Infection by Anisakid Nematodes *Contracaecum* spp. in the Mayan Cichlid Fish '*Cichlasoma* (*Nandopsis*)' *urophthalmus* (Günther 1862)

Author(s): Gaddy T. Bergmann and Philip J. Motta
Source: Journal of Parasitology, 90(2):405-407.
Published By: American Society of Parasitologists
DOI: http://dx.doi.org/10.1645/GE-104R
URL: http://www.bioone.org/doi/full/10.1645/GE-104R

RESEARCH NOTES

J. Parasitol., 90(2), 2004, pp. 405–407

Infection by Anisakid Nematodes *Contracaecum* spp. in the Mayan Cichlid Fish '*Cichlasoma* (*Nandopsis*)' *urophthalmus* (Günther 1862)

Gaddy T. Bergmann and Philip J. Motta, Department of Biology, University of South Florida, 4202 East Fowler Avenue, SCA 110, Tampa, Florida 33620. *e-mail: gaddyfl25@yahoo.com*

ABSTRACT: Larval nematodes that parasitize the Mayan cichlid fish '*Cichlasoma* (*Nandopsis*)' *urophthalmus* (Günther 1862) in southern Florida were identified as *Contracaecum* spp. (Nematoda: Anisakidae, Anisakinae). The objective of this study was to determine whether infection intensity and prevalence of these parasites differ between a brackish water and freshwater habitat or through ontogeny in the freshwater habitat only. The nematodes were removed from the abdominal cavity of the fishes and counted. Infection intensity was compared between habitats using analysis of covariance and evaluated through ontogeny using Spearman rank order correlation. Prevalence was compared between habitats and between adults and juveniles from the freshwater habitat using a *z*-test. Although infection intensity did not differ between habitats, infection prevalence was greater at the freshwater site (FWS). Both the prevalence and intensity of nematode infection increased through ontogeny at the FWS, and no nematode was found in fishes that were smaller than 93 mm standard length. Thus, the parasites appear to accumulate during the lifetime of the fishes.

Native to the Atlantic slope of tropical Central America (Miller, 1966), the Mayan cichlid '*Cichlasoma* (*Nandopsis*)' *urophthalmus* (Günther 1862) was first recorded in Florida in 1983 from the Everglades National Park (Loftus, 1987). It is referred to in this study as '*C.* (*Nandopsis*)' *urophthalmus* in accord with the convention for taxonomically undetermined cichlids (Stiassny, 1991). The means by which '*C.* (*Nandopsis*)' *urophthalmus* was introduced into Florida are unknown (Loftus, 1987), but it is likely that it was released by private aquarists. This species is euryhaline and can survive in a range of salinity from 0 to 40 ppt (Martinez-Palacios and Ross, 1992; Martinez-Palacios et al., 1993). '*Cichlasoma* (*Nandopsis*)' *urophthalmus* is susceptible to infection by parasites such as nematodes (roundworms), digenean trematodes, and poecilostomatoid copepods (Vidal-Martínez et al., 1994; Salgado-Maldonado and Kennedy, 1997). In the present study, '*C.* (*Nandopsis*)' *urophthalmus* was found to have larval *Contracaecum* spp. (Nematoda: Anisakidae, Anisakinae) (E. C. Greiner, pers. comm.) in the coelom. The species of *Contracaecum* infects '*C.* (*Nandopsis*)' *urophthalmus* in Mexico (Vidal-Martínez et al., 1994; Salgado-Maldonado and Kennedy, 1997). Lament (1999) found parasitic nematodes in the visceral cavity of '*C.* (*Nandopsis*)' *urophthalmus* collected in the Everglades region but did not identify them. The nematode described in Lament (1999) may also be *Contracaecum* spp.

Contracaecum spp. are widely distributed parasitic nematodes. Besides '*C.* (*Nandopsis*)' *urophthalmus*, they have also been recovered from other cichlids and many other fishes and aquatic birds (Deardorff and Overstreet, 1980; Banks and Ashley, 2000; Machado et al., 2000). Anisakids, such as *Contracaecum* spp., usually infect the alimentary tract of their definitive hosts, which are generally marine mammals. Marine crustaceans, cephalopod mollusks, or fishes often serve as intermediate or paratenic (transport) hosts. Anisakids are not found in the flesh of fishes, they infect the visceral organs instead. Thus, these parasites are not usually harmful to the fish they infect (Deardorff and Overstreet, 1980; Bush et al., 2001). In experimental infections of juvenile and adult chickens, mallard ducklings, mice, hamsters, and rats, *Contracaecum multipapillatum* was not harmful. However, in marine birds, *C. multipapillatum* can infect the proventriculus portion of the stomach and cause lesions. This parasite can also cause granuloma in dogs that eat fish. *Contracaecum multipapillatum* is also capable of infecting domestic cats (Deardorff and Overstreet, 1980; Vidal-Martínez et al., 1994). Anisakids are dangerous when ingested by humans, causing a condition known as anisakiasis, which causes violent abdominal pain, nausea, and vomiting (Bush et al., 2001; Plath et al., 2001).

