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Biological variation between two Brazilian geographical isolates of *Echinostoma paraensei*

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Abstract

The biological behaviour and morphometric data from two allopatric isolates of *Echinostoma paraensei* (Rio Bonito – RB and Sumidouro – SU) collected from naturally infected *Nectomys squamipes* from two secluded Atlantic Forest fragments were studied. Mice that had been experimentally infected with ten encysted metacercariae of each isolate were monitored weekly in two trials to analyse worm burden and the kinetics of worm distribution along the intestine. The total number of uterine eggs, wet weights and measurements of the worms and body, acetabulum, testes and ovaries were also analysed. The RB isolate showed a higher worm burden, 7.7 ± 0.8, and a longer life span, 16 weeks, compared to a worm burden of 5.8 ± 1.1 and life span of 9 weeks for the SU isolate. Worms of the RB isolate were clustered in the duodenum and in the bile duct while the SU isolate worms were dispersed along the small intestine of infected mice. Both isolates developed similarly as regards morphometric data and wet weight, although the total number of uterine eggs was greater in RB. The degree of intraspecific variation observed in the worm distribution along the intestine, worm burden and life span raises questions regarding the use of these criteria for species differentiation. These findings suggest that variation in biological parameters found between the *E. paraensei* isolates could result from geographical isolation and, in particular, the environmental conditions of transmission. Further studies on *E. paraensei* polulations from different forest fragments will contribute towards an understanding of the speciation of this parasite.

Introduction

The phylogeny of the genus *Echinostoma* within the digenetic trematodes is complex due to the great interspecific homogeneity of the taxonomic characters, particularly with reference to the aggregation of sibling species possessing 37 spines around the peristomic collar

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in a species-group named 'revolutum group' (Kostadinova & Gibson, 2001). The identification of the species has been supported by the morphology of the collar spines in adult worms (Maldonado *et al.*, 2003), cercarial chaetotaxy (Toledo *et al.*, 2000), biological criteria (Kanev *et al.*, 1995), and more recently through molecular sequence data (Morgan & Blair, 1998; Sorensen *et al.*, 1998). In Brazil, 25 nominal species of the genus *Echinostoma* have been described (Lutz, 1924; Travassos *et al.*, 1969; Yamaguti, 1971), of which eight are in the 37 collar-spined *Echinostoma revolutum* group (Kanev, 1994).

The existence of such a large number of biologically distinct and morphologically similar species results partly from the fact that *Echinostoma* species have low invertebrate and vertebrate host specificities, which increase the chance of colonizing new habitats and enhancing the conditions for speciation (Fried & Graczyk, 2000).

Echinostoma paraensei was first isolated from naturally infected *Biomphalaria glabrata* collected in Belo Horizonte, Brazil (Lie & Basch, 1967). More recently, Maldonado *et al.* (2001a) isolated *E. paraensei* in Sumidouro, Rio de Janeiro State, Brazil from its natural vertebrate host, the water rat *Nectomys squamipes*. The latter is a vagile rodent (Pires *et al.*, 2002), which could limit the spread of this parasite species especially as the host's geographical distribution is presently limited to fragments of Atlantic Forest that in the past extended from the northeast to the southeast of the Brazilian coast.

The present study aimed to investigate the intraspecific variability between two isolates of *E. paraensei* from two distant fragments of the Atlantic Forest through morphometric data and biological parameters of adult worms in experimentally infected mice.

Materials and methods

Parasite isolation and experimental infection

Adult worms of *E. paraensei* were collected from *Nectomys squamipes*, the water rat, in Sumidouro (SU), (22°02′46″ S; 42°41′21″ W), (Maldonado Jr *et al.*, 2001a) and Rio Bonito (RB), (22°42′30″ S 42°37′34″ W), both of which are rural locations in Rio de Janeiro State, Brazil. Metacercariae of *E. paraensei* were obtained after experimental infection of laboratory-reared *Biomphalaria glabrata* according to Maldonado Jr. *et al.* (2001b). Ten encysted metacercariae were administered orally to male albino mice (Swiss Webster) aged 6–8 weeks. Divided into two trials, a total of 150 mice were infected with the Sumidouro isolates and 156 mice with the Rio Bonito isolates.

