International Journal for Parasitology Vol. 22, No. 8, pp. 1143–1149, 1992
Printed in Great Britain

0020-7519/92 $5.00 + 0.00
Pergamon Press Ltd

CROSS-REACTIVITY BETWEEN *NECATOR AMERICANUS* AND *SCHISTOSOMA MANSONI* IN MICE

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(*Received* 20 *August* 1991; *accepted* 24 *June* 1992)

Abstract—TIMOTHY L. M., COULSON P. S., BEHNKE J. M. and WILSON R. A. 1992. Cross-reactivity between *Necator americanus* and *Schistosoma mansoni* in mice. *International Journal for Parasitology* **22**: 1143–1149. Poly-parasitism is common in endemic communities and reactivity of sera from hookworm-infected patients against schistosomular antigens has been reported. Protective cross-immunity between *N. americanus* and *S. mansoni* was investigated in NIH and BALB/c mice. Protective resistance to homologous challenge with both parasites was confirmed in this model, however, functional immunity to heterologous challenge was not demonstrated. Sera from animals which had received homologous challenge with *N.americanus* and from hookworm-infected mice, which had previously been exposed to radiation-attenuated *S. mansoni*, exhibited an enhanced IgGAM response to infective stage *N. americanus* somatic antigens. The implications of these results with respect to serodiagnosis are discussed.

INDEX KEY WORDS: Cross-reactivity; *Necator americanus; Schistosoma mansoni*; mice; serodiagnosis.

INTRODUCTION

HUMAN hookworm infections are endemic in most tropical and subtropical regions and rank among the 10 most common infections in the world (World Health Organization, 1987). It has been estimated that some 900 million people are affected globally (Banwell & Schad, 1978; Crompton, 1989; Keymer & Bundy, 1989; Schad, 1991). Of the two major species affecting man (*Necator americanus* and *Ancylostoma duodenale*) the former relies almost exclusively on skin penetration as the route of infection, whereas *A. duodenale* is known to establish more readily after oral ingestion of infective larvae (Hoagland & Schad, 1978; Komiya & Yasuraoka, 1966; Mizuno & Yanagisawa, 1963). Schistosomiasis, another major, human parasitic infection, is thought to affect 200 million people worldwide (Rollinson & Simpson, 1987). The most extensively studied of the schistosomes affecting man — *Schistosoma mansoni* —is endemic in much of Africa, Egypt, the Middle East, South America and the Caribbean (Rollinson & Southgate, 1987; Whitfield, 1982). In common with *N. americanus*, the cercariae of *S. mansoni* infect the human host by skin penetration and follow similar routes in the initial stages of migration, i.e. after penetration most enter the blood vasculature before migrating to the lungs.

It is apparent, therefore, that schistosomiasis and hookworm infections co-exist over a wide area and many of those affected by one may have been exposed to the other. Indeed numerous researchers have suggested that poly-parasitism in the field is the rule rather than the exception (e.g. Buck, Anderson & McRae, 1978a, b, c). In the light of this and recent work reporting serological responsiveness of individuals infected with hookworm to *S. mansoni* antigens (Correa-Oliveira, Dusse, Viana, Colley, Carvalho & Gazzinelli, 1988), it was considered important to determine whether protective immunity to *N. americanus* could be induced by exposure to *S. mansoni* (or vice versa) in an easily manipulable model system. The murine model of *S. mansoni* infection is well established and a model for the invasive and migratory stages of *N. americanus* infection in the mouse has been developed at the University of Nottingham (Behnke, Wells & Brown, 1986; Wells & Behnke, 1988a, b; Wilkinson, Wells & Behnke, 1990). In this paper, we describe experiments which were undertaken to establish whether protective cross-immunity could be demonstrated in a system where functional resistance to homologous challenge has been shown. Confirmation of serological cross-reactivity is also presented.