The goal of this study was to determine whether the pattern of infection by *Contracaecum* spp. in '*C.* (*Nandopsis*)' *urophthalmus* differs among habitats or through ontogeny. Because *Contracaecum* spp. parasitize both marine and freshwater fishes, it was hypothesized that neither prevalence nor infection intensity of the nematodes would differ significantly between adult '*C.* (*Nandopsis*)' *urophthalmus* from a brackish water site (BWS) and a freshwater site (FWS). Finally, on the basis of the notion that *Contracaecum* spp. might accumulate in '*C.* (*Nandopsis*)' *urophthalmus* with time, it was hypothesized that both prevalence and infection intensity would increase significantly through ontogeny at the FWS where juvenile fishes were collected.

The 2 sites chosen for this study, one of which is a brackish water habitat and the other a freshwater, are part of the canal system of southern Florida. The first site was located approximately at the intersection of US-41, the Tamiami Trail, and State Route 29. This is the site of intersection of the Tamiami Canal and the Main Barron River Canal in Carnestown, Collier County (25°54′41″N,81°21′53″W), at the western border of the Big Cypress National Preserve. This region of the Tamiami Canal passes through brackish water and consists mostly of salt marsh and some mangrove stands and naturally experiences seasonal saltwater influx from Chocoloskee Bay. Data from a nearby water monitoring station on the Tamiami Canal (25°57′40″N,81°30′60″W) are used in this study as indicators of the salinity fluctuations at this first collection site. In this region, salinity fluctuates seasonally from 0.00 to 34.30 ppt. Sampling at this site took place on 29 April 2000 and 27 May 2000. The second collection site was within the Big Cypress National Preserve in the canal along State Route 94 (Loop Road), east of Trail City, Monroe County (25°45′35″N,81°0′9″W). This area is a freshwater cypress swamp, and sampling took place on a monthly basis from 24 June 2000 to 19 October 2000. Data from a nearby water monitoring station on the Tamiami Canal (25°50′35″N,80°55′4″W) verify that this is a FWS, with salinity ranging from 0.00 to 0.29 ppt only.

Fishes were collected from the BWS and the FWS using seine and dip nets, hook and line angling, and minnow traps. Samples were taken on a monthly basis from 29 April 2000 to 19 October 2000 to minimize the confounding effect of changing seasons; 257 '*C.* (*Nandopsis*)' *urophthalmus* were collected for this study. Fifty-three adults were collected from the brackish water population. Sixty-eight adults and 136 juveniles were collected from the freshwater population. Only adult '*C.* (*Nandopsis*)' *urophthalmus* were considered when comparing the brackish and freshwater habitats. All individuals greater than 102 mm standard length (SL) were considered to be adults (Caso Chávez et al., 1986; Martinez-Palacios and Ross, 1992; Martinez-Palacios et al., 1993; Faunce and Lorenz, 2000). It was presumed that the 2 groups sampled are not subpopulations capable of intermixing because '*C.* (*Nandopsis*)' *urophthalmus* is philopatric or site tenacious (Caso Chávez et al., 1986; Salgado-Maldonado and Kennedy, 1997; Faunce and Lorenz, 2000).

Fishes were killed with an overdose of tricaine methanesulfonate (MS-222). They were then injected with 10% buffered formalin and placed on ice for preservation. On return to the laboratory, the fishes were cut from vent to pelvic girdle. All parasitic nematodes found in the coelomic cavity were removed and counted. The parasites were then fixed in 10% buffered formalin and stored in 70% ethanol. Twelve nematodes from both the brackish and freshwater populations were identified by E. C. Greiner, Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, Florida. The fact that only 1 site was used for each habitat type means that there is no replication for each treatment. Therefore, statistical power is low, and the results of the analysis presented in this study must be interpreted with caution. Parasite infection was quantified using both prevalence (the proportion of the host population infected) and intensity (the number of parasites per host). Descriptive statistics were computed using

the arithmetic mean as the measure of central tendency and the standard deviation as the measure of data dispersion. Because nematodes are individual organisms, descriptive statistics are reported in this study using whole numbers. Prevalence was compared between sites and between adults and juveniles at FWS using a *z*-test with an alpha level of 0.05. Comparison of infection intensity between sites was accomplished using analysis of covariance (ANCOVA), with SL as covariate and an alpha value of 0.05. Both the number of nematodes and the SL were square-root transformed for analysis. Infection intensity was examined through ontogeny at FWS using Spearman rank order correlation on untransformed data (Zar, 1996). The statistical programs used were SigmaStat 2.03 and SYSTAT 10.0 (SPSS Science).

Contracaecum spp. found in the visceral cavity of '*C.* (*Nandopsis*)' *urophthalmus* ranged from approximately 5 to 20 mm in length. They were less than 1 mm in cross-sectional diameter, light pink in color, and generally found in a coiled position, although some were extended. These larvae were probably all in the L3 stage (B. G. Chitwood and M. B. Chitwood, 1974; E. R. Noble and G. A. Noble, 1982; Olsen, 1986), and only these larvae were considered in this analysis. Of the 56 fish collected from BWS, 22 contained *Contracaecum* spp. (none among the 3 juvenile specimens, and all 22 among the 53 adults). This is equivalent to 39.3% of all BWS fish sampled or 41.5% of the adults. Of the 204 fishes collected at FWS, 47 were infected with the nematodes (3 among the 136 juveniles and 44 among the 68 adults). This is equivalent to 23.0% of all FWS fishes or 2.2% of juveniles and 64.7% of adults. The *z*-tests revealed that prevalence of infection was significantly higher in FWS adults than in either BWS adults ($z = 2.359$, $P = 0.018$) or FWS juveniles ($z = 9.850$, $P < 0.001$).

