An experimental study of the reproductive success of *Echinostoma friedi* (Trematoda: Echinostomatidae) in the golden hamster

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SUMMARY

Viable eggs produced weekly per infective stage was used as a measure of the reproductive success of *Echinostoma friedi* during the first 12 weeks of infection in hamsters. The weekly reproductive success was not constant during the experiment in relation to the egg output and the proportion of viable eggs produced. The egg release started during week 2 post-inoculation, attaining a maximum during week 3. A decline in egg output was observed from week 9. Viable eggs were only produced from week 3 post-inoculation and a maximum was attained at week 4 of the experiment. A decline in egg viability was observed from week 9. Considering together the egg output and the egg viability, the maximum weekly reproductive success was obtained during week 4 post-inoculation. The changes in the weekly reproductive success were not reflected in variations in worm numbers and body sizes during the course of the infection. The humoral immune response of golden hamsters during the infection with *E. friedi* was determined. Increases of IgG levels against somatic and excretory/secretory products of *E. friedi* were detected coinciding with the reduction in the reproductive success.

Key words: trematoda, *Echinostoma friedi*, golden hamster, reproductive success, ELISA serum antibody response.

INTRODUCTION

The elucidation of the host–parasite relationships in different *Echinostoma*–rodent models has been studied during recent years (Christensen *et al*. 1988, 1990; Odaibo, Christensen & Ukoli, 1988, 1989; Huffman & Fried, 1990; Hansen *et al*. 1991; Mahler, Christensen & Hindsbo, 1995). Nevertheless, several aspects determining the compatibility between the components of the system remain to be investigated. Much of the interest in describing and modelling the population dynamics of the echinostomes has concerned parasite fecundity in the definitive host. Quantitative approximations to the reproductive output have been limited to measurements on the basis of uterine egg counts (Christensen *et al*. 1990), eggs per gram of faeces (Odaibo *et al*. 1988, 1989), and total amount of eggs in the faeces (Mahler *et al*. 1995). However, egg counts may not reflect the dynamics of the reproductive output in trematode infections since other parameters such as the viability of the eggs should be taken into account (Whitfield, Anderson & Bundy, 1986). This suggested the need for further studies on the parasite reproductive output and to standardize the experimental procedures and criteria for elucidating the parasite fecundity in echinostome infections.

Similarly, the host immune response in echinostome infections has been poorly studied. It is known that echinostomes elicit detectable immune responses in vertebrate hosts, which converts this system to a suitable model for studying the immunological responses of experimental hosts to infection with intestinal trematodes. Nevertheless, most of the studies on this topic have focussed only on the antibody attack to the parasite surface (Simonsen & Andersen, 1986; Andresen *et al*. 1989; Simonsen *et al*. 1990). Studies on antibody responses in serum are limited to those of Simonsen, Estambale & Agger (1991), Agger, Simonsen & Vennervald (1993) and Graczyk & Fried (1994, 1995), though neither the antigens used in these studies nor the experimental procedures to detect the antibody responses have been standardized to an extent to make possible comparisons between different studies.

Echinostoma friedi Toledo *et al*. 2000 (Trematoda: Echinostomatidae) has been described as an intestinal parasite of mammals in Spain (Toledo, Muñoz-Antoli & Esteban, 2000). The aim of the present study is to provide a framework to analyse several aspects of the host–parasite relationships in the golden hamster–*E. friedi* system. We have focused the investigations on the parasite fecundity and the host immune response and their respective variations along the course of the infection. Such information provides an essential background for further development of the *Echinostoma*–rodent system as an experimental model.

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MATERIALS AND METHODS

Parasites

E. friedi (originally obtained from the Albufera Natural Park of Valencia, Spain) was maintained in the laboratory using *Lymnaea peregra* (Müller, 1774) as the first intermediate host. This snail species was used as second intermediate host and golden hamsters (*Mesocricetus auratus*) were used as definitive hosts. Further techniques for the maintenance of *E. friedi* in the laboratory were described in a previous study (Toledo *et al*. 2000).

