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# Free-Pool Amino Acids in *Biomphalaria glabrata* Infected with *Echinostoma caproni* as Determined by Thin-Layer Chromatography

**Elizabeth L. Ponder, Bernard Fried*†, and Joseph Sherma,** Department of Chemistry, Lafayette College, Easton, Pennsylvania 18042; *Department of Biology, Lafayette College, Easton, Pennsylvania 18042; †To whom correspondence should be addressed. *e-mail: friedb@lafayette.edu*

ABSTRACT: Thin-layer chromatography was used to analyze the free-pool amino acids of the digestive gland–gonad complex (DGG) of *Biomphalaria glabrata* infected with *Echinostoma caproni* and uninfected (control) snails. Qualitative analysis revealed the presence of histidine, lysine, serine, alanine, valine, and isoleucine or leucine in all samples. Quantitative analysis of lysine and valine gave mean weight percentages of 0.00699 ± 0.0022 and 0.00174 ± 0.00056, respectively, in the DGG of uninfected snails, and 0.00504 ± 0.0014 and 0.00254 ± 0.00033, respectively, in the DGG of infected snails. The differences in values between infected and uninfected snails were not statistically significant (Student's *t*-test, $P > 0.05$).

Most studies on the free-pool amino acid content of snails infected with larval digeneans have focused on *Biomphalaria glabrata* infected with *Schistosoma mansoni* (see review in Pachuski, Fried, and Sherma, 2002). These studies have indicated that *S. mansoni* infection qualitatively and quantitatively reduces the free-pool amino acids in the digestive gland–gonad complex (DGG) and hemolymph of *B. glabrata*. Recent work has been concerned with another snail–trematode relationship that involves the African 37-collar-spined echinostome *Echinostoma caproni* and experimental infections in *B. glabrata* snails (see review in Fried and Huffman, 1996).

Because information is not available on the effects of *E. caproni* infection on the free-pool amino acid content of *B. glabrata*, we used thin-layer chromatography (TLC) to examine qualitative and quantitative differences in free-pool amino acids of the DGG of uninfected *B. glabrata* versus those infected with *E. caproni*.

*Biomphalaria glabrata* snails were infected with miracidia of *E. caproni* as described by Idris and Fried (1996) and fed ad libitum on Romaine leaf lettuce. The DGG of 9 infected and 9 matched control snails was removed at 8 wk postinfection (PI) and pooled into 3 infected and 3 control samples (0.0932–0.137 g). At 8 wk PI, snails were patent and began to release hundreds of cercariae per day per snail. Each snail has about 200–300 daughter rediae, and histopathological studies show zones of lysis around the rediae and disruption of the tissues of the digestive gland and the ovotestis (Fried and Huffman, 1996). Infection was initially determined by isolating exposed snails in Stender dishes containing artificial spring water. Snails that released cercariae were considered infected. All snails used in the experiment were reexamined after they were dissected to expose the DGGs. All snails that were positive by isolation had 200–300 daughter rediae in their DGGs, whereas control snails had no redia. Amino acids were extracted from the pooled samples as described by Pachuski, Wagner et al. (2002), and the extract was blown down to dryness at 60 C and reconstituted in 200 μl of 70% ethanol before TLC analysis.

Qualitative TLC analysis by comparison of samples with standards of 19 amino acids (alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, threonine, tryptophan, tyrosine, serine, and valine) was performed on silica gel, cellulose, reversed-phase, and cation-exchange layers as described by Pachuski, Wagner et al. (2002). Quantitative analysis was conducted by visible-mode densitometry of sample and standard zones. The weights of amino acids in the sample zones were automatically interpolated from standard calibration curves relating densitometer scan areas to weights by linear regression for lysine and polynomial regression for valine. The weight percentages of amino acids in samples were calculated. Student's *t*-test was used to determine statistical differences between infected and control DGG samples. A *P* value of $<0.05$ was considered to be significant.

Qualitative analysis of free-pool amino acids of the DGGs of infected and control samples revealed the presence of histidine, lysine, serine, alanine, valine, and isoleucine or leucine because these are the only amino acids detected in all samples. There was also 1 zone on the reversed-phase plate at $R_f = 0.30$ that did not comigrate with any of the 19 amino acids examined. This zone may be 1 of the nonprotein amino acids found in animal and plant tissues as a result of metabolic processes (Fried and Sherma, 1999). No qualitative difference in free-pool amino acid content was apparent.