Infected mice were euthanized using a CO_2 chamber and examined weekly from the first week of infection onwards. To analyse the worm distribution along the intestine, the small intestine was divided into five equal sections (S1–S5), from the pyloric sphincter to the ileo valve, and the location of worms in each section was recorded (Kaufman & Fried, 1996). The large intestine, bile duct and pancreatic duct were also examined for worms. Worms recovered were washed several times in Locke's solution, dried briefly on filter paper (Whatman no. 3) to absorb surface buffer and then weighed. Subsequently, the same worms were individually teased with needles and the number of uterine eggs per worm counted.

Experiments were performed according to the rules of the Ethical Commission of Animals Testing of the Fundação Oswaldo Cruz.

Morphometric analyses

For light microscopy, every week three worms were randomly separated from both isolates and fixed under slight cover slip pressure at room temperature with an alcohol-formalin-acetic acid solution (AFA). Worms were then stained with acid carmine, dehydrated in a graded ethanol series and cleared with methyl salicylated and mounted in Canada balsam. Drawing was made with a Zeiss Axioskop 2 and the use of a camera lucida. All measurements are given in millimetres.

Statistical analysis

Data were analysed using a Mann-Whitney test to determine differences between the groups and a Spearman's correlation test was used to determine the levels of association between the wet weights of worms and number of eggs in the uteri. Values less than ($P < 0.05$) were considered statistically significant.

Results

Worm recovery and niche segregation

All of the Swiss Webster mice (100%) became infected with *E. paraensei* RB isolates in both trials and 91.6% and 100% of mice were infected with SU isolates in the first and second trials, respectively (table 1). The proportion of worms recovered from infected mice was 77% for *E. paraensei* RB isolate and 58% for *E. paraensei* SU isolate.

The *E. paraensei* RB isolate had a longer life span than the SU isolate, surviving for 16 weeks post-infection (p.i.). Worms were progressively eliminated from week 6 p.i. with significantly fewer worms being recovered when compared to the previous week ($P < 0.05$). Mice were negative for parasites for the first time in weeks 5 and 7 p.i. in the first and second trials, respectively.

The *E. paraensei* SU isolate showed a shorter life span, with infections being absent in mice after weeks 5 and 9 in the two trials. Significant worm elimination occurred between weeks 3 and 4 p.i. ($P < 0.05$).

Worms from the SU isolate were dispersed along the length of the small intestine throughout the infection, except at weeks 8 and 9 when they were restricted to the first three sections (table 2). As the infection progressed, worms migrated to the anterior region of the small intestine, with 80% of the woms aggregating in section 1 by week 4 p.i. From week 5 p.i. onwards, worms were again dispersed along length of the small intestine and this coincided with a gradual worm loss.

Worms derived from the RB isolate were not distributed along the length of the small intestine (table 2) as 85% of worms were aggregated in section 1 as early as the first week of infection. A further migration of worms was observed from week 5 onwards, with many

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Table 1. Weekly changes in the mean worm burden of *Echinostoma paraensei* from Swiss Webster mice after experimental infection with ten metacercariae of Sumidouro and Rio Bonito isolates, up to 17 weeks post-infection.

Isolates	Weeks after infection																
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Sumidouro																
First trial	5.9 ± 0.5 (11/12)	5.9 ± 1 (12/12)	2.2 ± 0.8 (7/12)	4.1 ± 0.9 (10/12)	1.7 ± 0.6 (8/12)	2.1 ± 1.6 (6/12)	0.4 ± 0 (1/12)	0.3 ± 0 (4/12)	0 (0/12)							
Second trial	5.7 ± 1.1 (6/6)	1.3 ± 0.9 (2/6)	1.2 ± 0.8 (2/6)	0.5 ± 0.3 (2/6)	0 (0/6)											
X ± SE	5.8 ± 0.1	3.6 ± 2.3	1.7 ± 0.5	2.3 ± 1.8	1.7 ± 0.9											
Rio Bonito																
First trial	7.6 ± 0.8 (6/6)	6.1 ± 1.0 (6/6)	6.1 ± 0.4 (6/6)	5.0 ± 0.9 (5/6)	3.3 ± 0.9 (6/6)	3.6 ± 1.3 (6/6)	3.0 ± 0 (3/6)	2.3 ± 1.0 (3/6)	2.0 ± 1.2 (4/6)	–	1.2 ± 1.0 (1/6)	–	5.0 ± 0.8 (6/6)	–		
Second trial	7.8 ± 0.8 (6/6)	8.5 ± 0.5 (6/6)	9.4 ± 1.2 (6/6)	7.2 ± 0.9 (6/6)	5.8 ± 1.2 (6/6)	3.2 ± 1.2 (5/6)	2.0 ± 1.2 (5/6)	1.8 ± 1.3 (3/5)	0.3 ± 0.2 (2/6)	3.2 ± 1.1 (3/6)	0.3 ± 0.2 (1/6)	1.3 ± 1.0 (1/6)	1.0 ± 1.1 (3/6)	2.2 ± 1.2 (1/4)	0.6 ± 1.0 (2/5)	0 (0/5)
X ± SE	7.7 ± 0.7	6.4 ± 1.2	7.7 ± 1.6	6.1 ± 1.1	4.5 ± 1.2	3.4 ± 0.1	2.5 ± 0.5	2.0 ± 0.2	1.2 ± 0.9	–	0.7 ± 0.5	–	3.2 ± 0	–	–	–