Experimental infections

Each of 47 outbred male golden hamsters (*M. auratus*), weighing 45–62 g, were infected by stomach tube with 100 metacercariae of *E. friedi* collected from the kidney and pericardial cavity of *L. peregra*. The hamsters were randomly allocated to groups A (6 hamsters), B (36), and C (5). Moreover, 5 hamsters were left uninfected and used as controls. Group A was used to study the kinetics of egg production of *E. friedi* in golden hamsters. Group B was used to study the worm recovery, the production of viable eggs over the course of the infection, and the variation of the morphological features of adult worms during the infection. The humoral immune responses against *E. friedi* were investigated in group C. All the infected hamsters were maintained under conventional conditions with food and water *ad libitum*.

Kinetics of egg release

This experiment was designed to investigate the kinetics of egg output of *E. friedi* during the course of the infection in golden hamsters. For this purpose, the egg output was determined daily in the hamsters of group A during 84 consecutive days post-experimental infection. The 24 h faecal production was collected individually from each animal according to the method described by Keymer & Hiorns (1986). The faecal samples from each day and hamster were individually pooled and weighed. The number of eggs of *E. friedi* per gram of faeces (EPG) was determined according to the method described by Brindley & Dobson (1981). Briefly, faecal samples were emulsified in the ratio 1 g/30 ml of 0·1 M NaOH. This mixture was shaken at room temperature for 2 h and the sediment homogenized in 2 ml. The eggs present in 200 μl were counted. Five replicated samples were analysed for each day and each hamster to determine the EPG and the average was considered to estimate the total number of eggs daily released per hamster (EPH).

Worm recovery and egg viability

This experiment was designed to investigate the worm establishment of *E. friedi* in golden hamsters and the effect of worm age on the viability of the eggs produced during the first 12 weeks after infection. The egg viability was measured in terms of miracidial hatching.

At each week post-inoculation (p.i.) 3 hamsters of group B were necropsied and the number of worms recovered per hamster was recorded. The uteri of 10 worms were teased to obtain eggs. Eggs obtained were placed in Petri dishes containing 10 ml of spring water. Egg cultures were maintained at 20 ± 1 °C in darkness. At days 10–15 of incubation, the egg cultures were exposed daily to artificial light (60 W) for 2 h. The number of swimming miracidia was counted at 15-min intervals. Four cultures containing 500 eggs each were studied for each week that comprised the experiment. Miracidial hatching was expressed as the percentage of miracidia hatched over the 2000 eggs studied each week.

Weekly reproductive success

In this section, we formulate a simple model to describe the population dynamics of *E. friedi* in golden hamsters from metacercaria to viable egg. Our aim is to provide a framework for measuring the reproductive success through this section of the cycle and its variations over the course of the infection. Using the concepts developed by Whitfield *et al*. (1986), the reproductive success of a parasite species in a particular host (R) is defined as:

$$R = E_{s+1}/C_0,$$

where E_{s+1} represents the total number of viable eggs produced by the cohort of adult parasites resulting from host exposure to C_0 cercariae (or other unities of inoculation).

In the present study we have calculated the reproductive success for each of the weeks p.i. to estimate the variations of the fecundity of *E. friedi* in golden hamsters during the infection. The weekly reproductive success (R_w) may be defined as the total number of viable eggs produced each week divided by the number of metacercaria to which the host was exposed. The total number of viable eggs produced each week per host (E_i) is calculated as:

$$E_i = N_i P_i / 100,$$

where N_i is the number of eggs of *E. friedi* released per host on week i and P_i is the percentage of egg viability during week i.

The weekly reproductive success for each week of the study (R_{wi}) was calculated as:

$$R_{wi} = E_i / C_0,$$

where C_0 is the number of metacercariae at which the hosts were exposed (100 metacercariae in the present study).

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Morphological features of adult worms

To evaluate the effect of ageing on adult worms and a possible relationship with the reproductive success, the surface areas of the body, ovary, anterior and posterior testes and uterine eggs were determined weekly. Five of the adult worms collected each week from the hamsters of group B were fixed in Bouin's fluid under cover-slip pressure and mounted in Canada balsam. Maximum length and width of each morphological feature were recorded and the product was used as a measurement of the respective surfaces.