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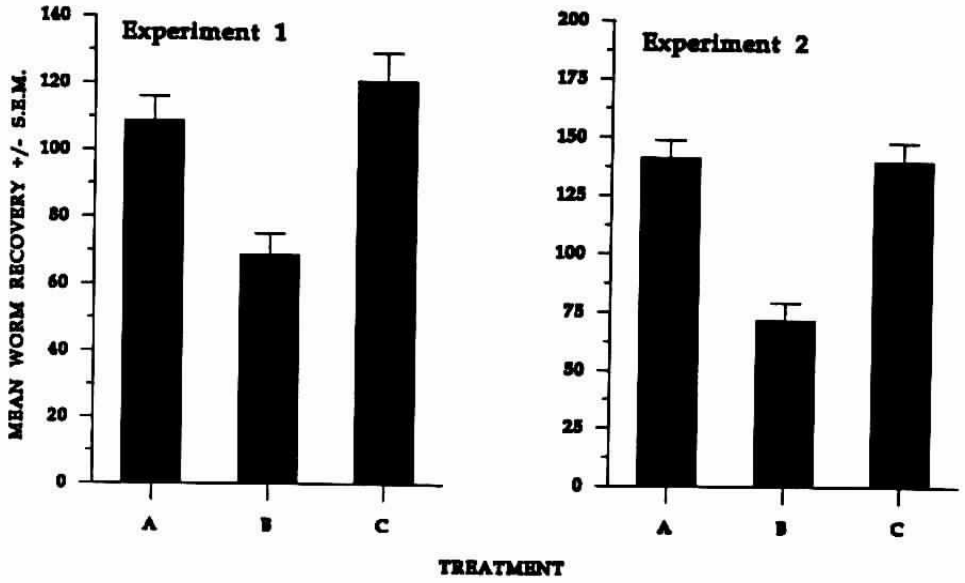

FIG. 1. *S. mansoni* does not induce protective cross-immunity to *N. americanus*. Mean number of active larvae recovered, 5 days post-infection with 250 *N. americanus* L3, from the lungs of male NIH (Experiment 1) and female BALB/c mice (Experiment 2). Treatment groups were as follows: A. control, no previous infection; B. previously infected with *N. americanus* L3; C. previously vaccinated with *S. mansoni* cercariae.

MATERIALS AND METHODS

Animals. Male NIH (Experiment 1) and female BALB/c (Experiment 2) mice were bred and maintained under conventional conditions in the Department of Zoology, University of Nottingham. Male, SPF, BALB/c mice (Experiment 3) were obtained from Harlan-Olac Ltd, Bicester, Oxon and housed, separately, under conventional conditions. Experimental groups comprised six to eight animals.

Parasites. Infective *N. americanus* larvae were obtained by routine passage through neonatal hamsters at the University of Nottingham (Behnke, Paul & Rajasekariah, 1986). The original isolate was obtained from Dr G. Rajasekariah of Hindustan CIBA-GEIGY Ltd, Bombay, India in 1983. A Puerto Rican strain of *S. mansoni* was maintained by serial passage through albino *Biomphalaria glabrata* and Lac A mice at the University of York. Radiation-attenuated *S. mansoni* cercariae were prepared by exposure to 200 Gy of radiation from a ^{60}Co source at the Department of Radiobiology, Cookridge Hospital, Leeds and used on the same day.

Infection of mice and worm recovery. Animals were infected with *N. americanus* L3 as described by Behnke, Wells & Brown (1986). Briefly, mice were anaesthetized with Sagatal (May and Baker, Veterinary Products), the dorsal thorax shaved and 250 infective larvae applied, to the exposed area, on a gauze and secured with adhesive tape. The gauzes were left in place for 24 h. Vaccination with 500 radiation-attenuated *S. mansoni* cercariae was also carried out on anaesthetized animals. The abdominal area was shaved and the attenuated cercariae administered percutaneously. Challenge *S. mansoni* infections, using individual suspensions of 200 freshly harvested cercariae, were carried out using the tail immersion method described by Wilson, Coulson & Dixon (1986). Enumeration of actively migrating, lung stage *N. americanus* larvae was carried out according to the method of Behnke, Paul & Rajasekariah (1986). After necropsy, the lungs were removed and incubated, at 37°C, in Hanks' Balanced Salt Solution. The lungs were minced with scissors and the larvae, which had migrated from the tissue, recovered at 2, 6 and 24 h. Total *N. americanus* burdens were determined by examination of lung tissue which had been squashed between two microscope slides (Wilkinson *et al.*, 1990). Adult *S. mansoni* were recovered, at autopsy, by perfusion of the hepatic portal system with heparinized saline (Wilson *et al.*, 1986).