Only lysine and valine had zones in sample chromatograms that were adequately separated to allow accurate quantitative analysis by densitometry. For the other amino acids, scans of sample zones were not baseline resolved and, therefore, their areas were not accurate measurements of quantities related to the scans of the standard zones. Resolution of all sample zones on all the layers was sufficient, however, for reliable qualitative identification based on comparison of $R_f$ values and colors with standard zones. Lysine was quantified on a reversed-phase layer ($R_f = 0.61$) and valine on a cellulose layer ($R_f = 0.52$). The mean weight percentages of lysine in control and infected DGGs were 0.00699 ± 0.0022 and 0.00504 ± 0.0014, respectively (n = 3). The mean weight percentages of valine in control and infected DGGs were 0.00174 ± 0.00056 and 0.00254 ± 0.00033, respectively (n = 3). No statistical difference was found between the weight percent of lysine or valine in infected versus uninfected DGG samples (Student's *t*-test, $P > 0.05$). In infected snails, the amount of lysine detected was reduced, whereas amount of valine was elevated. We do not know if these differences in amino acid concentrations indicate that the amino acid metabolism is altered in infected snails or if the larval echinostomes selectively take up and use more lysine.

*Biomphalaria glabrata* snails release amino acids in snail-conditioned water, which attract the miracidia of some trematode species (Haas and Haberl, 1997). However, the shift in lysine and valine levels reported in our study probably has no effect on the ability of *B. glabrata* to attract *E. caproni* miracidia because Haberl et al. (2000) found that only the high–molecular weight glycoprotein fraction in snail-conditioned water attracted miracidia of this echinostome.

Pachuski, Fried, and Sherma (2002), using the same type of TLC plates used in this study, found an array of amino acids in uninfected *B. glabrata* and those infected with *S. mansoni*, similar to those we found in our *B. glabrata–E. caproni* model. However, contrary to the results of our model, Pachuski, Fried, and Sherma (2002) noted that the *S. mansoni* infection caused a significant decrease in the concentration of lysine in the DGG of infected snails. Some differences might be expected in the 2 models because *S. mansoni* has sporocysts that obtain material from the DGG by tegumentary absorption, whereas *E. caproni* has rediae that actively feed on the DGG.

The effects of *E. caproni* infection on *B. glabrata* are different depending on the biological substance studied. Fried et al. (1989, 1993) and Shetty et al. (1992) reported significant reductions in the concentration of various lipids in *B. glabrata* infected with *E. caproni*, and Perez et al. (1994) and Wagner et al. (2001) noted similar reductions in several carbohydrates in *B. glabrata* infected with *E. caproni*. Layman et al. (1996), however, did not find significant differences in metallic-ion concentrations in uninfected *B. glabrata* versus those infected with *E. caproni*. Similarly, in our study on free-pool amino acids in *B. glabrata* infected with *E. caproni*, there was no significant difference in the amino acid concentrations between the infected versus uninfected snails.

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# The Epidemiological Investigation of *Trichinella* Infection in Brown Rats (*Rattus norvegicus*) and Domestic Pigs in Croatia Suggests That Rats are not a Reservoir at the Farm Level

**Dagny Stojcevic, Tatjana Zivicnjak, Albert Marinculic, Gianluca Marucci*, Gaspar Andelko†, Mate Brstilo‡, Lucic Pavo§, and Edoardo Pozio*‖,** Department of Parasitology and Parasitic Diseases, Veterinary Faculty, University of Zagreb, 10000 Zagreb, Croatia; *Laboratory of Parasitology, Istituto Superiore di Sanità, 00161 Rome, Italy; †Ministry of Agriculture and Forestry, 10000 Zagreb, Croatia; ‡Croatian Veterinary Institute, 10000 Zagreb, Croatia; §Veterinary Clinic ''Interna veterina,'' 32000 Otok, Croatia; ‖To whom correspondence should be addressed. *e-mail: pozio@iss.it*

ABSTRACT: Whether the brown rat (*Rattus norvegicus*) is a reservoir of *Trichinella* spp. infection or merely an accidental host, which may be vector of *Trichinella* spp., continues to be debated. We estimated the prevalence of *Trichinella* sp. infection in brown rat populations and in domestic pigs in 2 villages in Croatia, where *Trichinella* sp. infection in pigs has been endemic in the past 10 yr. *Trichinella spiralis* larvae, identified by a multiplex polymerase chain reaction analyses, were the only species detected in both rats and pigs. In 2001 and 2002, 2,287 rats were collected on 60 farms with different levels of sanitation and with, or without, *T. spiralis*–infected pigs. The prevalence of infection in rats ranged from 0.2 to 10.7%. Infected rats were detected only on farms with *T. spiralis*–positive pigs and low sanitation or formerly with low sanitation ($P = 0.007$, Fisher's exact test), yet no infected rat was detected on farms with *T. spiralis*–negative pigs. The finding that no infected rat was found on farms with *T. spiralis*–negative pigs suggests that, in the investigated area, the brown rat is not a reservoir but only a victim of improper pig slaughtering.