Sera and antigens

Blood was collected weekly from the hamsters of group C by cardiac puncture under anaesthesia. After clotting overnight at 4 °C, serum was separated from the clot by centrifugation.

Three different antigens were used to evaluate the antibody response of hamsters to *E. friedi* infections: whole worm extracts (WWE), excretory/secretory products from adult worms (ESP), and whole egg extracts (WEE). To prepare WEE, adult worms were collected from the intestines of hamsters 6 weeks p.i. with 100 metacercariae of *E. friedi*. After thorough washing with phosphate-buffered saline (PBS, pH 7·4), the worms were homogenized in culture medium of PBS containing 0·8 mM phenylmethylsulfonyl fluoride (Sigma), 100 U penicillin (Sigma), and 100 μg/ml streptomycin (Sigma). After initial centrifugation at low speed to remove larger particles, the supernatant fraction was centrifuged at 15 000 ***g*** for 30 min at 4 °C. The protein content was measured by the Bio-Rad protein assay and adjusted to 1 mg/ml. For isolation of ESP, adult worms were collected as described above and cultured at concentrations of 10 worms/ml of medium for 12 h at 37 °C. The medium was collected and centrifuged as before, and the supernatant was collected and concentrated to 1 mg/ml using an ultrafiltration membrane (YM-3, Amicon). To obtain WEE, eggs were obtained by teasing the uteri of adult worms collected as described above. After washings in PBS, eggs were transferred to medium culture at a concentration of 10 000 eggs/ml and homogenized. The homogenate was centrifuged and the supernatant concentrated to 500 μg/ml as before.

All the sera and the antigens were stored at −20 °C until use.

Indirect ELISA procedures

The optimal concentration of the antigens was determined by titration against known positive and negative sera. The antigen concentration was 3 μg/well. For the determination of the optimal dilution of the sera, anti-*E. friedi* hamster and pre-immune sera were titrated from 1 : 50 to 1 : 1600. The serum dilution that rendered the highest difference was chosen as the optimal dilution for subsequent assays.

Polystyrene microtitre plates (Nalgene) were coated overnight at 4 °C with 100 μl/well of 0·1 M carbonate coating buffer (pH 9·6) containing 30 μg/ml of antigen. The plates were washed 3 times with PBS 0·05% Tween 20 (PBST). Uncoated sites were blocked with 5% fat-free dry milk in PBST. After incubation for 1 h at 37 °C, 100 μl of diluted sera (1 : 100) were dispensed in each well. The plates were incubated for 2 h at room temperature and subsequently washed 5 times with PBST. Then 100 μl of horseradish peroxidase goat anti-hamster IgG (ICN/Cappel) diluted 1 : 3000 in PBS were added per well and incubated for 2 h at room temperature. Ortho-phenylendiamine (Sigma) was used at 0·4 mg/ml in 0·05 phosphate-citrate buffer (pH 5·0). The plates were incubated in darkness for 15 min at room temperature. The enzyme reaction was stopped by addition of 50 μl/well of 3 M HCl. The plates were read at 492 nm with a Bio-Rad 550 Microplate Reader. Each serum was examined in triplicate and given as the mean optical density (OD) with the correspondent standard deviation. The positive cut-off levels for each antigen were established as OD greater than the mean +3 S.D. of the pre-immune sera.

Statistical analysis

Analysis of variance (ANOVA) was performed to compare the daily egg output by the hamsters of the group A. Spearman's correlation test was used to estimate the relationship between different parameters of the study. Student's *t*-test was used for statistical analysis of the results obtained by indirect ELISA. $P<0{\cdot}05$ was considered as significant.

RESULTS

Pre-patent period and worm recovery

All the hamsters experimentally exposed to 100 metacercariae of *E. friedi* became infected. The duration of the pre-patent period, studied on the hamsters of the group A, was homogeneous. Egg release began 10–13 ($11{\cdot}3 \pm 1{\cdot}3$) days p.i.

The number of worms recovered weekly in the hamsters belonging to group B ranged from 9–25 ($15{\cdot}7 \pm 3{\cdot}9$) worms/hamster. No relation was found between the number of worms recovered and the age of the infection during the first 12 weeks post-infection (Fig. 1).