Infection intensity per individual fish at BWS ranged from 1 to 16 nematodes, whereas it ranged from 1 to 43 nematodes at FWS. If the individual fish that contained 43 nematodes is discarded from the analysis, infection intensity at FWS ranged from 1 to 6 nematodes. Mean infection intensity among adults at BWS was 2 ± 4 worms/fish. At FWS, mean infection intensity was 2 ± 5 worms/fish for adults and 0 ± 0 worms/fish for juveniles. ANCOVA indicates that infection intensity is not significantly different between sites ($F = 2.621$, $P = 0.108$).

Nematode infection showed an increase through ontogeny at FWS. Very small fish showed no evidence of infection. Ninety-three millimeters was the first SL at which infection was recorded. Beyond this length, there was no clear relationship between SL and infection intensity as the fish continued to grow. A Spearman rank order correlation was performed to evaluate *Contracaecum* spp. infection through ontogeny. Two separate tests were performed, 1 including the outlying data point of 43 nematodes in the coelom of 1 fish and 1 excluding that data point. If the outlier was included, there was a significant and positive correlation between number of nematodes in the coelom and SL ($R^2 = 0.612$, $P < 0.001$). If the outlier was excluded, a similar result was obtained ($R^2 = 0.605$, $P < 0.001$).

The broad ranges of *Contracaecum* spp. appear to be naturally occurring, probably because of migratory birds (Deardorff and Overstreet, 1980). It is presently unclear whether '*C.* (*Nandopsis*)' *urophthalmus* in Florida are infected by a *Contracaecum* spp. from Florida, from Central America, or by species from both locations (Hoffman and Schubert, 1984; Lament, 1999). This study shows that although infection intensity among adults did not differ significantly between sites, adults at FWS exhibited a significantly higher prevalence of infection by *Contracaecum* spp. than adults at BWS. The cause for this higher prevalence of infection at FWS could be attributed to a greater density of fishes at this site, but this remains to be determined. This study also shows that smaller '*C.* (*Nandopsis*)' *urophthalmus* harbors fewer parasites than do larger individuals. The smallest infected fish from BWS was 115 mm SL, and the smallest infected fish from FWS was 93 mm SL. However, there is no clear relationship between size and infection intensity beyond the minimum age of infection.

Larger individuals may harbor more parasites because they have had more time to accumulate them. '*Cichlasoma* (*Nandopsis*)' *urophthalmus* are carnivorous, feeding on decapod crustaceans such as crayfish *Procambarus* sp. and shrimp *Palaemonetes paludosus*. They also prey on fish such as mosquitofish *Gambusia holbrooki*, sailfin mollies *Poecilia latipinna*, and juvenile '*C.* (*Nandopsis*)' *urophthalmus* (Caso Chávez et al., 1986; Martinez-Palacios and Ross, 1988; Bergmann, 2002). Consuming these prey items could have led to postcyclic parasitic transmission (Scott, 1954; Smith and Wootten, 1975). However, '*C.* (*Nandopsis*)' *urophthalmus* is also detritivorous (Caso Chávez et al., 1986; Martinez-Palacios and Ross, 1988; Bergmann, 2002). In the detritivorous striped mullet, *Mugil cephalus*, fish size is likewise positively correlated with the intensity of infection by *Contracaecum* spp. and other parasites combined (Valles-Rios et al., 2000). Thus, detritivory may lead to an increase in infection intensity through ontogeny, perhaps because of repeated exposure to the parasite. Further investigation would be necessary to determine whether predation, detritivory, or other factors are the primary sources of infection by *Contracaecum* spp. in '*C.* (*Nandopsis*)' *urophthalmus*.

The Department of Biology at the University of South Florida provided generous support. We thank S. S. Bell, E. D. McCoy, W. F. Font, and anonymous reviewers for their contributions to this publication. We are grateful to E. C. Greiner for identifying the parasitic nematodes found within the Mayan cichlids. We also thank S. Connor, J. Hill, S. Huskey, J. Lament, B. Loftus, and K. O. Winemiller for their guidance. We are grateful to K. Dugan of Collier County for providing us with water quality data. Finally, we acknowledge A. Cockburn, S. Jacobson, D. Klemm, J. Krebs, H. Porter, and M. Robeson for their critical technical assistance. The contents of this paper are from a thesis submitted to the University of South Florida by G.T.B. in partial fulfillment of the requirements for an M.S. degree in zoology. This research was conducted with approval by the University of South Florida Institutional Animal Care and Use Committee, file 1714.

LITERATURE CITED

BANKS, S. M., AND D. C. ASHLEY. 2000. Observations on the internal helminth parasite fauna of largemouth bass, *Micropterus salmoides*, from Smithville Reservoir, Missouri. Journal of Freshwater Ecology **15:** 299–306.

BERGMANN, G. T. 2002. Trophic ecomorphology of the nonindigenous Mayan cichlid '*Cichlasoma* (*Nandopsis*)' *uropthalmus* (Günther, 1862) in southern Florida. M.S. Thesis. University of South Florida, Tampa, Florida, 171 p.