-, not investigated; number in brackets indicate rodents infected in total sampled.

moving up to the bile duct, amounting to 64% of worm establishment by week 12 and resulting in enlargement of the duct circumference or associated with liver necrosis.

Uterine egg production

Eggs first appeared in the uterus of SU worms in week 2 p.i. and in week 3 p.i. in the RB worms. The highest egg count in SU worms (2744 ± 908) occurred in week 6 p.i., followed by a significant decrease from week 8 onwards (fig. 1).

For RB worms the highest egg count (3910 ± 298) occurred in week 8 p.i., followed by a gradual reduction in the mean number of eggs from week 10 p.i. ($P < 0.05$), and variable egg outputs thereafter.

Weekly comparisons made between egg output from both isolates showed significantly more eggs being produced by the SU isolate by week 6 p.i., but this trend was reversed by week 8 (fig. 1), when more eggs were present in RB worms ($P < 0.05$). Overall, the number of uterine eggs from RB isolate was significantly ($P < 0.05$) greater than that in the SU isolate.

Worm development

Wet weights of worms of both isolates increased in a similar manner up to week 4 p.i. ($P < 0.05$), when the weight of RB worms began to decrease, with variable levels being observed during the last 8 weeks of infection (fig. 2). On the other hand, the wet weight of SU worms showed a decline from weeks 6 to 8, before rising again. In summary, the SU demonstrated a more rapid gain in wet weight compared with the RB isolate, with a maximum weight observed in week 6 for SU worms, compared to weeks 4, 9 and 12 in RB worms (fig. 2). In both cases, however, the increase and decrease in wet weights of worms were correlated with the number of eggs in the uteri ($P < 0.05$).

Morphometric data showed that worm growth rates in terms of body length and width were similar and no differences were observed in the sizes of the acetabulum, oral sucker, ovary and testes (table 3).

Morphological features of the peristomic collar surrounding the oral sucker of both RB and SU isolates were similar, with 37 spines distributed along the collar.

Discussion

Speciation of echinostomes has previously been supported by intraspecific differences observed in excretory–secretory polypeptide products from African isolates of *Echinostoma caproni* (Trouvé & Coustau, 1998) and by variation in the regions of internal transcribed spacers of the ribosomal DNA between *Echinostoma revolutum* and *Echinostoma trivolvis* (Sorensen *et al.*, 1998). Speciation has also been indicated in *Echinoparyphium recurvatum* living in sympatric conditions, i.e. different niches in the small intestine of the vertebrate host and by the possession of a distinct, first intermediate host (McCarthy, 1990). The present results clearly indicate that *Echinostoma paraensei* isolates exhibited distinct

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Table 2. Distribution (%) of *Echinostoma paraensei* in five sections (S1 to S5) of the small intestine and in the bile duct of Swiss Webster mice infected with ten metacercariae of Sumidouro and Rio Bonito isolates up to 16 weeks post-infection.

	Weeks after infection															
Intestinal section	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Sumidouro																
S1	32	31	41	80	78	59	64	66	50							
S2	18	9	11	10	7	14	9	17	25							
S3	14	12	23	4	3	14	9	17	25							
S4	9	16	10	3	6	4	9									
S5	27	32	15	3	6	9	9									
Rio Bonito																
Bile duct					7	8	15	8	13	25	26	64	12			33
S1	85	85	95	97	90	90	75	92	87	71	74	36	88	94	100	67
S2	15	10	5	3	3	2	10			4				6		
S3		5														
S4																
S5																

biological characteristics, but were indistinguishable by morphological features.