Kinetics of egg release

The egg release was continuous from the first day of the patent period until the end of the study in each

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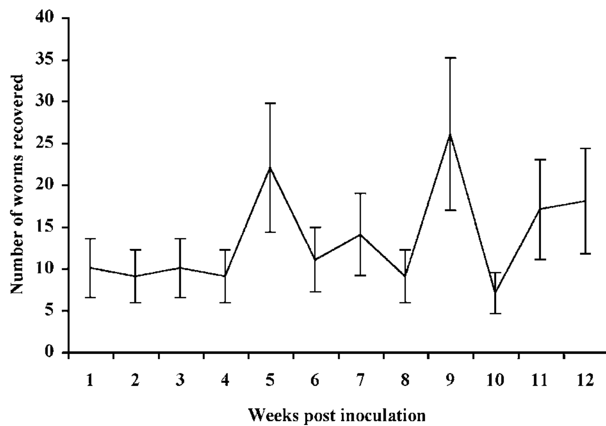

Fig. 1. *Echinostoma friedi* worm recovery over the course of infection (weeks) with 100 metacercariae in golden hamsters. Vertical bars represent the standard deviation.

hamster. Eggs were found in all the faecal samples analysed. Analysis of variance demonstrated a similar pattern in the daily egg counts in all the hamsters of the group A. However, the daily egg output was not homogeneous over time and there was an important variation in daily egg counts (Fig. 2A). The maximum number of daily released eggs was 10 070 eggs/hamster/day, at day 21 p.i. The first days of the patent period are characterized by a progressive increase in the egg output, to reach a maximum egg release between the 19th and 34th day p.i. Furthermore, the egg output steadily declined until the end of the experiment at day 84 p.i.

The analysis of the number of eggs released weekly per hamster showed that the maximal output occurred on the 3rd week after infection (Fig. 2B). From this week the egg output continuously decreased, although the number of eggs released remained at a high level until the 6th week, when a sudden decrease occurred.

Egg viability

The egg viability was measured in terms of miracidial hatching. Overall viable eggs constituted about 37% of the total egg production. However, the percentage of viable eggs varied with worm age (Fig. 3A). Although gravid worms were found from the second week p.i., viable eggs only were detected from the 3rd week p.i. The weekly percentage of egg viability observed ranged from 0% to 61%. The period of maximum miracidial hatching was observed between the 4th and 8th weeks p.i., with a peak at week 4 p.i. A marked reduction in egg viability occurred during the 9th week of the experiment. No viable eggs were detected at weeks 10 and 12 p.i. The weekly egg output and the egg viability showed a significant degree of correlation ($r = 0{\cdot}94$).

Weekly reproductive success

The weekly reproductive success over the course of the experiment, expressed as the number of viable eggs produced each week per metacercaria, is presented in Fig. 3B. Overall, the mean value of the R_w was 82·8±78·0 viable eggs/metacercaria. However, the weekly reproductive success was not homogeneous over time and a marked peak occurred on week 4 p.i. (Fig. 3B). The values of the R_w ranged from 0 to 274 viable eggs/metacercaria. However, high values of the R_w were only obtained between week 3 and week 8 after infection. R_w was 0 at weeks 1, 2, 10 and 12 p.i. Within the first 12 weeks of infection transmission of the parasite was likely between the weeks 3 to 8 p.i. according to the R_w observed.

Morphological characteristics of the adult worms

The body area of adult worms of *E. friedi* over the course of the experiment is shown in Fig. 4A. Body area increased rapidly from the first week after infection up to week 7 p.i. Thereafter, the body area was variable and the maximum was observed at week 11 p.i. (43·8±0·4 mm^2). Mean gonad areas of the same worms are represented in Fig. 4B. Ovarian area ranged from 3999±2409 to 530 150±237 413 μm^2. Mean testicular areas followed a similar pattern to that of body areas, ranging from 5963±528 to 1 719 317±856 364 and from 5339±592 to 1 328 954±55 223 μm^2 for anterior and posterior testis, respectively. The mean uterine egg areas observed weekly remained constant over the course of the study ranging from 5457±337 to 9239±3254 μm^2 (Fig. 4C).