BUSH, A. O., J. C. FERNÁNDEZ, G. W. ESCH, AND J. R. SEED. 2001. Parasitism: The diversity and ecology of animal parasites. Cambridge University Press, New York, 566 p.

CASO CHÁVEZ, M., A. YÁÑEZ-ARANCIBIA, AND A. L. LARA-DOMÍNGUEZ. 1986. Biologia, ecologia y dinamica de poblaciones de *Cichlasoma urophthalmus* (Gunter) (Pisces: Cichlidae) en habitat de *Thalassia testudinum* y *Rhizophora mangle*, Laguna de Terminos, sur del Golfo de Mexico. Biotica **11:** 79–111.

CHITWOOD, B. G., AND M. B. CHITWOOD. 1974. Introduction to Nematology. University Park Press, Baltimore, Maryland, 334 p.

DEARDORFF, T. L., AND R. M. OVERSTREET. 1980. *Contracaecum multipapillatum* (=*C. robustum*) from fishes and birds in the northern Gulf of Mexico. Journal of Parasitology **66:** 853–856.

FAUNCE, C. H., AND J. J. LORENZ. 2000. Reproductive biology of the introduced Mayan cichlid, *Cichlasoma urophthalmus*, within an estuarine mangrove habitat of southern Florida. Environmental Biology of Fishes **58:** 215–225.

HOFFMAN, G. L., AND G. SCHUBERT. 1984. Some parasites of exotic fishes. *In* Distribution, biology, and management of exotic fishes, W. R. Courtenay Jr. and J. R. Stauffer Jr. (eds.). The Johns Hopkins University Press, Baltimore, Maryland, p. 233–261.

LAMENT, J. J. 1999. Life history variation in introduced populations of the Mayan cichlid, *Cichlasoma urophthalmus* in the Everglades. Ph.D. Dissertation, University of Miami, Coral Gables, Florida, 153 p.

LOFTUS, W. F. 1987. Possible establishment of the Mayan cichlid, *Cichlasoma urophthalmus* (Günther) (Pisces: Cichlidae), in Everglades National Park, Florida. Florida Scientist **50:** 1–6.

MACHADO, P. M., S. C. DE ALMEIDA, G. C. PAVANELLI, AND R. M. TAKEMTO. 2000. Ecological aspects of endohelminths parasitizing *Cichla monoculus* Spix, 1831 (Perciformes: Cichlidae) in the Parana River near Porto Rico, State of Parana, Brazil. Comparative Parasitology **67:** 210–217.

MARTINEZ-PALACIOS, C. A., C. CHAVEZ-SANCHEZ, AND M. A. OLVERA NOVOA. 1993. The potential for culture of the American Cichlidae with emphasis on *Cichlasoma urophthalmus*. Recent Advances in Aquaculture **4:** 193–232.

———, AND L. G. ROSS. 1988. The feeding ecology of the Central

American cichlid *Cichlasoma urophthalmus* (Gunther). Journal of Fish Biology **33:** 665–670.

———, AND ———. 1992. The reproductive biology and growth of the Central American cichlid *Cichlasoma urophthalmus* (Günther). Journal of Applied Ichthyology **8:** 99–109.

MILLER, R. R. 1966. Geographical distribution of Central American freshwater fishes. Copeia **1966:** 773–802.

NOBLE, E. R., AND G. A. NOBLE. 1982. Parasitology: The biology of animal parasites. Lea and Febiger, Philadelphia, Pennsylvania, 522 p.

OLSEN, O. W. 1986. Animal parasites, their life cycles and ecology. Dover Press, New York, 562 p.

PLATH, F., A. HOLLE, D. ZENDEH, F. W. MOLLER, M. BARTEN, E. C. REISINGER, AND S. LIEBE. 2001. Gastric anisakiasis—A case report from Germany: Conclusion. Zeitschrift für Gastroenterolgie **39:** 177–180.

SALGADO-MALDONADO, G., AND C. R. KENNEDY. 1997. Richness and similarity of helminth communities in the tropical cichlid fish *Cichlasoma urophthalmus* from the Yucatan Peninsula, Mexico. Parasitology **114:** 581–590.

SCOTT, D. M. 1954. Experimental infection of Atlantic cod with larvae of the marine nematode from smelt. Journal of the Fisheries Research Board of Canada **11:** 894–900.

SMITH, J. W., AND R. WOOTTEN. 1975. Experimental studies on the migration of *Anisakis* sp. larvae (Nematoda: Ascaridida) into the flesh of herring, *Clupea harengus* L. International Journal for Parasitology **5:** 133–136.

STIASSNY, M. L. J. 1991. Phylogenetic intrarelationships of the family Cichlidae: An overview. *In* Cichlid fishes: Behavior, ecology, and evolution, M. H. A. Keenleyside (ed.). Chapman and Hall, New York, p. 1–35.

VALLES-RIOS, M. E., G. RUIZ-CAMPOS, AND L. GALAVIZ-SILVA. 2000. Parasite prevalence and intensity in *Mugil cephalus* (Pisces: Mugilidae), from Colorado River, Baja California, Mexico. Revista de Biologia Tropical **48:** 495–501.

VIDAL-MARTÍNEZ, V. M., D. OSORIO-SARABIA, AND R. M. OVERSTREET. 1994. Experimental infection of *Contracaecum multipapillatum* (Nematoda: Anisakinae) from Mexico in the domestic cat. Journal of Parasitology **80:** 576–579.