The life cycle of trematode parasites of vertebrates in the final host frequently reveals a phase of migration that begins with their entrance into the host in a passive or active way and is completed in a specific niche (Fried & Graczyk, 1997). Some trematodes follow a migratory route which comprises passing through several organs, as observed for *Schistosoma mansoni* and *Fasciola hepatica* (Doy & Hughes, 1984). Sukhdeo (1990) and Sukhdeo & Sukhdeo (2002) hypothesized that habitat selection by helminths is based on specific environmental conditions within the host and concluded that releaser responses determine worm migration and if a specific behaviour is extremely adaptive it becomes fixed.

Echinostomes are intestinal trematodes and their development in the vertebrate host commences after metacercariae have been ingested (Fried & Huffman, 1996). Irwin (1997) demonstrated *in vitro* that excystment of metacercariae was dependent on the presence of bile salts and trypsin in an alkaline medium that acts in a synergistic way to promote larval release. Meece & Nollen (1996) reported that after the initial period of infection, *E. paraensei* and *E. caproni* differed in their preference of habitat, the former being located in the duodenum and the latter in the ileum, in both mice and hamsters. Lie & Basch (1967) had already noted the migration behaviour of *E. paraensei* within the small intestine of rodents during the course of infection, with worms aggregating in the duodenum.

In the present study, the *E. paraensei* SU isolate showed similar migration patterns to those observed by Lie & Basch (1967) and Meece & Nollen (1996) in the *E. paraensei* isolate from Belo Horizonte. However, adult worms of the RB isolate are aggregated in the duodenum of mice from week 2 p.i., with some worms migrating to the bile duct. This migration coincides with the beginning of worm expulsion.

As demonstrated by Fujino & Fried (1993), the process of expulsion of *E. caproni* from the small intestine in the mouse model is dependent upon non-specific, immunological, inflammatory reactions. It is possible that

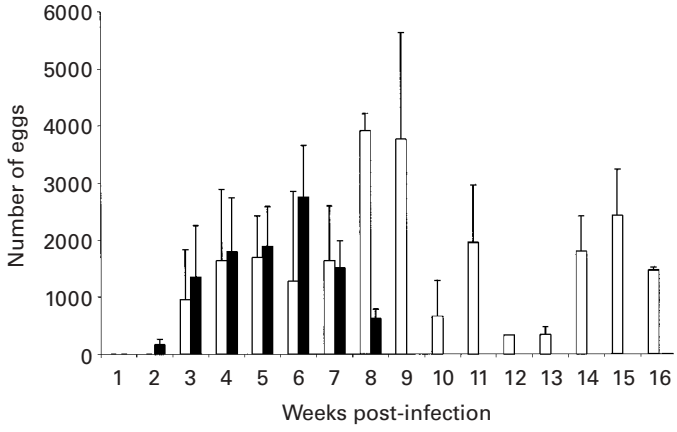

Fig. 1. Mean number of uterine eggs per worm in Rio Bonito (□) and Sumidouro (■) isolates of *Echinostoma paraensei* from 2 to 16 weeks post-infection.

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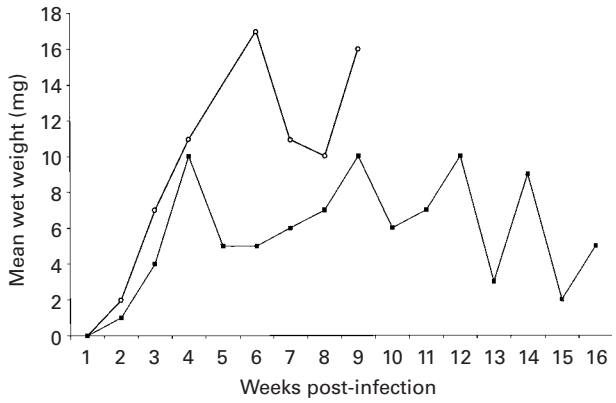

Fig. 2. Mean wet weights of Sumidouro (○) and Rio Bonito (■) isolates of *Echinostoma paraensei* from 2 to 16 weeks post-infection.

E. paraensei RB worms evade these immunological responses in the duodenum and small intestine by migrating to the bile duct. In the case of the tapeworm *Rodentolepis microstoma*, migration from the small intestine to the bile duct may be attributed to an escape from expulsion promoted by the inflammatory response in the host's intestine (Howard *et al.*, 1978). Another explanation for *E. paraensei* RB worms aborting the classical behaviour of migration in the vertebrate host may be linked to a strategy to enhance survival under stress conditions during migration.