Indirect ELISA

The cut-off OD values to differentiate positive from negative sera were 0·175 and 0·339 when WWE and ESP were used as antigens, respectively. The OD values followed a similar pattern throughout the weeks after infection using both antigens (Fig. 5A, B). These figures show that golden hamsters develop significant IgG responses against both antigens of *E. friedi*. In both cases, the OD values were positive from week 4 p.i. and remained positive until the end of the experiment. The maximum OD values were detected at week 11 after infection (0·310±0·082 using WWE and 0·461±0·049 using ESP). Statistically significant differences between OD values of the sera from infected hamsters and the pre-immune and control sera were detected from the 4th week after infection until the end of the study using both antigens ($P \leqslant 0{\cdot}02$).

The cut-off OD value was 0·313 when WEE was used as antigen. The weekly mean OD values of sera from infected hamsters are shown in Fig. 5C. This

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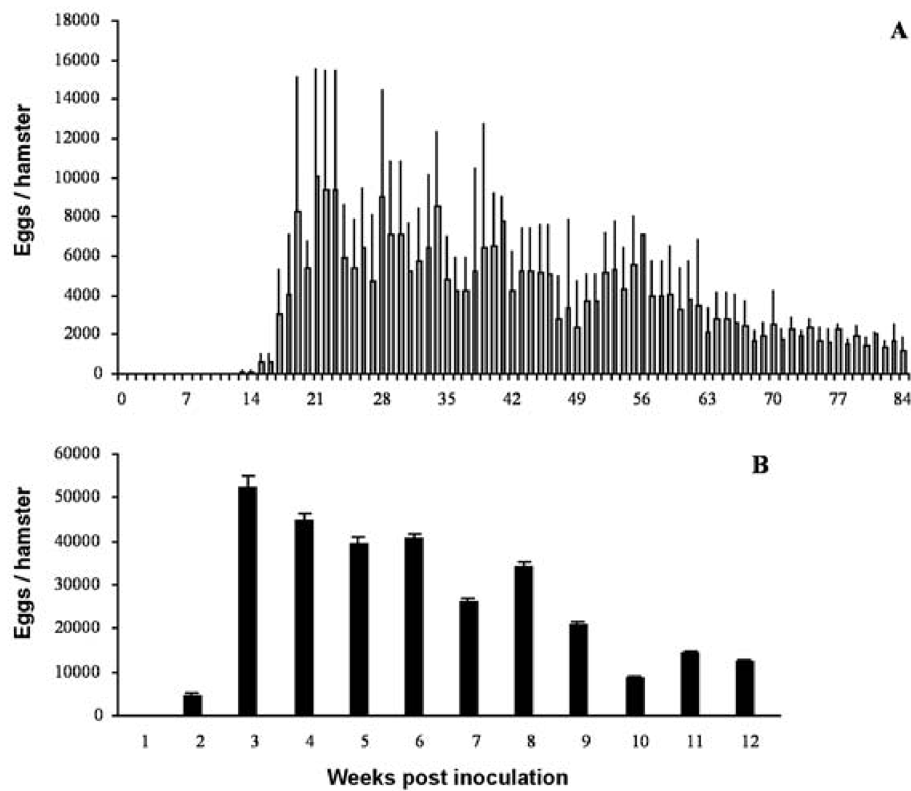

Fig. 2. Average daily (A) and weekly (B) egg output of *Echinostoma friedi* with increasing age in infections with 100 metacercariae in golden hamsters. Vertical bars represent the standard deviation.