Whether the brown rat (*Rattus norvegicus*) is a reservoir of *Trichinella spiralis* infection or merely an accidental host, which may be vector of *T. spiralis*, continues to be debated. In the 19th century, Leuckart (1866, 1876), with his ''Rat Theory,'' was the first to propose that rats were the reservoir of *T. spiralis* infection for domestic pigs, whereas according to Zenker (1871), the infection in rats can be considered as a symptom of the infection in pigs, and the real source of infection for both animals is scrap and offal of pig carcasses. Although *T. spiralis* infection in pigs is often associated with infection in rats living in abattoirs, farms, and garbage dumps, in these environments, there is no report of *T. spiralis* infection in brown rats where pig populations have been found to be negative, suggesting that brown rats are merely an accidental host, which may be vector of *T. spiralis*.

In the Republic of Croatia, the prevalence of *T. spiralis* infection in domestic pigs has been quite high in the past 10 yr because of the previously uncontrolled migration of humans and domestic animals as result of the war conflict. Before this event, the infection was present only in the District of Vukovar, which is located near the Serbian border, with a prevalence of about 0.05% in domestic pigs (Marinculic et al., 2001). After the conflict, the prevalence in pigs increased from 0.25% in 1995 to 1.52% in 1999, and although the prevalence decreased in 2002, it is still quite high, i.e., 0.21%.

The objective of this study was to estimate the prevalence of *T. spiralis* infection in brown rat and pig populations in this highly endemic area and to contribute to the debate on whether or not these animals are a reservoir of infection for domestic pigs. In this article, the term ''reservoir'' refers to an animal species that can maintain *T. spiralis* worms in the environment for a long period of time, i.e., for several


FIGURE 1. Map of investigated areas in the village of Otok. **A.** Investigation carried out in 2001 and map of Croatia showing the Vukovar district (black area) and the Otok and Komletinci villages. **B.** Investigation carried out in 2002. *Trichinella spiralis*–positive pigs, ●; *T. spiralis*–negative pigs, ○; *T. spiralis*–positive rats, ▲; and *T. spiralis*–negative rats, △.

years, without new infections being introduced from another source. The term ''accidental host'' refers to an animal species or pig offal that harbors *T. spiralis* worms, which may be vector of *T. spiralis*, yet is unable to maintain the parasite in the environment for a long period of time.

The study was conducted in the villages of Otok (6,000 inhabitants) and Komletinci (2,000 inhabitants), which are located about 10 km from each other and are both near the town of Vinkovci (eastern Croatia) (Fig. 1). Most of the inhabitants raise pigs outdoors, either in their backyard (Fig. 1) or on small private farms. The pigs are generally fed with vegetable waste, corn, and leftovers and are raised for personal consumption, with no more than 10 pigs per site. The total pig population in the 2 villages was about 10,000 in 2001.

In the past 10 yr, both villages have been the foci of several outbreaks of human trichinellosis, which occurred as a result of the consumption of undercooked pork and smoked pork products at Christmastime. The products had been prepared from pigs slaughtered at home, in the absence of veterinary controls. The occurrence of these foci led local veterinary authorities to perform rat control, beginning in January 1999. Every year, in spring and autumn, poisoned bait is placed in all pig-breeding areas. Information campaigns have also been conducted with the aim of discouraging the practice of scattering pork scraps, i.e., edible trimmings after slaughtering, and offal on farms or in backyards. For the purposes of the present study, those farms where pork scraps are no longer scattered after slaughtering and where veterinary controls are performed to identify and eliminate infected pigs are referred to as farms with ''good sanitation.'' However, in these cases, the level of sanitation is actually well below the standard; on the other farms, referred to as having ''low sanitation,'' pigs are slaughtered without any veterinary controls, and pork scraps are scattered in backyards.

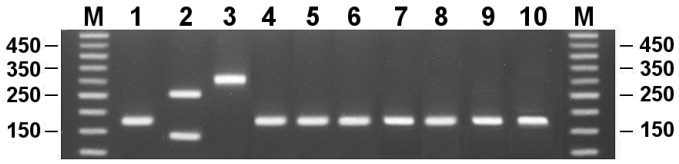


FIGURE 2. Electrophoretic pattern after multiplex-polymerase chain reaction amplification of *Trichinella* larvae of brown rats and domestic pigs of Otok and Komletinci villages. Lanes M, 100 bp ladder (Amersham-Pharmacia biotech); lane 1, *Trichinella spiralis* reference larva (code ISS3); lane 2, *T. britovi* reference larva (code ISS2); lane 3, *T. pseudospiralis* reference larva (code ISS13); lanes 4–7, *T. spiralis* larvae from representative brown rats (*Rattus norvegicus*); lanes 8–10, *T. spiralis* larvae from representative domestic pigs.

