Ichthyophthiriasis in carp *Cyprinus carpio*: infectivity of trophonts prematurely exiting both the immune and non-immune host

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ABSTRACT: *Ichthyophthirius multifiliis* exposed to naturally immunised carp established short-term infections, the majority of parasites actively emerging within 2 h of entering the epidermis. A small, but significant, number of these expelled parasites were shown to retain theront-like properties with the capacity to directly re-invade a further fish host. Infectivity fell rapidly with time in the host and was comparable to that of trophonts of a similar age artificially induced to emerge from non-immune hosts with the aid of MEM (minimal essential medium). Trophonts recovered with MEM from immune carp 2 to 8 h post infection rarely established infections upon exposure to susceptible new hosts and no infections resulted from older trophonts recovered after 8 to 24 h exposure; older trophonts, however, represented only a small percentage of the original parasite population. A low level of infectivity was recorded in trophonts collected with the aid of MEM from non-immune carp after up to 24 h of infection. The results are discussed in relation to theront transformation and evasion of the host immune response.

KEY WORDS: *Ichthyophthirius multifiliis* · Carp · Infectivity · Immune evasion · Premature exit

INTRODUCTION

The ciliate *Ichthyophthirius multifiliis* Fouquet invades the epithelia of the skin and gills of a wide variety of freshwater fish in tropical, subtropical and temperate regions (Nigrelli et al. 1976, Valtonen & Keränen 1981) and accounts for economic losses in cultured freshwater species (Matthews 1994). As early as 1910 Buschkiel reported that fish which had experienced and survived an *I. multifiliis* infection were at least partly resistant to re-infection (Buschkiel 1910). These early findings have since been confirmed by several authors in a wide range of fish (Bauer 1958, Beckert & Allison 1964, Hines & Spira 1973, 1974, Dickerson et al. 1984, Wahli & Meier 1985, Houghton & Matthews 1986, McCallum 1986, Subasinghe & Sommerville 1986). Serum from immunised fish was shown

to immobilise parasites *in vitro* (Hines & Spira 1974, Wahli & Meier 1985, Houghton & Matthews 1986, Clark et al. 1987); however no similar effect has been demonstrated *in vivo* so far. Hines & Spira (1974) reported a complete lack of ciliates in the mucus of immune carp. Based on this finding, they suggested that mucus of immune fish has a barrier function directed against *I. multifiliis*. Cross & Matthews (1992) demonstrated that theronts exposed to the skin of immune carp penetrated the epidermis in the normal way but re-emerged within 2 h after penetration without completing development. Monoclonal murine antibodies directed against surface membrane antigens of *I. multifiliis* immobilised the parasite *in vitro*. When these antibodies were passively transferred to catfish, no immobilisation was achieved but rapid exit of the parasites from the epithelium resulted (Clark et al. 1996). After exit from the host, parasites appeared viable in all respects although antibodies were detectable on their surface. Matthews et al. (1996) induced

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premature exit of *I. multifiliis* from hosts by altering the external environment by exposing the fish to Eagle's minimal essential medium (MEM).

The fate of parasites induced to exit the skin prematurely either by the MEM method or by the hosts immune system is not known. The present study aimed (1) to determine whether trophonts prematurely recovered from a non-immune host retain an ability to directly re-infect new hosts or to complete encystment and develop infectious theronts and (2) to assess the viability of 'post theronts' prematurely exiting or induced to exit immune fish.

MATERIALS AND METHODS

Fish. Juvenile carp *Cyprinus carpio* L. (2 to 6 cm length), naive to ichthyophthiriasis, were obtained from the Hampshire College of Agriculture, Sparsholt, UK. These were kept in de-chlorinated tap water at $20 \pm 2°C$ and held in 5 l glass aquaria. Water quality was maintained by aeration, biological filtration and partial water changes. All fish were fed daily on goldfish flakes (King British, Bradford, UK). When necessary, fish were anaesthetised with MS 222 (3-aminobenzoic acid ester, Sigma Chemical Co., St. Louis, USA) at a concentration of 50 mg l^{-1}.

Parasite isolate. *Ichthyophthirius multifiliis* was isolated from rainbow trout *Oncorhynchus mykiss* L. obtained from a local fish farm on a tributary of the river Erne, Devon. It was routinely maintained through serial primary infections in carp and goldfish *Carassius auratus*. All procedures concerning handling of parasitic and free-living stages were as previously described by Wahli (1985, p 47–49).