Alterations in the spatial distribution of *E. revolutum* sin. *E. caproni* (Franco *et al.*, 1988) or the presence of worms in extra intestinal locations in experimentally infected golden hamsters have also been associated with the administration of high doses of metacercariae (Huffman *et al.*, 1988). Meece & Nollen (1996) reported that *E. parensei* worms were found only in the duodenum of infected mice despite being infected with 25 metacercariae. In the present study, the number of metacercariae (10) in the infection procedures was too low to be responsible for dislodging the worms to extra intestinal sites, indicating that the RB isolate presents biological features that distinguish it from the SU isolate.

Brunet *et al.* (2000) suggested that *Echinostoma caproni* modulates its host-immune response in order to facilitate survival. An increase in the survival rate of worms in primary *E. caproni* infections in two mouse strains was directly related to the higher number of worms harboured by the rodent (Christensen *et al.*, 1988). Therefore, reduced migration of *E. paraensei* RB worms to the gut may result in high worm burdens, suggesting the adoption of a survival strategy by the RB isolate.

Despite the *E. paraensei* RB infection producing higher numbers of adult worms in mice, it always produced lower numbers of uterine eggs per worm than *E. paraensei* SU isolate, suggesting intraspecific competition. Fried *et al.* (1990) observed a greater number of eggs per worm from single worm infections compared with multiple worm infections, and pointed out that a single worm of *E. caproni* was capable of self-fertilization. Moreover, the aggregation behaviour of *E. paraensei* could increase the chance of heterologous insemination (Nollen, 1990).

Table 3. Weekly morphometric comparison of *Echinostoma paraensei* adult worms of Rio Bonito and Sumidouro isolates. Measurements in micrometres.

	Weeks post-infection						
	1	2	3	4	5	6	7
Rio Bonito							
Body length	2000 ± 78	6367 ± 538	7763 ± 2204	10 333 ± 2554	6515 ± 544	6297 ± 851	8207 ± 805
Body width	323 ± 133	1050 ± 135	1400 ± 180	1823 ± 93	1765 ± 113	1360 ± 280	1503 ± 76
Oral sucker	110 ± 30	250	223 ± 71	317 ± 29	195 ± 21	340 ± 113	380 ± 62
Acetabulum	240 ± 20	467 ± 23	650 ± 156	793 ± 11	625 ± 134	817 ± 133	933 ± 76
Testes	20	350 ± 79	367 ± 125	506 ± 95	255 ± 64	283 ± 75	330 ± 30
Ovary	55 ± 7	183 ± 35	230 ± 50	397 ± 46	210	127 ± 30	197 ± 11
Sumidouro							
Body length	3033 ± 907	4867 ± 1595	10 977 ± 2228	11 233 ± 1662	11 800 ± 2893	12 777 ± 1940	8433 ± 681
Body width	633 ± 115	993 ± 11	1543 ± 471	2000	1740 ± 242	1900 ± 173	1533 ± 115
Oral sucker	148 ± 72	226 ± 23	233 ± 49	310 ± 35	286 ± 75	330 ± 12	340 ± 35
Acetabulum	393 ± 120	533 ± 58	733 ± 58	820 ± 72	883 ± 107	917 ± 35	817 ± 38
Testes	104 ± 61	186 ± 40	516 ± 185	443 ± 98	540 ± 130	616 ± 112	406 ± 70
Ovary	71 ± 23	153 ± 41	250 ± 134	283 ± 105	360 ± 120	367 ± 11	293 ± 168

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Egg production in digenetic trematodes has been related to the nutritional requirements of adult worms as well as its definitive host's compatibility and intrinsic features of the trematode species involved (Christensen *et al.*, 1988). In addition, egg production was variable over the period of infection of *Echinostoma friedi* in the hamster model (Toledo *et al.*, 2003). The different rates of fecundity of *Echinostoma caproni* suggest the link between nutritional dependence and maximum egg production in the mouse model (Reddy & Fried, 1996).

The present results confirmed that growth rates measured through morphometry and gain in weight, were similar in both *E. paraensei* isolates. In fact, the number of uterine eggs from worms after experimental infection of four different species of rodent hosts with *Echinostoma paraensei* revealed much interspecific variation (Maldonado *et al.*, 2001b). In conclusion, further experiments are required to determine if variability in *E. paraensei* populations will reflect a transient adaptation to environmental conditions or genetic variation.

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