ZAR, J. H. 1996. Biostatistical analysis, 3rd ed. Prentice Hall, Inc., Upper Saddle River, New Jersey, 662 p.

J. Parasitol., 90(2), 2004, pp. 407–409

Ex Vivo Anthelmintic Activity of Albendazole-Sulphoxide Enantiomers

F. Bolás-Fernández, S. Rama-Iñiguez, and J. J. Torrado*, Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain; *Deparmento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain. *e-mail: francisb@farm.ucm.es*

ABSTRACT: The antiparasitic activity of racemic albendazole-sulphoxide (Ricobendazole = *rac*RBZ) and its (+) and (−) enantiomers was tested in an ex vivo murine model for *Trichinella spiralis* infection. Larvae were isolated from the muscle of infected mice and exposed to concentrations between 0.01 and 1 μg/ml of the racemic mixture or to each of its enantiomers. The activity of each compound was then assayed by measuring the ability of the treated larvae to infect naive mice (larval viability). At a concentration of 0.5 μg/ml, all 3 compounds were highly effective in reducing the viability of the larvae, achieving reductions of 91.26% (*rac*RBZ), 96.7% (+), and 89.2% (−), when compared with untreated controls. At lower concentrations (0.1 μg/ml), only treatment with (+)RBZ rendered a significant reduction in larval viability in comparison with controls (84.3%; $P < 0.01$), whereas at 0.01 μg/ml, none of the compounds altered larval viability ($P > 0.05$).

Albendazole (ABZ), methyl (5-(propyl-thio)-1-*H*-benzimidazole-2yl) carbamate, is a broad-spectrum drug that acts against the most important animal and human helminth parasite species (Horton, 2000). After administration, ABZ is rapidly transformed by 2 distinct hepatic microsomal enzyme systems: the flavin-containing monooxygenase system (Lanusse et al., 1993) and the cytochrome P-450 chain (Souhaili-El Amri et al., 1987). These modifications produce albendazole-sulphoxide (ABZSO) and albendazole-sulphone, the main metabolites that can be recovered from the plasma of sheep (Lanusse et al., 1995), cattle (Sanchez et al., 1997), mice (Rueda-Polo et al., 1998), and humans (Prochanzkova et al., 2000). Because of their affinity for the parasite's beta-tubulin, both ABZ and ABZSO exhibit antiparasitic activity (Lacey, 1988). However, ABZ can be further metabolized and is thus only found at low levels in plasma. Hence, the ABZSO product is thought to be the more active of the 2 in combating tissue-dwelling parasites (Marriner and Bogan, 1980). The chiral core in the sulfur atom of ABZSO permits 2 enantiomers to be generated, (+)ABZSO and (−)ABZSO. The pharmacokinetic disposition of ABZSO enantiomers after oral administration of ABZ in distinct animal species and humans (Delatour, Benoit et al., 1991; Delatour, Garnier et al., 1991; Garcia et al., 1999) reveals differences that possibly reflect the selective metabolism of ABZ and ABZSO enantiomers (Marques et al., 1999; Solana et al., 2000; Virkel et al., 2002). Indeed, even sex-related differences in the pharmacokinetic behavior of ABZSO enantiomers have been documented in sheep (Capece et al., 2000). Furthermore, the selective uptake of ABZSO enantiomers and their selective binding to cytosolic proteins isolated from different helminth parasites has also been demonstrated (Alvarez et al., 2000; Solana et al., 2002). Thus, these factors must be taken into account because they may contribute significantly to the pharmacological properties of this chiral molecule.

However, despite the interest in these agents, the antiparasitic activity of the (+)ABZSO and (−)ABZSO enantiomers has never been directly tested. Using optimized chiral high-performance liquid chromatography (HPLC), we have been able to obtain very pure samples of (+)ABZSO and (−)ABZSO. This has enabled us to test the anthelmintic activity of these enantiomers in an ex vivo model system, using the well-established murine model of *Trichinella spiralis* infection.

To purify ricobendazole enantiomers from a racemic ABZSO mixture (Ricobendazole = RBZ), a modular liquid chromatography apparatus was used, equipped with: a Jasco PU-1580 isocratic pump (Tokyo, Japan); an automatic sampler (Gilson 231 XL, Villers le Bels, France) fitted to a 100-μl sampler loop (Rheodyne, Rohnert Park, California); a variable wavelength detector (UV-1575, Jasco); and a PC integrator (Borwin 1.5, JMBS developments [Jasco]). Before injection, the samples were filtered through a polyvinylidene fluoride Durapore® 0.45-μm filter (Millipore Co., Boston, Massachusetts). Subsequently, the racemic ricobendazole (*rac*RBZ) sample mix was injected into the HPLC system at a concentration of 0.1 mg/ml, and chiral HPLC separation was performed according to a modified version of the method described previously by Delatour et al. (1990) (Garcia et al., 1999). Briefly, a chiral–alpha 1-acid glycoprotein column (100 × 4 mm, 5 μm) and a mobile phase containing sodium phosphate buffer (8 mM, pH 7.0), at a flow rate of 0.9 ml/min, were used. The samples were analyzed at 290 nm, and under these conditions, the retention times for (−) and (+)RBZ were 3.1 and 10.5 min, respectively. The liquid samples were collected in accordance with the retention times of the enantiomers. The samples were concentrated to dryness under vacuum at 70 C using a Savant Speedvac® concentrator (Holbrook, New York). The samples recovered were quantified by the same HPLC method described above but included 2-propanol (1 ml/L) as an additive in the mobile phase. Under these conditions, the retention times after injection were 2.6 min for

TABLE I. Ex vivo larvicidal effect of RacRBZ.