In 1999, the national veterinary authorities instituted compulsory trichinelloscopy, which consists of the analysis of 0.5 g of diaphragm pillars of all slaughtered pigs. Muscle samples found to be positive for *T. spiralis* are sent to the Department of Parasitology and Parasitic Diseases of the University of Zagreb (Croatia), where the larvae are collected after digestion and stored in absolute ethyl alcohol for identification at the species level, as described below. In the present study, the data on the prevalence of *T. spiralis* infection in pigs at the national level and at the level of the entire village in the 2 villages are those provided by the routine trichinelloscopy.

In both villages, the survey on rats was carried out in the areas with the highest concentration of *T. spiralis*–positive pigs. Brown rats were trapped from 2001 to 2002. The rats were killed with ether, and the carcasses were transported to the laboratory in Zagreb and kept at 4 C until examination, which was performed within 24 hr of trapping. For

TABLE I. Prevalence of *Trichinella* infection in domestic pig populations in Croatia, at the national level and in the villages of Otok and Komletinci, by year.

| | Prevalence (%) (positive/examined) | | | |
|---|---|---|---|---|
| | 1999 | 2000 | 2001 | 2002 |
| Croatia | 0.06 (693/1,180,752) | 0.32 (4,478/1,392,347) | 0.1 (1,378/1,385,673) | 0.07 (1,128/1,565,064) |
| Otok | | | | |
| Entire village | 1.2 (135/10,380) | 2.1 (198/8,846) | 0.6 (50/8,581) | 0.1 (7/6,678) |
| Investigated farms (n = 49) | NR* | NR | 25.5 (50/196) | 7.5 (7/93) |
| Komletinci | | | | |
| Entire village | 4.9 (139/2,827) | 3.2 (83/2,616) | 2.1 (37/1,725) | 1.1 (19/1,759) |
| Investigated farms (n = 11) | NR | NR | 32.4 (37/114) | 19.4 (19/98) |

* NR, not reported.

each rat, the whole diaphragm and the musculature of rear limbs (for a total of 10 g of muscle) were digested according to previously published protocols (Pozio, 1987; Gamble et al., 2000).

Larvae from positive samples were washed several times in cold water and counted in triplicate under a microscope. Ten larvae from each animal were collected separately in a conical tube with 5 μl of water and then frozen at −30 C.

To identify the larvae at the species level, 5 to 10 muscle larvae (ML) from each positive animal were identified using a multiplex polymerase chain reaction (Zarlenga et al., 1999), following a previously published protocol (Pozio and La Rosa, 2003). ML of reference strains of *T. spiralis* (code ISS3), *T. britovi* (ISS2), and *T. pseudospiralis* (ISS13) were used as controls, i.e., the only 3 species potentially present in the area (Marinculic et al., 2001; Pozio, 2001).

Table I shows the prevalence of *Trichinella* infection in pig populations at the national level and at the level of the entire village (taken from routine trichinelloscopy), for the years 1999–2001, and on the farms investigated in the present survey, for the years 2001 and 2002. At the national level, the prevalence increased from 1999 to 2000 and then decreased in the successive year. A similar trend was observed both in Otok, where the prevalence increased from 1999 to 2000 and then decreased in 2001 and 2002, and in Komletinci, where the prevalence decreased from 1999 to 2002.

Table II shows the prevalence of infection in rats and domestic pigs on the 49 farms in Otok and the 11 farms in Komletinci, for the years 2001 and 2002, by level of sanitation. In 2001, 29 farms in Otok had low sanitation, and infected pigs were found on all. On these farms, an infected rat was also found. Neither infected pigs nor infected rats were found on the 20 farms with good sanitation. In 2002, 23 of the 29 farms that had had low sanitation in 2001 had good sanitation in 2002, in addition to the 20 farms with consistently good sanitation. On none of these farms was an infected pig or infected rat found, whereas on 1 of the 6 farms that continued to have low sanitation, 7 infected pigs and 3 infected rats were detected (Fig. 1). In Komletinci, 1 of the 11 farms had low sanitation in 2001; 37 infected pigs and 14 infected rats were detected on this farm. No infected rat or pig was found on the 10 farms with good sanitation. In 2002, the 1 farm with low sanitation had improved; although 19 infected pigs were found, there was no infected rat (Fig. 1).