Infection of carp with *Ichthyophthirius multifiliis*. Isolated tomonts were allowed to encyst in crystallising bowls and were incubated at room temperature (18 to 20°C) for 24 h. In establishing experimental infections, theronts were used within 3 to 4 h of excystment; viability was tested on naive carp prior to challenge of immune fish. Theronts and trophonts were enumerated either in a 1 ml sample of suspension with the aid of a Sedgewick-Rafter cell on immobilisation by the addition of 1 drop of formalin or by counting the parasites in 24 × 5 µl suspension after adding 1 drop of formalin and calculating the mean for 1 ml. As results of the 2 methods never differed more than 10%, the faster drop method was preferred.

MEM induced exit of trophonts from infected fish. Premature exit of trophonts from primary infections was induced by immersion of carp in MEM (Eagle's Minimum Essential Medium; Gibco BRL, Life Technologies Ltd., Paisley, Scotland) for 50 min following the method described by Matthews et al. (1996). Here,

however, the effect of the MEM was also investigated at 1:1 and 1:2 dilutions in distilled water. The number of exiting trophonts was scored as low (<10 parasites per view at 25× magnification), medium (10 to 20 parasites per view) or high (>20 parasites per view) respectively.

Immunisation of carp against *Ichthyophthirius multifiliis*. 60 juvenile carp, 4 to 6 cm in length were exposed to 5000 theronts fish^{-1} in 250 ml of gently aerated water for 1 h. Following infection, fish were maintained in aquaria and infections allowed to proceed to conclusion. Fish were re-exposed to theronts after 1 mo, following clearance of previous infection, and challenged on 3 further occasions, at 10 to 20 d intervals, with increasing numbers of theronts (5000 to 10 000 fish^{-1}). These immunised fish were maintained for a minimum period of 14 d before use in experiments (Houghton & Matthews 1986).

Experimental procedure. *Primary infections (Fig. 1):* 100 carp were infected with *Ichthyophthirius multifiliis* following exposure to theronts as described above. Two to 4 fish (donors) were removed at timed intervals after exposure, passed through 3 containers of clean water to remove any free theronts from the fish surface, and placed in 7 ml of MEM in glass crystallising bowls for 50 m to induce trophont emergence. Timed intervals included 1, 2, 3, 4, 6, 8, 10 and 12 h and thereafter every 24 h up to the 6th day of infection. All fish investigated at intervals later than 8 h post exposure were killed with an overdose of MS222 and immediately washed in clean water prior to treatment with MEM. The number of exiting trophonts was estimated following removal of infected fish from glass containers by a stereomicroscope (25× magnification). The MEM containing the parasites was then diluted by addition of 70 ml of aerated water and a single carp (recipient), naive to *I. multifiliis*, was introduced to the system for a period of 1 h, providing opportunities for infections to be established by the exited trophonts. After removal of the recipient to a new aquarium any free trophonts remaining within the crystallising bowls were monitored for survival, encystment and ability to complete development with the production of viable theronts. The exposed recipient was maintained in isolation for 5 d at 19 to 20°C. The number of white spots detected provided a rough comparison of infection level; these foci might include more than 1 trophont. Spots were scored as follows with respect to infection, in parentheses: + = 1 to 5 parasites (low), ++ = 6 to 10 parasites (moderate) and +++ = >10 parasites (heavy).

The following procedure was adopted in order to determine the percentage of exiting parasites capable of re-invading another host. 10 carp were exposed individually to *Ichthyophthirius multifiliis* as described above (infection dose: 22 000 theronts fish^{-1}). After 1 h,