Concentration (μg/ml)	Mean adult worms (SD)	% Reduction*
	66.80 (21.49†)	—
Control	44.16 (10.20)	—
1	0.33 (0.47)	92.50‡
0.5	0.16 (0.37)	99.63‡
0.25	0.66 (1.10)	98.50‡
0.12	11.33 (7.27)	74.34§
0.06	39.16 (16.20)	11.32

* Compared with untreated controls.
† Nonincubated larvae.
‡ $P < 0.01$.
§ $P < 0.05$.

(−)RBZ and 4.6 min for (+)RBZ. For use in biological assays, the concentrated samples were dissolved in acidified deionized water (pH 2) and then diluted to the appropriate concentrations in culture medium.

Trichinella. spiralis L1 larvae (MFEL/SP/62/GM-1 ISS48 strain; *Trichinella* Reference Centre, Istituto Superiore di Sanitá, Rome, Italy) were obtained from the carcasses of experimentally infected mice by standard artificial digestion. After isolation, the larvae were separated using a Baermann apparatus and then washed 10 times by passive sedimentation in phosphate-buffered saline containing added antibiotics. The larvae were then suspended in culture media (500 ± 50 larvae/ml), which consisted of a basic Hank's balanced salt solution (HBSS) with added antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin; Sigma Chemical Co., St. Louis, Missouri). Under these conditions, 66.8% of incubated larvae retain their capacity to infect mice when compared with nonincubated controls (Table I). Interestingly, we noted that the addition of L-glutamine or *N*-2-hydroxythylpiperazine-*N*′-2-ethane-sulfonic acid buffer significantly affected further larval viability (data not shown).

To test the different compounds, 0.2 ml of the larval suspension was added to each well of a 24-well culture plate (Costar, Corning, Albany, New York) and mixed with 1.8 ml of either *rac*RBZ or one or other of the purified enantiomer solutions. Different concentrations of each solution were tested in 6 wells of each plate, whereas, as controls, the larvae in the remaining 6 wells were exposed to medium alone. After 24 hr at 37 C in a conventional 5% CO_2 incubator, the larvae were observed using an inverted light microscope. Thereafter, 1.5 ml of medium was carefully removed from each of the wells, and the larvae in the remaining 0.5 ml (100 ± 10) were used to orally infect 6 Swiss-CD-1, 8-wk-old male mice. As controls, another group of 6 age-matched mice was infected 24 hr beforehand with freshly isolated larvae. Five days after infection (6 days for the fresh larval controls), the mice were sacrificed with an excessive dose of chloroform anesthesia, and their small intestines were removed and opened longitudinally. Each intestine was placed on sanitary gauze and incubated in saline solution for 4 hr at 37 C. The tissues were removed, and the adult worms released were allowed to settle before being counted using a stereomicroscope. The mean number of worms from the animals infected with ex vivo–treated larvae was compared with those obtained from animals infected with incubated but untreated larvae using Student's *t*-test. Significance was set at $P < 0.05$ and $P < 0.01$.

After the 24-hr incubation in medium alone, there was a 33.9% reduction in larval viability, as seen in Table I. Whereas 66.8 worms were recovered from animals infected with fresh isolated larvae, this figure dropped to 44.1 when the animals were infected with control larva that had been incubated for 24 hr in medium alone. This reduction may reflect the noxious effects of maintaining the larvae in an atmosphere of 5% CO_2 because an earlier study has shown that totally anaerobic conditions are necessary to retain the full capacity of infection of *Trichinella* larvae cultured in conventional cell culture media (Bolas-Fernandez, 2002). However, under these anaerobic conditions, larvae are not sensitive to drugs (probably because of a hypobiotic condition); therefore, some degree of aerobiosis is needed to be able to detect anthelmintic activity.

TABLE II. Ex vivo larvicidal effect of RBZ enantiomers.

	Mean adult worms	SD	% Reduction
Experiment 1			
Control (0.5 μg/ml)	70.50	10.59	
Compounds			
*rac*RBZ	6.16	2.85	91.26*
(+)RBZ	2.30	2.21	96.7*
(−)RBZ	7.60	2.21	89.2*
Experiment 2			
Control (0.1 μg/ml)	49.00	7.18	
Compounds			
*rac*RBZ	15.03	5.19	69.32**
(+)RBZ	7.66	2.98	84.36*
(−)RBZ	34.26	25.18	30.08
Control (0.01 μg/ml)	49.00	7.18	
Compounds			
*rac*RBZ	56.10	10.15	0.00*
(+)RBZ	32.81	26.8	33.04
(−)RBZ	38.41	24.56	21.61

* $P < 0.01$.
** $P < 0.05$.