There was a significant association between farms with low sanitation and the presence of infected rats ($P = 0.007$, Fisher's exact test). Of the 18 infected rats (0.78% of examined rats) collected in 2001 and 2002, 83.3% were adults (8 males and 7 females), and 16.7% were juveniles (2 males and 1 female). The average number of larvae per gram was 234.4 (range: 0.2–974). All larvae collected from rats and pigs were identified as *T. spiralis* (Fig. 2).

To define the brown rat as reservoir of *Trichinella* for pigs in a given area, 4 criteria need to be met: (1) the 2 animals should be infected with the same *Trichinella* species; (2) rats must be eaten by pigs; (3) the area where infected rats are detected should be similar in size to or

TABLE II. Prevalence of *Trichinella* infection in brown rats (*Rattus norvegicus*) and domestic pigs on farms with different levels of sanitation in the villages of Otok and Komletinci, Croatia, 2001–2002.

| | 2001 | | | 2002 | | |
|---|---|---|---|---|---|---|
| Level of sanitation | No. of examined farms | No. of positive pigs/total (%) | No. of positive rats/total (%) | No. of examined farms | No. of positive pigs/total (%) | No. of positive rats/total (%) |
| Otok (49 farms) | | | | | | |
| Low sanitation* | 29 | 50/128 (39.0) | 1/583 (0.2) | 6 | 7/22 (31.8) | 3/145 (2.0) |
| Formerly with low sanitation† | — | — | — | 23 | 0 | 0/315 |
| Good sanitation‡ | 20 | 0/68 | 0/310 | 20 | 0/71 | 0/178 |
| Komletinci (11 farms) | | | | | | |
| Low sanitation* | 1 | 37/64 (57.8) | 14/130 (10.7) | — | — | — |
| Formerly with low sanitation§ | — | — | — | 1 | 19/42 (45.2) | 0/143 |
| Good sanitation‡ | 10 | 0/50 | 0/228 | 10 | 0/56 | 0/255 |

* Farms where pigs are slaughtered without any veterinary control and where pork scraps are not properly destroyed after slaughtering; these farms had at least 1 *Trichinella*-positive pig.

† Farms where the level of sanitation improved between 2001 and 2002 (i.e., pork scraps are no longer scattered after slaughtering, and veterinary controls are performed to identify and eliminate infected pigs).

‡ Farms with good sanitation: farms where, since 2000, pork scraps are no longer scattered after slaughtering and where veterinary controls are performed to identify and eliminate infected pigs.

§ Farms on which sanitation improved between 2001 and 2002, yet where there were still *Trichinella*-positive pigs.

larger than the area of infected pigs; and (4) the rat populations should maintain the infection for years, independently of the presence of *T. spiralis* infection in pigs or other animals living in the same area. In our survey, the first criterion was met. With respect to the second criterion, we cannot exclude that rats were eaten by pigs (Schad et al., 1987). However, the third and fourth criteria were not met. In fact, infected rats were detected on far fewer farms than infected pigs and only on farms with both infected pigs and low sanitation or formerly with low sanitation. On farms where the scattering of pork scraps and offal had been discontinued, infected rats were no longer detected after 1 yr. No infected rat was detected on farms where all the pigs were negative, independent of the level of sanitation. That infected rats were only found in the presence of infected pigs is consistent with the absence of reports of *T. spiralis* infection in brown rats collected on farms where the pig population was negative. In a study carried out in Pennsylvania, *T. spiralis*–free pigs introduced in a farm with a low level of sanitation, i.e., with infected pigs and synanthropic rats, acquired the infection within 3–4 mo. Moreover, the prevalence of infection in these newly introduced pigs was related to the level of exposure to rats (Schad et al., 1987). Although the authors debated whether or not the presence of infected pigs is necessary for establishing *T. spiralis* infection in a rat population living among the pig herd, they still concluded that rats were a reservoir of infection. However, this conclusion can only be reached if positive rats are found in the presence of negative pigs, which was not the case.

The above data strongly suggest that brown rats are only accidental hosts of *T. spiralis* and do not constitute a reservoir. If so, then it is likely that rats living on farms, in garbage dumps, or in slaughterhouses with low sanitation acquire the infection and then act as a source of infection for pigs bred nearby or for synanthropic wild animals, i.e., rats can only play the role of vector of *T. spiralis*. This distinction between accidental host and reservoir is necessary for better understanding the role of animals (in this case rats) in the epidemiology of *Trichinella* spp. The importance of such a distinction has been emphasized by Haydon et al. (2002), who proposed that a reservoir be defined as 1, or more, epidemiologically connected populations in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population.