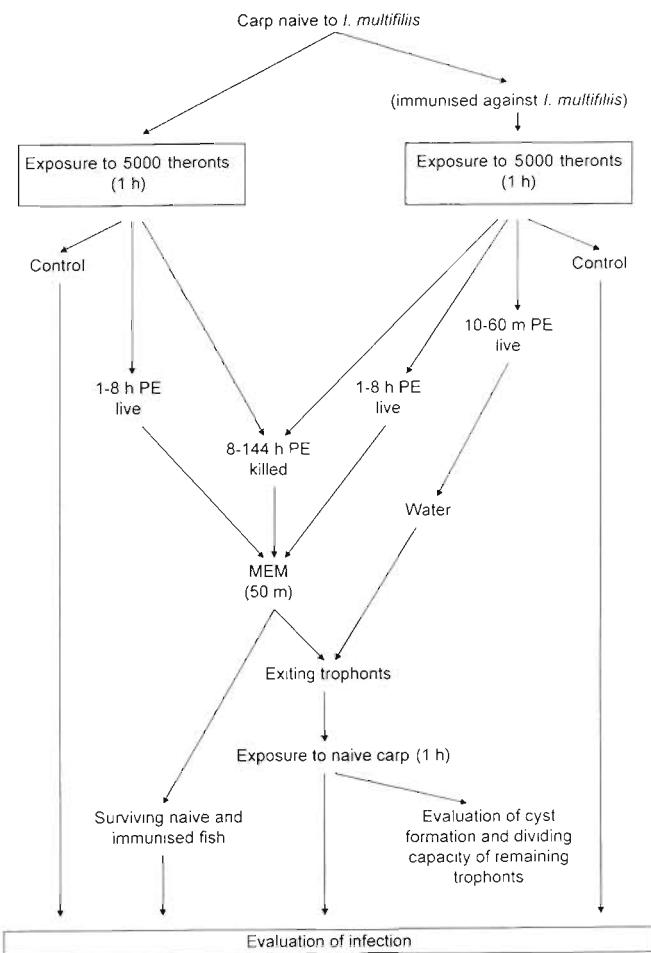Fig. 1 *Ichthyophthirius multifiliis* infecting *Cyprinus carpio*. Procedure for testing infectivity of prematurely recovered tomonts. PE: post exposure

fish were carefully rinsed 3 times in clean water and transferred to MEM for 50 min. Parasites remaining in the infection bath and in the MEM after removal of fish were counted. Naive fish were exposed to the recovered parasites for 1 h then transferred to holding tanks. After 5 d developing parasites on these latter carp were enumerated.

Recovery of trophonts from immunised carp (Fig. 1):
60 carp immunised against *Ichthyophthirius multifiliis* were exposed to theronts for 1 h as in primary infections above. At known times post exposure (PE), 1 fish was examined for exiting trophonts, these being recovered for viability tests as described for primary infections. Investigations of emerged trophonts up to 1 h PE, including at intervals of 10, 20, 30, 50 and 60 min, however, were undertaken on fish maintained in water without the addition of MEM; trophonts are known to exit immunised carp within 2 h of infection (Cross & Matthews 1993). Immediately following exposure, these fish were quickly passed through 3 containers of

clean water to remove free theronts and finally to 7 ml of water contained in crystallising bowls, from which exiting trophonts were recovered over a 90 min period. Those immunised carp examined at later intervals, 2, 4, 6, 8, 10 and 12 h and daily up to Day 6 post exposure, were maintained in MEM for 50 min, as in primary infections; those examined after 8 h were killed with an overdose of MS222 prior to treatment. Treatment with MEM aimed to induce the emergence of developing trophonts which might have evaded the immune response. After careful removal of carp, the MEM was diluted by the addition of 70 ml of aerated water and the infectivity of recovered trophonts investigated following the procedure shown in Fig. 1.

RESULTS

MEM induced exit of trophonts in primary infections with time PE to theronts

MEM was more effective in stimulating premature exit of trophonts when used at full strength, fewer parasites being recovered at 1:1 and 1:2 dilution in water. Undiluted MEM was therefore used in all subsequent experiments. No adverse effects were recorded in carp as a result of short-term exposure to MEM as used in the present study and all survived treatment. The highest number of parasites was recovered from live fish sampled within 4 h PE, the first trophonts exiting immediately upon treatment with the undiluted MEM. Relatively lower yields of trophonts were obtained from fish at 8 h PE, although recovery was marginally greater from freshly killed fish. However only single parasites left a freshly killed fish within 1 h when incubated in water whereas clearly more parasites could be recovered from a MEM-incubated dead fish in the same period. Parasites induced to exit up to 8 h PE still resembled the theront stage in shape, having an elongated body, and in behaviour, actively swimming in the upper water layer. In contrast, those emerging later were spherical, with considerable variation in diameter by 3 d PE, and tended to settle on the bottom of the container shortly after leaving the fish. Some trophonts recovered after 3 d PE resembled theronts in size, shape and behaviour. All surviving fish showed a moderate to heavy infection 5 to 6 d PE even when they had previously been exposed to MEM.