We tested concentrations of *rac*RBZ ranging from 0.06 to 1 μg/ml. Visual observation of the larvae at the light microscopic level after incubation showed no difference between treated and untreated larvae at any of the concentrations analyzed. The larvae exhibited a semicoiled ''serpentine'' shape and were actively motile. However, in the larval viability assay, it was apparent that *rac*RBZ was highly active in reducing the viability of the larva at concentrations of 0.25, 0.5, and 1 μg/ml. At these concentrations, the viability and capacity of infection of the larvae was reduced by 92.5–99.6%, when compared with untreated controls (Table I). Lower, but still significant, activity was recorded at 0.12 μg/ml (74.3%), whereas at 0.06 μg/ml, no significant reduction was observed ($P > 0.05$).

We compared the antilarval activity of *rac*RBZ, (+)RBZ, and (−)RBZ at concentrations of 0.5 μg/ml (experiment 1) and 0.1 and 0.01 μg/ml (experiment 2). All 3 compounds were highly active at 0.5 μg/ml, producing a significant reduction ($P < 0.01$) in larval viability when compared with controls (Table II). The (+)RBZ was still highly active at 0.1 μg/ml, producing a reduction of 84.4% in larval viability ($P < 0.01$), which was greater than that observed for *rac*RBZ (69.3% reduction, $P < 0.05$). At this concentration, (−)RBZ did not produce a significant reduction in larval viability (30.1%). None of these 3 compounds was active at 0.01 μg/ml, provoking reductions of 0.00, 33.4, and 26.61% for *rac*RBZ, (+)RBZ, and (−)RBZ, respectively ($P > 0.05$). Nevertheless, we did observe some evidence that (+)RBZ at 0.01 μg/ml may provoke a significant response, and this is being analyzed further (data not shown).

These results suggest that the (+) enantiomer of ABZSO is likely to be responsible for it's activity against helminth parasites. However, more definitive proof of this hypothesis would require the total separation of these enantiomers or their chemical synthesis, which is now in progress. Nevertheless, we believe that pending this achievement, it is of interest to advance these findings from our ex vivo model system of anthelmintic activity. Total purification of the enantiomers was not achieved in the HPLC separation because each contained ≈15% of the opposite isomeric moiety. Thus, it seems likely that the activity shown by (−)RBZ at 0.5 μg/ml may actually be due to the (+)RBZ contamination that was present (equivalent to 0. 075 μg/ml).

The suitability of our ex vivo model for testing anthelmintic activity is supported by the fact that helminths, unlike mammals, oxidize ABZ in a nonenantiomer-selective fashion (Solana et al., 2001). On the other hand, it seems unlikely that *T. spiralis* larvae might convert the *rac*RBZ

and the (+) or (−) enantiomers to ABZ, as has been shown for the cestode *Moniezia expansa* with ABZSO (Solana et al., 2001), because no activity was recorded for the (−) enantiomer. Although the selective retroconversion of (+)RBZ to ABZ in detriment of (−)RBZ cannot be ruled out, the weak antilarval activity of (−)RBZ would again be confirmed. As mentioned above, variability in the pharmacokinetic disposition of ABZ and its metabolites is well documented in different animal species. Thus, a few minutes after oral administration of ABZ, (−)ABZSO becomes the dominant enantiomer in mice (reaching > 60%), and by 1,400 min, the proportion of this enantiomer increases to 75% (García et al., 1999). This pharmacokinetic enantiomer selectivity may explain why high doses of ABZ are necessary to achieve an effect in mice (50–100 mg/kg/day; García et al., 2003), when taken into consideration along with the results obtained in our study. In contrast, in other species such as sheep and cattle (Fetterer, 1982), in which the (+) enantiomer is the dominant form (Delatour, Garnier et al., 1991), a dose of 5 mg/kg is effective for treatment of tissue-dwelling parasites. However, another reason may relate to the pharmacokinetic differences between monogastric and ruminant species, which may influence the solubility and absorption rates of ABZ. For instance, it has been demonstrated that the rumen may act a reservoir, prolonging the duration of benzimidazole absorption and its outflow down to the gastrointestinal tract in sheep (Hennessy et al., 1994), thus favoring a sustained action. Moreover, in addition to host pharmacokinetic variability, variation in the uptake and accumulation of ABZSO and its enantiomers by different helminth species has also been demonstrated (Alvarez et al., 2000, 2001), and this variation is also relevant to the efficiency of anthelmintic activity.

In summary, knowledge of pharmacokinetic behavior of ABZSO and its enantiomeric forms in production animals and in the parasites they host, together with the data provided in this study on pharmacological activity of these compounds, will be of use for optimizing chemotherapy of parasitic diseases.

We thank C. Picornell (Chemo Ibérica, Madrid, Spain) for kindly supplying us with racemic ricobendazole.

LITERATURE CITED

ALVAREZ, L. I., F. A. IMPERIALE, S. F. SANCHEZ, G. A. MURNO, AND C. E. LANUSSE. 2000. Uptake of albendazole and albendazole sulphoxide by *Haemaonchus contortus* and *Fasciola hepatica* in sheep. Veterinary Parasitology **94:** 75–89.

———, M. L. MOTTIER, S. F. SANCHEZ, AND C. E. LANUSSE. 2001. Ex vivo diffusion of albendazole and its sulphoxide metabolite into *Ascaris suum* and *Fasciola hepatica*. Parasitology Research **87:** 929–934.

BOLAS-FERNANDEZ, F. 2002. Total anaerobiosis during in vitro culture in conventional cell culture media is required for retaining infectivity of *Trichinella spiralis* L1 larvae. Journal of Parasitology **88:** 794–796.