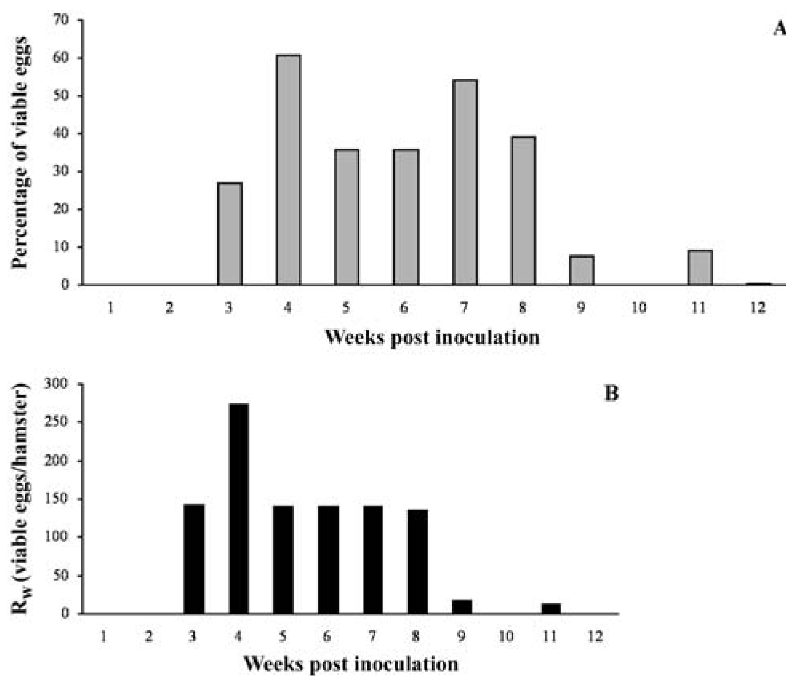

Fig. 3. (A) Proportional egg viability of *Echinostoma friedi* at increasing age (weeks) in infections with 100 metacercariae in golden hamsters. (B) Weekly reproductive success (R_w) estimated for *Echinostoma friedi* with increasing age in infections with 100 metacercariae in golden hamsters.

figure indicates that hamsters only develop a weak antibody response against egg antigens of *E. friedi*. The OD values were below of the cut-off most of the weeks p.i. Only a slight rise in OD values was observed on weeks 5 and 6 after infection. The maximum OD value was observed at week 6 after infection ($0{\cdot}333 \pm 0{\cdot}056$). Statistically significant differences between OD values of the sera from infected

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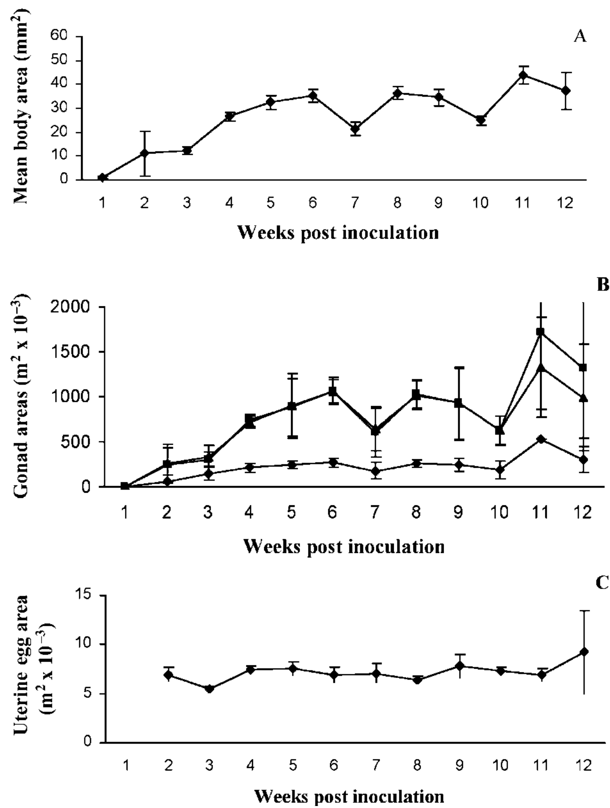

Fig. 4. Morphological characteristics (mean areas $\pm$ S.D.) of *Echinostoma friedi* with increasing age (weeks) in infections with 100 metacercariae in golden hamsters. (A) Mean body area (mm^2). (B) Anterior testis (■); posterior testis (▲) and ovarian (●) areas. (C) Uterine eggs area (mm^2).

hamsters and the pre-immune and control sera only were detected at week 6 after infection ($P<0{\cdot}05$).