The spread of infected pork scraps in the environment by humans seems to always be at the basis of *T. spiralis* infection in rats. Rat control campaigns and farm renovations can force rats to migrate and to spread the infection to neighboring farms and villages, and the use of pesticides against rats can favor transmission because poisoned rats are easy prey for pigs. The role of the brown rat as vector of *T. spiralis* was clearly shown by Smith et al. (1976) in some swine herds of the Atlantic provinces of Canada. In these herds, a rat control program forced rats to migrate from *Trichinella*-positive herds to *Trichinella*-negative herds. A few months later, pigs of the *Trichinella*-negative herds became positive. Thus, infected rats represent an offshoot of the domestic cycle, being recipients of infection from that cycle (Campbell, 1983). This is consistent with the finding in the United States that the occurrence of *T. spiralis* infection in domestic pigs greatly decreased once feeding with uncooked garbage and offal was ended, which is usually done to control bacterial and viral infections (Hall, 1937). This is also consistent with Zenker's (1871) affirmation that the source of infection for both pigs and rats is scrap and offal of pig carcasses.

That we detected only *T. spiralis* in rats and pigs is consistent with the findings of previous studies, which have shown that this species is the main parasite transmitted in the domestic environment (Murrell et al., 1987). However, *T. britovi* and *T. pseudospiralis* have also been detected in both rats and pigs, suggesting that other species can be transmitted in particular epidemiological situations (Britov, 1997; Pozio, 2000, 2001). Moreover, the role of humans is apparently not limited to the domestic environment, as shown by the detection of infected animals in sylvatic environments where hunters leave animal carcasses in the field after skinning (Pozio et al., 2001). In an area of central Italy where sylvatic *T. britovi* infection has been found to be endemic, i.e., 25% of foxes positive for *T. britovi* in the winter, 5 brown rats (3.2% of the rats tested) in 2 garbage dumps were found to have been infected with the parasite. The dumps were used by hunters to dispose of the fox carcasses during the hunting season (November–February). In June, none of the 112 rats collected in these garbage dumps was found to have been infected, given that the hunting season had ended (and no carcass was being disposed of) and that *T. britovi* does not survive for long periods of time in rats (Pozio et al., 1992, 1996).

The present results suggest that, in the investigated endemic area of Croatia, the brown rat acts not as a reservoir of *T. spiralis* in pigs but as an accidental host, which can play the role of vector of *T. spiralis*. Consequently, the hypothesis that rats are a reservoir of *T. spiralis* in the domestic environment should be revised not only in the investigated area but also in other regions of the world, distinguishing the concept of reservoir from that of accidental host. Nonetheless, regardless of these considerations, the control of rat populations at the farm level remains of utmost importance in reducing the occurrence of infection in pigs because of their potential role of vector of *T. spiralis* and less frequently of *T. britovi* and *T. pseudospiralis*.

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# High Prevalence of *Hepatozoon* Spp. (Apicomplexa, Hepatozoidae) Infection in Water Pythons (*Liasis fuscus*) From Tropical Australia

**Beata Ujvari***†**, Thomas Madsen***†**, and Mats Olsson**‡§**,** *Department of Animal Ecology, Ecology Building, Lund University, S-223 62 Lund, Sweden; †School of Biological Sciences A08, University of Sydney, New South Wales 2006, Australia; ‡Department of Zoology, Animal Ecology, University of Gothenburg, Box 463, S+405 30 Gothenburg, Sweden; §School of Biological Sciences, Institute of Conservation Biology, University of Wollongong, New South Wales 2522, Australia. *e-mail: beata.ujvari@zooekol.lu.se*

ABSTRACT: Molecular methods were used to identify blood parasites frequently observed in blood smears of water pythons (*Liasis fuscus*) captured in our study area in the Northern Territory of Australia. A nested polymerase chain reaction (PCR) using primers amplifying the 18s ribosomal RNA (rRNA) nuclear gene resulted in a short PCR product (180 bp) matching this region in the genus *Hepatozoon*. However, because of the short sequence obtained, 2 new primers were designed based on 18s rRNA sequences of 3 *Hepatozoon* taxa available in GenBank. Using these primers, approximately 600 bp of the parasite's 18s rRNA gene was amplified successfully and sequenced from 2 water python samples. The new primers were used to investigate the prevalence of blood parasites in 100 pythons. In 25 of these samples we did not observe any blood parasites when examining stained slides. All the samples revealed a 600-bp PCR product, demonstrating that pythons in which we did not visually observe any parasites were infected by *Hepatozoon* spp. We also analyzed the nucleotide sequences of blood parasites in 4 other reptile taxa commonly encountered in our study area. The sequences obtained from water pythons and from 1 of these taxa were identical, suggesting that the parasite is capable of infecting hosts at different taxonomic levels.