Antibody responses. Serum antibody titres to a PBS-soluble fraction of infective, *N. americanus* L3 homogenate were assessed by a standard enzyme-linked immunosorbent assay (ELISA) (Wells & Behnke, 1988b). Relative IgGAM responses were calculated from the resulting optical densities of test sera (TS), with reference to those of known, high titre (HTS) and naïve, control (CS) sera as:

$$\text{Relative response} = \left[\frac{\text{TS} - \text{CS}}{\text{HTS} - \text{CS}} \right]$$

Statistical analysis. Data are presented as group means ± S.E.M. Analysis of data was carried out according to a specific unified rank analysis (Meddis, 1984) and $P \leqslant 0.05$ was considered significant.

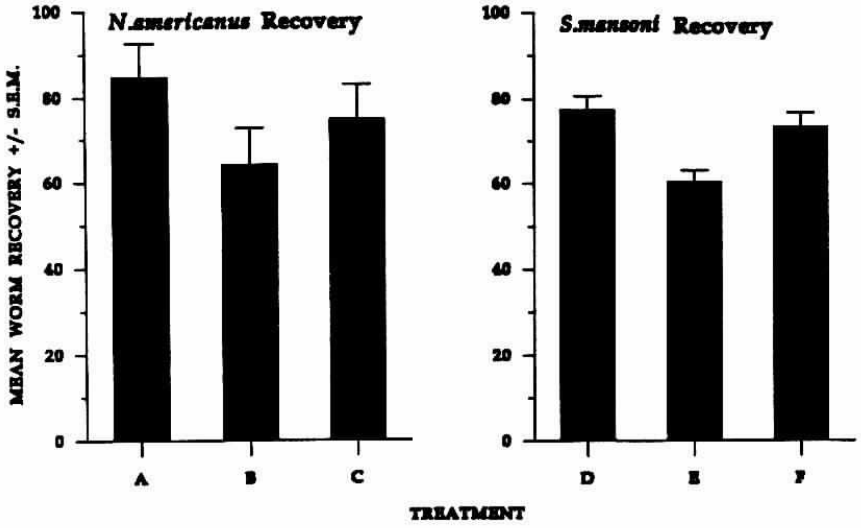

FIG. 2. Reciprocal, protective cross-immunity between *N. americanus* and *S. mansoni* is not elicited in male BALB/c mice (Experiment 3). Mean recoveries of active larvae, from the lungs, 5 days post-infection with 250 *N. americanus* L3 (left). Treatment groups A, B and C correspond to those in Fig. 1. Mean recoveries of *S. mansoni* 6 weeks post-infection with 200 cercariae (right). Treatment groups were as follows: D. control, no previous infection; E. previously vaccinated with irradiated *S. mansoni* cercariae; F. previously infected with *N. americanus* L3.

RESULTS

The effect of prior vaccination with S. mansoni *on recovery of* N. americanus *viable lung stage larvae following heterologous challenge*

Protective immunity to *N. americanus* infection was assessed in terms of actively migrating lung worm numbers on day 5 post-infection. Two experiments were carried out to determine whether functional cross-immunity could be induced by vaccination with radiation-attenuated *S. mansoni* cercariae and these results are presented in Fig. 1. It can be seen that, in each experiment, the lung worm recoveries from three groups of mice were compared. Treatment group A received a single dose of 250 *N. americanus* L3; group B was treated with primary and secondary infections of 250 *N. americanus* L3, each 6 weeks apart; group C was vaccinated with 500 radiation-attenuated *S. mansoni* cercariae and, 6 weeks later, challenged with 250 *N. americanus*. In Experiment 1, significantly fewer viable larvae (41.4%) were recovered from the secondary *N. americanus* infection group B when compared to the primary infection group A ($L=70.0$, $Z=3.0$, $P=0.0016$). The vaccinated group C did not differ significantly from group A. Similarly in Experiment 2, the number of lung worms recovered from the secondary infection group B was significantly reduced (49.2%) in comparison to group A ($L=63.0$, $Z=3.0$, $P=0.0016$). Groups A and C were not significantly different. It is clear from these results that, although protective immunity resulted from homologous challenge with *N. americanus* by day 5 post-infection, no functional immunity was induced by prior vaccination with *S. mansoni*.