Viability of trophonts following MEM-induced emergence from primary infections

Trophonts recovered up to 24 h PE were able to re-infect naive carp, the degree of infectivity decreasing
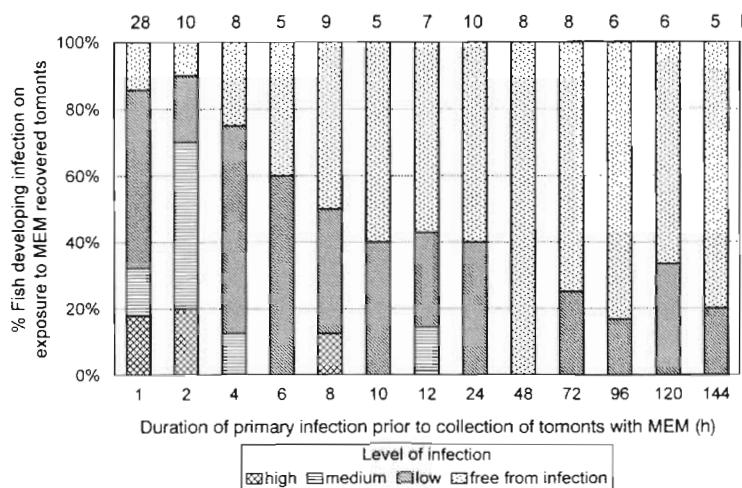
Fig. 2. *Ichthyophthirius multifiliis* infecting *Cyprinus carpio*. Infection levels of *I. multifiliis* recorded in carp following exposure to tomonts prematurely recovered from a primary infection in carp with the aid of MEM. N = carp exposed at each time interval

with time PE during this period (Fig. 2). No trophonts recovered 48 h PE were able to re-infect carp directly. However some of these trophonts completed the aquatic phase of development, encysting and producing viable theronts. Parasites exiting after 72 h PE and on Days 5 and 6 PE following MEM treatment produced low level infections on re-exposure to naive carp. The few trophonts detected in such cases were significantly smaller compared to those observed in the control carp from the primary infection.

Eighty percent of the theronts exposed to carp in primary infections were estimated to have entered the tissues within the 1 h exposure period. On treatment with MEM 1 h PE, 11 to 29% of these were induced to exit prematurely within 50 min. Infections, although in the low category (+), were established in 8 of the 10 fish exposed to these emerged trophonts. This represented approximately 0.1% of the original trophonts population of the primary infection (Table 1).

Exit of trophonts from immunised carp with time PE to theronts

A significant number of trophonts exited the immunised carp within 1 h PE without MEM treatment. In contrast, only 1 or 2 trophonts emerged from non-immune control carp exposed to similar numbers of theronts within the same time period. Trophonts could still be recovered from immune carp up to 48 h PE with the aid of MEM. The appearance of these trophonts was identical to that of corresponding parasites collected from non-immunised fish, although the former

showed a more active swimming behaviour. Few parasites emerged after 2 d, although slight infections confined to the periphery of fins were detected on 1 or 2 carp. Non-immune controls developed ichthyophthiriasis and some died during the second phase of infection.

Viability of trophonts following premature emergence from immunised carp

A small number of trophonts recovered up to 8 h PE were able to re-infect naive carp (Fig. 3). The degree of infection decreased with time PE. Trophonts exiting immunised carp later than 8 h PE were not able to re-infect a fish except in one single case in which few parasites recollected 48 h PE developed to mature trophonts within the fish. Highest infection degrees were found with parasites sampled $^1/_2$, 1 and 2 h after the initial infection.