DISCUSSION

Parasite fecundity in echinostome infections has not been adequately studied. In an attempt to complete and standardize the studies on this topic, Mahler *et al.* (1995) defined the reproductive capacity of *E. caproni* in a particular host species considering the percentage of parasite establishment, the survival of the adult worms and the egg production rates. It is obvious that these three components influence the success of a parasite species in a host, though other parameters, such as the egg viability, need to be taken into account (Whitfield *et al.* 1986). The latter authors defined the overall reproductive success of the digenean ectoparasite *Transversotrema patialense* in a particular host species as the total number of viable eggs produced during the life-span of the parasite per cercaria to which the host was exposed. This measure has been applied to compare the compatibility of a parasite species to different host species (Whitfield *et al.* 1986). From the parasite population point of view, however, the reproductive fitness should not be considered as a constant over time. We have calculated the parasite reproductive success of *E. friedi* in golden hamsters for each of the weeks that comprised our study in an attempt to analyse the dynamics of the parasite fecundity. This has

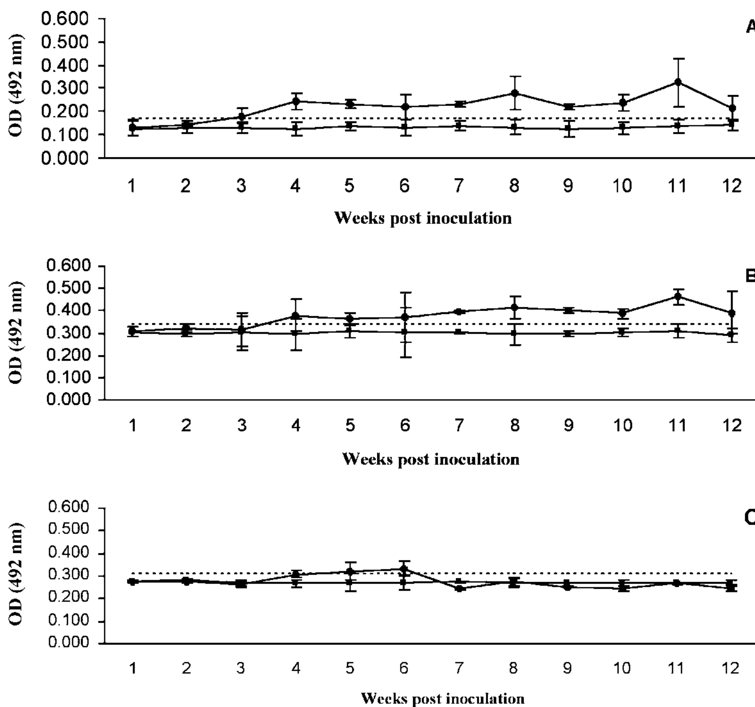

Fig. 5. Mean OD values obtained by indirect ELISA for sera of golden hamsters infected with 100 metacercariae of *Echinostoma friedi* during the first 12 weeks of the infection. (A) Using whole worm extracts as antigen. (B) Using excretory/secretory products as antigen. (C) Using whole egg extracts as antigen. Infected hamsters (●). Uninfected control hamsters (■). (- - - -) Cut-off point value defined as mean value of the pre-immune sera + 3 standard deviations. Vertical bars represent the standard deviation.

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been termed weekly reproductive success of the parasite in the particular host (R_w) in order not to be confused with the terminology of Whitfield *et al*. (1986). The introduction of the concept of weekly reproductive success allows estimation of the variations of the fecundity of a parasite per unit of inoculation (metacercaria).

The results obtained herein show that the reproductive success of *E. friedi* in hamsters is not constant over the course of the infection, attaining its maximum at week 4 after infection. This variation seems to be related to changes in the egg output and the viability of the eggs produced. Previous studies on the fecundity of echinostomes have been based on egg counts (Odaibo *et al*. 1988, 1989; Christensen *et al*. 1990; Mahler *et al*. 1995). However, total egg production does not constitute *per se* a measure of the reproductive success and only the output of viable eggs should be considered. In fact, we have found that the maximum weekly reproductive success is not concomitant with the maximum egg output. Thus, the common use of the egg counts to measure the reproductive capacity in echinostome infections should be reevaluated. The kinetics of egg release in echinostome infections has been studied on the basis of uterine egg counts (Christensen *et al*. 1990) and EPG (Odaibo *et al*. 1988, 1989). In the present study, daily EPG and EPH are correlated and both parameters can be used to determine the fecundity of *E. friedi*. We prefer the use of the daily EPH to obtain a more accurate estimation of the total egg output and also to determine the number of viable eggs produced. The egg output of *E. friedi* was not constant during the experiment. The egg release rapidly increased during the first weeks of infection, probably in relation to the progressive maturation of the adult worms. The maximum egg output was observed on week 3 p.i. and thereafter the egg release steadily decreased to almost zero at week 9 p.i. Notwithstanding the difficulty entailed in such comparisons, this is in contrast to the dynamics of EPG in infections with 10 and 25 metacercariae of *E. caproni* in mice (Odaibo *et al*. 1988, 1989). The curves presented by these authors show a progressive increase in EPG until weeks 7 and 13 p.i., respectively. Probably the different host–parasite system may explain, at least partly, the different results.