In 1949, John Haldane initiated the idea that parasites could act as significant selective agents on their hosts, e.g., parasites could mediate competitive interactions between hosts, produce and maintain rare host genotypes, and even drive host speciation (Haldane, 1949). During the past decades Haldane's ideas have resulted in numerous publications focusing on parasite–host interactions. Parasites have been demonstrated to regulate host population size (Anderson and May, 1979), reduce host fecundity (Møller, 1993), increase host morbidity (weakness or other debility) (Clayton and Moore, 1997), and even cause host mortality (Coltman et al., 1999; Pampoule et al., 1999). At the genetic level parasites may structure genotypic polymorphism (Clark, 1979) and thus determine host genetic structure (Schykof and Schmid-Hempel, 1991). Furthermore, parasites are frequently invoked as a major factor driving the evolution of the tremendous complexity of the vertebrate immune system (Hedrick, 1994).

Since 1989, we have been conducting fieldwork on a large water python (*Liasis fuscus*) population living on the Adelaide River floodplain situated 60 km southeast of Darwin in the Northern Territory of Australia (Madsen and Shine, 2000). During the course of the study we discovered that the pythons are frequently infected by a wide range of gatstrointestinal and blood parasites that in numerous cases have resulted in death of the hosts (T. Madsen, pers. obs.). Thus, during the last 2 yr our work has focused on quantifying individual variation in parasite prevalence and developing methods to identify the hemogregarine parasites observed in python blood smears. However, relying only on scanning stained blood smears may mean that low levels of parasite infections will not be detected (Macdonald, 1926; Perkins and Martin, 1999). Therefore, in recent years molecular methods, both mitochondrial DNA, e.g., cytochrome *b,* and nuclear DNA, e.g., ribosomal RNA (rRNA), have been used increasingly to detect the presence and identity of hematozoan parasites (Escalante et al., 1998; Mathew et al., 2000; Perkins and Schall, 2002). In the present study we used polymerase chain reaction (PCR) and sequencing to identify and determine the taxonomic status of the blood parasites infecting the pythons and 4 other squamate taxa frequently encountered in our study area. We provide data on the prevalence of these parasites in the python population, and the possible pathogenic effects of the blood parasites on water pythons are discussed.

Water pythons are large, nonvenomous snakes that occur over a wide area of tropical Australia (Cogger, 1992). Blood samples (100 μl) were collected from 102 pythons, and genomic DNA was isolated from whole blood by phenol–chloroform extraction (Sambrook et al., 1989). Initially, we tested the set of primers, described by Li et al. (1995) and Perkins et al. (1998), amplifying the 18s rRNA nuclear gene of blood parasites. A nested PCR was performed according to the protocol of Perkins et al. (1998). The PCR product was resolved by electrophoresis on 2% agarose gel stained with ethidium bromide. The product was sequenced using the dye terminator cyclic sequencing (big dye) system on an ABI PrismTM 310 automated DNA sequencer (Perkin–Elmer, Applied Biosystems, Foster City, California). The amplification of the PCR product resulted in a sequence spanning approximately 180 bp. The sequence was sent to GenBank and matched the 18s rRNA gene region in *Hepatozoon* spp. by 97%. However, because of the short sequence obtained, we decided to design a new set of primers based on *Hepatozoon* spp. 18s rRNA gene sequences available from GenBank. The sequences of 3 taxa were aligned; *H. catesbeiana* (AF176837), *H. canis* (AF176835), and *H. americanus* (AF176836) and the conservative regions were used to design 2 new primers: HepF300: 5′-GTT TCT GAC CTA TCA GCT TTC GAC G-3′, Hep900 5′-C AAA TCT AAG AAT TTC ACC TCT GAC-3′.

The PCR reactions were run in a 50-μl reaction mixture containing 50 ng total genomic DNA, 1 U of AmpliTaq polymerase (Perkin–Elmer, Applied Biosystems), 1.5 mM $MgCl_2$, 0.125 mM of each nucleotide, 5 μl of Perkin–Elmer GeneAmp 10× PCR buffer (100 mM Tris–HCl, 500 mM KCl, and 0.01% gelatine), and 0.6 μM of each primer. The reaction mixture was heated to 94 C for 3 min, and then amplification was performed through 35 cycles at 94 C for 30 sec, 60 C for 30 sec, and 72 C for 1 min. After the 35 cycles there was a final 10-min extension at 72 C. The PCR products of the 2 water pythons were resolved and sequenced as described above.