DISCUSSION

Although Cross & Matthews (1992) described the expulsion of *Ichthyophthirius multifiliis* from immune carp, they did not investigate the viability of the exiting parasites. In the present study these are shown to retain theront-like properties for up to 8 h following the invasion of carp epidermis with the potential, upon premature exit, to re-invade and complete development within a further fish host. The majority of trophonts had actively emerged within 2 h of infection and parasites remaining within immune hosts after this period and all those from non-immune carp were recovered only with the aid of MEM. Infectivity of trophonts emerging in direct response to immune hosts

Table 1. *Ichthyophthirius multifiliis* infecting *Cyprinus carpio*. Fate of *I. multifiliis* exposed to carp in primary infections and following induced emergence in MEM 1 h PE. Carp for secondary infection: N = 15

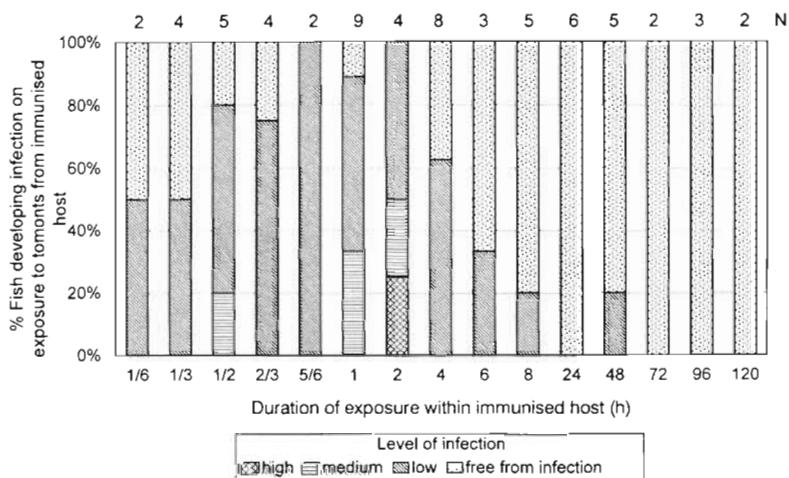| Stage of infection	% of initial theront population		
	Mean	Minimum	Maximum
Theronts entering carp at 1st exposure	82.5	81.8	83.2
Trophonts recovered in MEM	19.8	11.3	29.3
Trophonts completing development after second invasion	0.098	0	0.55

Fig. 3. *Ichthyophthirius multifiliis* infecting *Cyprinus carpio*. Infection levels of *I. multifiliis* recorded in carp following exposure to tomonts prematurely exited from immunized hosts of the same species. Recovery method from primary infected host: within 1 h post infection active exit, thereafter with the aid of MEM. N = carp exposed at each time interval

was comparable to that of trophonts of similar age recovered from non-immune carp with MEM although in both groups viability was significantly higher in parasites emerging within 1 h of infection. That few infections were established by post 2 h trophonts from immune carp and none by post 8 h trophonts was not unexpected as the majority of the parasite population would already have exited in response to immune factors. Nevertheless viable trophonts were recorded up to 8 h post infection irrespective of the immune status of the host from which the parasites emerged or whether the emergence was induced by MEM treatment. Previous studies of *I. multifiliis* suggest a commitment to trophont transformation and maturation immediately on host entry, with the sequential release of the theront's secretory products from mucocysts and the organelle of Lieberkühn. Mucocysts are thought to play a major role in the infection process as their discharge coincides with contact and penetration of the host surface (Matthews & Matthews 1984, Ewing et al. 1985, Ewing & Kocan 1992). It is possible that depletion of these organelles contributed to the rapid decline in viability of prematurely emerging parasites. Mucocysts are present in all stages of the life cycle of *I. multifiliis* although the question of whether they are chemically distinct in theronts and trophonts is unresolved. Their reappearance within 5 h trophonts (Matthews & Matthews 1984) could account for the infectivity recorded in parasites recovered up to 24 h PE from non-immune fish. The fact that only a very small number of infections were established from these older parasites, however, is consistent with trophont maturation, including the disappearance of the orga-

nelle of Lieberkühn over a time scale of 1 to 18 h post infection (Matthews et al. 1996). The small number of infective 8 to 24 h trophonts recovered from non-immune fish might, therefore, be attributed to slow developers and delayed transformation. There was no evidence of post 24 h trophonts directly invading new fish hosts, and by 72 h the parasites could have attained the threshold size for encystment and theront production within the aquatic environment (Maclennon 1937, 1942, Ewing et al. 1986). We suggest that the infections established from post 72 h trophonts in the present study resulted from such a new generation of theronts.