To verify that protective immunity to *S. mansoni* was induced by the vaccination procedure, two additional groups were included in Experiment 2: group D was vaccinated with *S. mansoni*, as before, and challenged, 6 weeks later, with 200 cercariae; group E received the challenge infection only. On perfusion, 6 weeks after challenge, 93.8 ± 3.0 adult schistosomes were recovered from the primary infection group E in comparison to 58.2 ± 8.0 from the vaccinated group D. Thus a significant reduction (38%) in *S. mansoni* recovery was achieved by vaccination ($L=40.0$, $Z=2.62$, $P=0.0046$).

The effect of a primary N. americanus *infection on the recovery of* S. mansoni *following heterologous challenge*

It was necessary to determine whether the failure, of vaccination with *S. mansoni*, to induce protective immunity to *N. americanus* infections was reciprocal (Experiment 3). An experiment was therefore carried out in which mice were immunized by prior exposure to *N. americanus* and challenged with *S. mansoni*. Protective immunity to *S. mansoni* infection was assessed in terms of adult worm recovery 6 weeks after challenge. In Fig. 2, treatment groups A–C correspond to those in Fig. 1 and protective immunity to *N. americanus* was assessed as before. Group D was administered a single dose of 200 *S. mansoni* cercariae; group E was vaccinated with 500 attenuated cercariae

TABLE 1—VACCINATION WITH *S. mansoni* DOES NOT ENHANCE LARVAL ENTRAPMENT IN THE LUNGS

Experiment*	Treatment	Mean worm recovery ± S.E.M.		Index of trapping§ ± S.E.M.	Statistical significance‖ ($P<$)
		Active migrating larvae (left lung)†	Total larvae (right lung)‡		
1	A	61.0 ± 3.4	43.3 ± 3.7	1.2 ± 1.2	
	B	26.5 ± 3.6	34.8 ± 2.6	26.0 ± 6.5	0.01
	C	53.0 ± 3.6	58.0 ± 8.4	12.4 ± 6.5	n.s.

* Experimental and treatment groups correspond with those in Fig. 1.

† Migrating *N.americanus* larvae recovered, 9 days post-infection, from minced lungs during a 24 h incubation in Hanks' Balanced Salt Solution.

‡ Total *N.americanus* burdens of the left lungs were determined, 9 days post-infection, by counting worms *in situ* following examination of squashed tissue.

§ The index of trapping was calculated, for each animal, as:

$$100 - \left[\frac{\text{migrating larvae in right lung}}{\text{total larvae in left lung}} \times 100 \right]$$

‖ Significance according to a specific unified rank analysis as described by Meddis (1984), the null hypotheses were that neither treatments B or C showed greater larval trapping than A.

and, after 6 weeks, challenged with 200 cercariae: group F was infected with 250 *N. americanus* L3 and challenged, 6 weeks later, with 200 cercariae. In this experiment, there was a reduction (24.4%) in the recovery of *N. americanus* following homologous challenge (group B), when compared to the primary infection group A, although this was not significant. Groups A and C did not differ significantly. When the primary and vaccinated *S. mansoni* groups (D and E, respectively) were compared a significant reduction (21.9%) in recovery of adult schistosomes was recorded ($L=468.5$, $Z=3.04$, $P=0.0014$). Groups D and F were not significantly different. It is evident, therefore, that while significant protective immunity was elicited to homologous challenge, with either *N. americanus* (see Fig. 1) or *S. mansoni*, protective cross-immunity could not be demonstrated.

The effect of vaccination with S. mansoni *on the pulmonary entrapment of* N. americanus *larvae following heterologous challenge*

Since vaccination with *S. mansoni* failed to elicit a protective response to *N. americanus* by day 5 post-infection, a further index of functional immunity was examined. In Experiment 1, the number of actively migrating larvae in the left lung was compared to total larval burdens in the right lung, of infected animals, and an index of trapping calculated (see Table 1). No significant difference was found between larval entrapment in the primary *N. americanus* infection group A and the vaccinated group C, but significantly greater entrapment ($L=53.5$, $Z=2.48$, $P=0.0067$) was seen in the secondary *N. americanus* infection group B, when compared to group A.