Perkins and Martin (1999) demonstrated that 18s rRNA primers may amplify host DNA and thus give rise to false-positive results. The following 3 steps were undertaken to reduce this risk: (1) to avoid contamination, negative controls were run with each set of reactions, using 1 μl of sterile Milli-Q water in place of the template (all other reagent concentrations remained the same); (2) our primers were submitted to Genbank, and the alignment demonstrated that our primers matched

only the 18s rRNA gene of *Hepatozoon* spp.; and (3) DNA of 2 great reed warblers (*Acrocephalus arundinaceus*) known to be infected with both *Plasmodium* spp. and *Haemoproteus* spp. were amplified, but both amplifications failed to reveal any PCR products. These results suggest strongly that our primers amplified only the 18s rRNA gene in *Hepatozoon* spp.

The new primers were used to investigate the prevalence of blood parasites in 100 pythons (none of these samples was sequenced). In 25 of these samples we had not observed any blood parasites when examining stained blood smears (2,000 erythrocytes were counted on each slide).

The primers were also used to investigate whether the 4 other squamate taxa frequently encountered in our study area were infected by *Hepatozoon*. We sampled 1 brown tree snake (*Boiga irregularis*), 2 slaty-grey snakes (*Stegonotus cucullatus*), 2 spotted tree goannas (*Varanus scalaris*), and 2 northern sand goannas (*V. panoptes*).

In all the 100 water pythons examined, we obtained a PCR product spanning approximately 600 bp. Thus, the 25 pythons in which we did not detect any parasites when scanning stained blood smears were infected by *Hepatozoon* spp., confirming that relying only on visual methodology may indeed produce false negatives. Furthermore, all the 7 specimens of the other 4 taxa revealed a similar PCR product, demonstrating that these squamates were also infected by *Hepatozoon* spp.

The 581-bp parasite sequences obtained from the 2 water pythons and the 2 northern sand goannas were identical. Pairwise comparisons (Kimura, 1980) demonstrated that all the 9 *Hepatozoon* haplotypes from the 5 host species were very similar (genetic distance ranging from 0.000 to 0.029), compared with the haplotypes of amphibian and canid *Hepatozoon* parasites (*H. catesbianae*, *H. americanum*, and *H. canis*; pairwise genetic distance comparisons ranged from 0.047 to 0.073). The sequences of the 5 host species have been deposited in GenBank (accession numbers: AY252103, AY252104, AY252105, AY252106, AY252107, AY252108, AY252109, AY252110, AY25211).

Hemogregarines of the Hepatozoidae have been reported to be the most common group of intracellular protozoan blood parasites found in snakes (Telford, 1984; Wozniak et al., 1996). These findings are supported by the results from the present study. Furthermore, the prevalence of *Hepatozoon* spp. infections of squamate reptiles in our study area appears to be very high because all the 100 water pythons and all the 7 specimens of the 4 other squamate taxa were infected.

On the basis of morphological studies, Telford et al. (2001) suggested that different *Hepatozoon* taxa often exhibit high levels of host specificity. However, our results demonstrate that *Hepatozoon* spp. exhibit identical nucleotide sequences, which indicates that the parasites are probably in the same taxon and are able to infect not only different host species but also different squamate families, i.e., Boidae and Varanidae.

Several workers have suggested that hemogregarine parasites exhibit low pathogenetic effects on their reptilian hosts (Nadler and Miller, 1984; Wozniak et al., 1994). However, blood parasite infections may cause leukocyte and erythrocyte dysfunction, with the host becoming more susceptible to systemic infections (Smith et al., 1999; Inokuma et al., 2002). Furthermore, our results suggest that *Hepatozoon* spp. with identical nucleotide sequences are able to infect different host organisms, suggesting that host shifts may occur among some of the squamate taxa found in our study area. Such host shifts have been reported in association with a change in parasite virulence (Toft and Karter, 1990). The findings by Toft and Karter (1990) were supported by Wozniak et al. (1996), who showed that lizards previously unexposed to *Hepatozoon* spp. suffered severe pathological effects when infected with *H. mocassini*. Another example of increased virulence caused by host shifting is the severe pathology, often fatal, associated with infection by *H. americanum* in dogs, as reported by Baneth et al. (2003). Our preliminary analyses suggest that *Hepatozoon* spp. may exert a significant effect on water python growth rates, i.e., high infection levels are significantly correlated with reduced growth rates as compared with snakes with low parasite numbers (T. Madsen, pers. obs.). Thus, the possible pathogenic effect of these hemogregarines may play a complex role in the evolution of their reptilian hosts; this potential phenomenon warrants further research.

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