Recent studies by Clark et al. (1996) and Clark & Dickerson (1997) indicate the involvement of specific antibodies in triggering premature exit in the immune host, 3 d old trophonts being induced to emerge from channel catfish following passive immunisation with monoclonal antibodies (mAb) against surface antigens (i-antigens). The expulsion of trophonts was shown to be dependent upon mAb binding to the ciliary surface at sub-lethal levels as parasite development proceeded normally with the production of theronts on entering water. Results presented in this study support a similar role for antibody in natural immunity against ichthyophthiriasis as theronts both entered and exited the epidermis of immune hosts yet retained the potential to re-infect a further fish host. The ability to respond rapidly to specific components of the immune host ensures a high probability that infectivity will be retained by a significant proportion of the expelled parasite population. Immobilising antibodies are a characteristic feature of the humoral response of fish to *Ichthyophthirius multifiliis* and are known to inactivate both theronts and trophonts *in vitro* (Wahli & Meier 1985). The question of whether these play a role in the protection of fish against ichthyophthiriasis has yet to be resolved.

The fact that a small number of trophonts remained within the immune hosts after 2 h might have resulted from normal healing processes, their escape being impeded by tissue entrapment. In primary infections epidermal regeneration quickly isolates the trophont from the external environment. According to Ewing et al. (1985), gill epithelium regains its normal appearance within 45 min of invasion by *Ichthyophthirius multifiliis* whereas Iger & Abraham (1990) reported advanced stages of healing in carp epidermis within 3 h of artificially inflicting wounds. To what extent the survival of *I. multifiliis* is affected by prolonged exposure

to specific components of the immune response is far from clear as live trophonts were recovered here from immune hosts after 2 d following the MEM treatment. Houghton & Matthews (1993) have also described the survival of this parasite in immune carp for up to 3 d. Following administration of the synthetic corticosteroid triamcinolone acetonide, trophonts developed to completion on the immune host, revealing that the cell-mediated immune response was suppressed. Matthews (1994) suggested that evasion of host response might be attributed to the trophont's ability to modify its immediate environment by destruction and phagocytosis of host cells. In the light of recent studies by Clark et al. (1996) localised antibody depletion resulting from antigen saturation of the epidermis might also account for the survival of small numbers of trophonts within the immune host. The fact that parasite numbers dwindled with day of infection and ciliates could not be detected in most immune hosts after 4 d both here and by Houghton & Matthews (1993) could also be explained on the basis of recovering antibody levels within the epidermis. That surviving parasites represent latent stages seems unlikely as trophont growth and development did not appear to be compromised within the immune host in the present study although those recovered after 2 d PE had not reached a sufficient stage of maturity for further development in the aquatic environment. Nevertheless, the presence of such concealed parasites would be significant in the transmission of ichthyophthiriasis in stressed fish populations. The small number of trophonts developing full-time infections within the peripheral regions of the fins of immunised carp here was probably indicative of a decline in the protective response (Houghton & Matthews 1993).

How MEM induces premature exit of *Ichthyophthirius multifiliis* was not investigated; however, it seems possible that osmotic factors are involved because full strength medium, as recommended by Matthews et al. (1996), was most effective in recovering parasites from the fish epithelium. It is assumed that MEM, being iso-osmotic with the fish epithelium, might equalise the concentration between host and aquatic environment, permitting the highly motile trophonts to freely move between the two. In support of this view, only a limited proportion of the parasite population was recovered in MEM and success was greatest within 4 h of infection when trophonts might still have been in contact with the exterior via entry routes prior to epidermal regeneration (Iger & Abraham 1990). Such artificial means of inducing emergence may not be comparable to normal events within the host. Nevertheless, it may be relevant that Ewing & Kocan (1992) suggested that the pressure of fluid expulsions from contractile vacuoles might aid in the

normal escape of this parasite from host tissues whereas excretion of tissue hydrolases from these organelles has also been suggested by Clark & Dickerson (1997). The fact that percent recovery diminished with time in the host suggests that trophonts become decreasingly attracted to high osmolarities with maturation, such that exit is assured in the tomont. Modulation of a response to osmolarity with parasite growth could provide a simplistic explanation for the behaviour observed in *I. multifiliis*. Ciliates are known to respond to a wide variety of environmental stimuli, behavioural responses being mediated through membrane bound receptors (Machemer & Dietmer 1987, Lukas et al. 1989). The temporary occlusion of such receptors by antigen antibody complexes at the cell surface could possibly account for the premature emergence of *I. multifiliis* from the immune host.

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