In Experiment 2, migrating lung worm burdens from the three groups were compared, on day 9, and although a greater mean number of active larvae (8.33 ± 3.61) were recovered from the secondary *N. americanus* infection group B when compared with the primary infection group A (4.33 ± 0.84), too few larvae were recovered to show a significant difference. When group A worm recoveries were compared with those from the vaccinated group C (6.5 ± 1.38), no significant difference was found. Protective immunity to homologous challenge with *N. americanus* was, then, clearly demonstrated, when pulmonary entrapment of larvae was used as an index, on day 9 post-infection, but functional cross-immunity between *S. mansoni* and *N. americanus* was not observed.

Antibody response to N. americanus *larval antigens*

The serum antibody response of mice to larval antigens of *N. americanus* was assessed on days 5 and 9 after infection with *N. americanus* (Experiment 1). From Fig. 3, it is clear that the relative IgGAM response of the primary *N. americanus* infection group

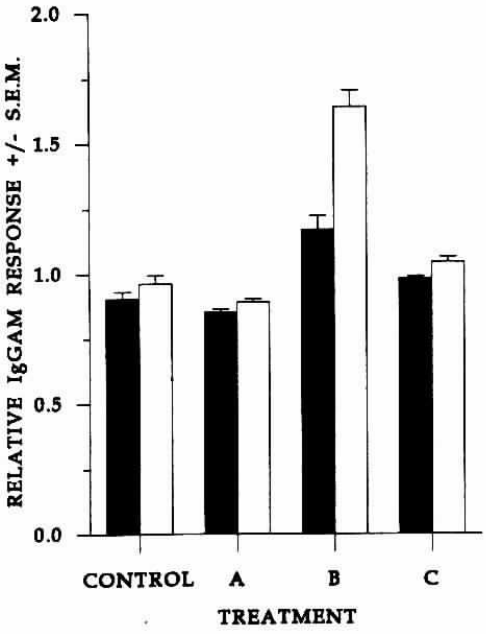

FIG. 3. The serum antibody response to *N. americanus* L3 homogenate is significantly enhanced, in male NIH mice, during a secondary infection (group B) and by vaccination with irradiated *S. mansoni* cercariae (group C). Mean relative IgGAM response on days 5 (■) and 9 (□) post-infection (Experiment 1). The control groups were uninfected, treatment groups A, B and C correspond to those in Fig. 1.

A was not enhanced when compared to the uninfected control and that the response of the secondary *N. americanus* infection group B was significantly increased, on both days 5 ($L=51.0$, $Z=2.74$, $P=0.0033$) and 9 ($L=51.0$, $Z=2.74$, $P=0.0033$) post-infection. Moreover, the group which had previously been vaccinated with *S. mansoni* (group C) exhibited a small but significant increase in relative IgGAM response over the uninfected control on both days 5 ($L=70.0$, $Z=2.05$, $P=0.0201$) and 9 ($L=45.0$, $Z=1.64$, $P=0.05$) post-infection with *N. americanus*.

DISCUSSION

Exposure to multiple parasitic infections is common in endemic areas. Of specific interest is the fact that hookworm infections frequently occur in regions where *S. mansoni* is endemic. In a recent report, Correa-Oliveira *et al.* (1988) assayed the antibody responses of hookworm (*Ancylostoma sp.*)-infected patients to schistosome-derived antigens and found significant cross-reactivity as adjudged by ELISA and the lethal activity of these sera against intact schistosomula *in vitro*. In conclusion, these researchers highlighted the need for a greater understanding of the effect that previous exposure to hookworm might have on resistance to subsequent infection with *S. mansoni*. In answer to this, the aim of the present work was to investigate whether protective immunity could be demonstrated as a result of heterologous challenge with either hookworm or *S. mansoni*. Since compatible, murine models for both *S. mansoni* and one of the highly anthropophilic hookworm species (*N. americanus*) have been defined, this system was chosen.

The data presented clearly confirm that protective resistance to homologous challenge, with both species, can be induced in this model (Figs. 1 and 2, Table 1). In addition, an enhanced relative IgGAM response to *N. americanus* L3 antigens was potentiated by previous exposure, of hookworm-infected mice, to *S. mansoni* (Fig. 3). However, functional immunity, after heterologous challenge, was not detected for either parasite. In this model, *N. americanus* only survive, in the gut, for around 9 days having undergone an apparently normal migration (Wells & Behnke, 1988a) and radiation-attenuated cercariae of *S. mansoni* are known to perish during their migration, i.e. before reaching maturity (Mountford, Coulson & Wilson, 1988). Thus, in the present work true secondary — and not concomitant — infections were investigated. There remains, then, the possibility of protective resistance or regulation of this resistance during concurrent heterologous infection. It is worth noting however that, after sensitization with both *N. americanus* and attenuated *S. mansoni*, challenge infections were administered when peak resistance to homologous challenge would have been expected to have developed (Dean, 1983; Timothy, Wells & Behnke, unpublished data).