The overall findings of infections with *E. friedi* in golden hamsters confirm that egg output is not necessarily a perfect measure of the parasite transmission potentials in a host species. The percentage of viable eggs varied over the course of the infection and needs to be included in the determination of the parasite reproductive success. No viable eggs were detected until the week 3 p.i. though the egg release started at week 2 p.i. The maximum viable egg output occurred on week 4 p.i. Furthermore, the production of viable eggs significantly declined on week 9 p.i. The introduction of the weekly reproductive success has allowed us to determine the kinetics of fecundity of *E. friedi* in hamsters over the course of the infection and to establish the maximum reproductive success at week 4 p.i. Under the experimental conditions used, our results suggest that transmission of *E. friedi* is only likely between weeks 3 and 8 after infection.

The egg output of a parasite at any point in time is the result of 2 separate influences (i) the number of reproducing parasites after the effect of the mortality, and (ii) age specific changes in the fecundity of the surviving worms (Whitfield *et al*. 1986). The results obtained in the present study indicate that the decline in egg output appears to be due to changes in the fecundity of the adult worms since neither worm mortality nor decline in worm recovery have been observed during the first 12 weeks of the infection. This suggestion is supported by the dynamics observed in the percentage of viable eggs produced, which does not depend on the number of surviving worms. The decline of egg output and egg viability may reflect functional changes in the adult worms as a consequence of the worm ageing and/or even the host response. Although the effect of worm ageing on parasite fecundity is difficult to evaluate, the functional changes observed are not reflected by significant changes in the morphology of the parasite. The growth curves of body, gonadal and uterine areas are similar to those observed for other echinostome species such as *E. trivolvis* and *E. caproni* during the first weeks of the infection (Odaibo *et al*. 1988, 1989; Isaacson, Huffman & Fried, 1989; Hosier & Fried, 1991; Yao, Huffman & Fried, 1991; Meece & Nollen, 1996) and do not decrease when fecundity declines late in the course of infection.

As a measure of the host response, we have investigated the humoral antibody response by indirect ELISA. The results obtained show that *E. friedi* adult worms induce weak, though detectable, IgG responses in golden hamsters. Significantly increased levels of IgG against WWE and ESP were detected from the week 4 p.i. Previous studies have shown that the hamster responds weakly against other echinostome species (Mabus, Huffman & Fried, 1988; Simonsen *et al*. 1991), although other host species (e.g. mice) develop stronger responses (Agger *et al*. 1993; Graczyk & Fried, 1994, 1995). Most of the studies on the role of the immune responses on the development of echinostome infections have been focused on the resistance to secondary infections (Christensen, Knudsen & Andreassen, 1986; Brunet *et al*. 2000), but other resistance-related factors have not been investigated. We observed that decline in reproductive success coincides with a rise in IgG levels. Previous studies on *Schistosoma* spp. have shown that reductions in egg output and hatchability of the eggs are related to the host immune response (Boulanger *et al*. 1991; Xu *et al*. 1993; De Bont, Vercruysse & Massuku, 1996). Although other

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immune effector mechanisms (e.g. local response at the mucosa level) should be considered and studied further, it is tempting to suggest that the immune response could affect any particular function of the worms affecting their fecundity. However, further studies are now in progress to elucidate the role of antibodies in this host–parasite relationship, especially with respect to the parasite reproductive success.

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