138:** 103–111.

KUMAR, S., K. TAMURA, I. B. JAKOBSEN, AND M. NEI. 2001. MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics **17:** 1244–1245.

MCLENNAN, J. A., M. A. POTTER, H. A. ROBERTSON, G. C. WAKE, R. M. COLBOURNE, L. DEW, L. JOYCE, A. J. MCCANN, J. MILES, P. J. MILLER, AND J. REID. 1996. Role of predation in the decline of kiwi, *Apteryx* spp., in New Zealand. New Zealand Journal of Ecology **20:** 27–35.

MERINO, S. 1998. *Babesia bennetti* n. sp. from the yellow-legged gull (*Larus cachinnans*, Aves, Laridae) on Benidorm Island, Mediterranean Sea. Journal of Parasitology **84:** 422–424.

PEIRCE, M. A. 1975. *Nuttalia franca*, 1909 (Babesiidae) preoccupied by *Nuttallia dall*, 1898 (Psammobiidae): A re-appraisal of the taxonomic position of the avian piroplasms. International Journal for Parasitology **5:** 285–287.

———. 2000. A taxonomic review of avian piroplasms of the genus *Babesia* Starcovici, 1893 (Apicomplexa: Piroplasmorida: Babesiidae). Journal of Natural History **34:** 317–332.

———. 2005. A checklist of the valid avian species of *Babesia* (Apicomplexa: Piroplasmorida), *Haemoproteus*, *Leucocytozoon*, (Apicomplexa: Haemosporida), and *Hepatozoon* (Apicomplexa: Haemogregarinidae). Journal of Natural History **39:** 3621–3632.

———, R. M. JAKOB-HOFF, AND C. TWENTYMAN. 2003. New species of haematozoa from Apterygidae in New Zealand. Journal of Natural History **37:** 1797–1804.

REICHARD, M. V., R. A. VAN DEN BUSSCHE, J. H. MEINKOTH, J. P. HOOVER, AND A. A. KOCAN. 2005. A new species of *Cytauxzoon* from Pallas' cats caught in Mongolia and comments on the systematics and taxonomy of piroplasmids. Journal of Parasitology **91:** 430–436.

ROBERTSON, H. A., R. M. COLBOURNE, P. GRAHAM, P. J. MILLER, AND R. J. PIERCE. 1999. Survival of brown kiwi (*Apteryx mantelli*) exposed to brodifacoum poison in Northland New Zealand. New Zealand Journal of Ecology **23:** 225–231.

STATTERSFIELD, A. J., D. R. CAPPER, AND G. C. L. DUTSON. 2000. Threatened birds of the world: the official source for birds on the IUCN Red List. BirdLife International and Lynx Edicons, Cambridge, U.K., 864 p.

VAN DE PEER, Y., AND R. DE WACHTER. 1993. TREECON: A software package for the construction and drawing of evolutionary trees. Computer Applications in Biosciences **9:** 177–82.

WORK, T. M., AND R. A. RAMEYER. 1997. Description and epizootiology of *Babesia poelea* n. sp. in brown boobies (*Sula leucogaster* (Boddaert)) on Sand Island, Johnston Atoll, Central Pacific. Journal of Parasitology **83:** 734–738.

YABSLEY, M. J., WORK, T. M., AND R. A. RAMEYER. 2006. Molecular phylogeny of *Babesia poelea* from brown boobies (*Sula leucogaster*) from Johnston Atoll, central Pacific. Journal of Parasitology **92:** 423–425.

YOKOYAMA, N., S. BORK, M. NISHISAKA, H. HIRATA, T. MATSUO, N. INOUE, X. XUAN, H. SUZUKI, C. SUGIMOTO, AND I. IGARASHI. 2003. Roles of the Maltese cross form in the development of parasitemia and protection against *Babesia microti* infection in mice. Infection and Immunity **71:** 411–417.

ZAHLER, M., H. RINDER, AND R. GOTHE. 2000. Genotypic status of *Babesia microti* within the piroplasms. Parasitology Research **86:** 642–646.

J. Parasitol., 94(2), 2008, pp. 560–562

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

Robert L. Smith, Juan A. Schnack*, Eduardo F. Schaefer†, and Arturo I. Kehr†, Department of Entomology, University of Arizona, Tucson, Arizona 85721; *División Entomología, Museo de La Plata, Universidad Nacional de La Plata, Paseo del Bosque s/n, 1900 La Plata, Argentina; †Centro de Ecología Aplicada del Litoral (CECOAL-CONICET), C.C. 140, C.P. 3400, Corrientes, Argentina.
e-mail: bobsmith@cals.arizona.edu

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.

LITERATURE CITED

BURRIDGE, M. J., AND L. A. SIMMONS. 2003. Exotic ticks introduced into the United States on imported reptiles from 1962 to 2001 and their potential roles in international dissemination of diseases. Veterinary Parasitology **113:** 289–320.

KEIRANS, J. E., AND L. A. DURDEN. 1998. Illustrated key to nymphs of the tick genus *Amblyomma* (Acari: Ixodidae) found in the United States. Journal of Medical Entomology **35:** 489–495.

———, AND J. H. OLIVER, JR. 1993. First description of the male and redescription of the immature stages of *Amblyomma rotundatum* (Acari: Ixodidae), a recently discovered tick in the U.S.A. Journal of Parasitology **79:** 860–865.

KLOMPEN, J. S. H., W. C. BLACK, J. E. KEIRANS, AND J. H. OLIVER. 1996. Evolution of ticks. Annual Review of Entomology **41:** 141–161.

LABRUNA, M. B., F. A. TERRASSINI, AND L. M. A. CAMARGO. 2005. First report of the male *Amblyomma rotundatum* (Acari: Ixodidae) from a field collected host. Journal of Medical Entomology **42:** 945–947.

LAMPO, M., AND P. BAYLISS. 1996. The impact of ticks on *Bufo marinus* from native habitats. Parasitology **113:** 199–206.

OBA, M. S. P., AND T. T. S. SCHUMAKER. 1983. Estudo da biologia de *Amblyomma rotundatum* (Koch, 1844), em infestações experimen-

tais de *Bufo marinus* (L. 1758) sob condições varidas de umidade relativa e de temperature do ar. Memorias do Instituto Butantan **47/48:** 195–204.

OLIVER, J. H., JR., M. P. HAYES, J. E. KEIRANS, AND D. R. LAVENDER. 1993. Establishment of the foreign parthenogenetic tick *Amblyomma rotundatum* (Acari: Ixodidae) in Florida. Journal of Parasitology **79:** 786–790.

ROBINSON, L. E. 1926. Ticks. A monograph of the Ixodidae. Part IV. The genus *Amblyomma*. Cambridge University Press, London, U.K., 302 p.

J. Parasitol., 94(2), 2008, pp. 562–564

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

Eszter Szöllősi, Olof Hellgren*, and Dennis Hasselquist*, Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/c, 1117 Budapest, Hungary; *Department of Animal Ecology, Lund University, Ecology Building, Sölvegatan 37, 22362 Lund, Sweden. *e-mail: sz_eszter@ludens.elte.hu*

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-