CAPECE, B., G. CASTELLS, F. PEREZ, M. ARBOIX, AND C. CRISTOFOL. 2000. Pharmacokinetic behaviour of albendazole sulphoxide enantiomers in male and female sheep. Veterinay Research Communications **24:** 339–348.

DELATOUR, P., E. BENOIT, S. BESSE, AND A. BOUKRA. 1991. Comparative enantioselectivity sulphoxidation of albendazole in man, dog and rats. Xenobiotica **21:** 217–221.

———, ———, F. GARNIER, AND S. BESSE. 1990. Chirality of the sulphoxide metabolites of fenbendazole and albendazole in sheep. Journal of Veterinary Pharmacology and Therapeutics **13:** 361–366.

———, F. GARNIER, E. BENOIT, AND I. CAUDE. 1991. Chiral behaviour of the metabolite albendazole sulphoxide in sheep, goats and cattle. Research in Veterinary Sciences **50:** 134–138.

FETTERER, R., R. S. REW, AND R. KNIGHT. 1982. Comparative efficacy of albendazole against *Fasciola hepatica* in sheep and calves: Relationship to serum drug metabolite levels. Veterinary Parasitology **11:** 309–316.

GARCÍA, J. J., F. BOLAS, AND J. J. TORRADO. 1999. Quantitative determination of albendazole and its main matabolites in plasma. Journal of Chromatography B **723:** 265–271.

———, ———, AND ———. 2003. Bioavailability and efficacy characteristics of two different oral liquid formulations of albendazole. International Journal of Pharmaceutics **250:** 351–358.

HENNESSY, D., D. ALI, AND S. TREMAIN. 1994. The partition and fate of soluble and digest particulate associated oxfendazole and its metabolites in the gastrointestinal tract of sheep. International Journal for Parasitology **24:** 327–333.

HORTON, J. 2000. Albendazole: A review of anthelmintic efficacy and safety. Parasitology **121:** S113–S132.

LACEY, E. 1988. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. International Journal for Parasitology **18:** 885–936.

LANUSSE, C., L. GASCON, AND R. PRICHARD. 1995. Comparative plasma disposition kinetics of albendazole, fenbendazole, oxfendazole and their metabolites in adult sheep. Journal of Veterinary Pharmacology and Therapeutics **18:** 196–203.

———, B. NARE, AND R. PRICHARD. 1993. Comparative sulphoxidation of albendazole by sheep and cattle liver microsomes. Xenobiotica **23:** 285–295.

MARQUES, M. P., O. M. TAKAYANAGUI, P. S. BONATO, S. SANTOS, AND V. L. LANCHOTE. 1999. Enatioselective kinetic disposition of albendazole sulphoxide in patients with neurocysticercosis. Chirality **11:** 218–223.

MARRINER, S., AND J. BOGAN. 1980. Pharmacokinetics of albendazole in sheep. American Journal of Veterinary Research **41:** 1126–1129.

PROCHANZKOVA, A., M. CHOUKI, R. THEURILLAT, AND W. THORMANN. 2000. Therapeutic drug monitoring of albendazole: Determination of albendazole, albendazole sulphoxide and albendazole sulphone in human plasma using non aqueous capillary electrophoresis. Electrophoresis **21:** 729–736.

RUEDA-POLO, S., J. J. TORRADO, F. BOLAS, AND S. TORRADO. 1998. A selective and simple RP-HPLC assay to quantify albendazole metabolites in plasma. Journal of Liquid Chromatography and Related Technologies **21:** 2327–2340.

SANCHEZ, S., L. ALVAREZ, AND C. LANUSSE. 1997. Fasting induced changes on the pharmacokinetic behaviour of albendazole and its metabolites in cattle. Journal of Veterinay Pharmacology and Therapeutics **20:** 38–47.

SOLANA, H., J. A. RODRIGUEZ, AND C. E. LANUSSE. 2001. Comparative metabolism of albendazole and albendazole sulphoxide by different helminth parasites. Parasitology Research **4:** 275–280.

———, J. SALLOVITZ, R. NAJLE, J. A. RODRIGUEZ, AND C. E. LANUSSE. 2000. Liver sulphoxidation metabolism of albendazole in rat: Enantioselectivity and effect of methimazole. Methods and Findings in Experimental and Clinical Pharmacology **22:** 83–88.

———, J. M. SALLOVITZ, C. E. LANUSSE, AND J. A. RODRÍGUEZ. 2002. Enantioselective binding of albendazole sulphoxide to cytosol proteins from helminth parasites. Methods and Findings in Experimental and Clinical Pharmacology **24:** 7–13.

SOUHAILI-EL AMRI, H., X. FARGETTON, P. DELATOUR, AND A. BATT. 1987. Sulphoxidation of albendazole by the FAD-containing and cytochorme P-450 dependent mono-oxygenases from pig liver microsomes. Xenobiotica **17:** 1159–1168.

VIRKEL, G., A. LIFSCHITZ, A. PIS, AND C. LANUSSE. 2002. In vitro ruminal biotransformation of benzimidazole sulphoxide anthelmintics: Enantioselective sulphoxidation in sheep and cattle. Journal of Veterinary Pharmacology and Therapeutics **25:** 15–23.