In man, the major pathologies resulting from infection with hookworm are generally attributed to the adult stages of this parasite. Similarly in schistosomiasis, significant pathology is usually identified with the adult worms in that it is associated with the deposition of ova. Resistance induced by 'vaccination' with the irradiated cercariae of *S. mansoni* is, however, known to be directed towards migrating or immature worms in mice (Wilson *et al.*, 1986) and Wells & Behnke (1988b) identified both the skin and lungs as sites of resistance to homologous challenge with *N. americanus* in the murine model. Our use of both adult *S. mansoni* and, of necessity, lung stage hookworm larvae as indices of protective resistance would then seem justified.

During secondary *N. americanus* infections in mice, increased larval entrapment in the lungs has been shown (Wilkinson *et al.*, 1990) and this was confirmed in Experiment 1 (see Table 1), although no increase in trapping was found after heterologous challenge. In Experiment 2, we compared the recoveries of actively migrating larvae on day 9 post-infection, by which time the majority of larvae would be expected to have

progressed from the lungs to the intestine. While a greater number were recovered from the secondary *N. americanus* infection group, suggesting a delay in their migration, this was not significant. Overall, however, very few larvae were recovered from any of the three groups and the absence of a significant difference is perhaps not surprising.

The enhanced relative IgGAM response to *N. americanus* L3 antigens, exhibited by NIH mice after heterologous challenge (Fig. 3), is all the more striking in light of the fact that this strain develops a relatively weak humoral response to both primary and secondary *N. americanus* infections (Timothy, Wells & Behnke, unpublished data). However, this response does not contribute to functional resistance in the present model. This, together with the failure of passive transfer studies, using homologous sera in mice, to establish a clear role for antibody in resistance to *S. mansoni* (see Vignali, Bickle & Taylor, 1989), or in resistance to *N. americanus* (see Wells & Behnke, unpublished data), suggest that humoral responses may not be a necessary component of resistance to these parasites. Indeed, it has been proposed that protective immunity in helminth infections may result from the induction of TH_1 activity (James & Sher, 1990), which, in broad terms, is thought to mediate several functions associated with local inflammatory responses and cytotoxicity.

The serological cross-reactivity demonstrated in this model does, however, cast further doubt on the efficacy of serodiagnostic procedures using crude antigen preparations. Shetty, Dilawari, Virk, Sehgal, Ganguly & Mahajan (1988) have reported 8–20% false positivity using an ELISA technique to detect infective-stage *Ancylostoma duodenale* excretory/secretory (ES) antigen, although a slightly lower percentage of false positives was recorded using somatic antigen. However, given the results of the field study by Correa-Oliveira *et al.* (1988), it is suggested that the percentage of false positives found might have been higher if *S. mansoni*-infected patients had been included in the test sample. In this paper we have investigated cross-reactivity between *N. americanus* (a species closely related to *A. duodenale*) and *S. mansoni*; our results underline the necessity of species-specific antigens for diagnosis of parasitic diseases. In the case of *N. americanus* infection, the 17 kDa major accumulated protein (Pritchard, McKean, Rogan & Schad, 1990) and the 28–33 kDa ES antigens of adult worms (Pritchard, McKean, Tighe & Quinnell, 1991) might be suitable candidates. Should compatible laboratory models for the study of *A. duodenale* and *S. mansoni* become available, a controlled investigation of cross-reactivity between these species would undoubtedly provide further useful data.

Acknowledgements—We would like to express our gratitude to the Trustees of the Sir Halley Stewart Trust for the provision of generous funds for this study and also acknowledge a contribution from the Sir Samuel Scott of Yews Trust. We thank Professors P. N. R. Usherwood and D. Wakelin for the provision of facilities within the Department of Zoology, University of Nottingham.

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