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The swimbladder nematode *Anguillicola crassus* in the European eel *Anguilla anguilla* and the Japanese eel *Anguilla japonica*: differences in susceptibility and immunity between a recently colonized host and the original host

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Abstract

The swimbladder nematode *Anguillicola crassus* originates from Asia where it is a parasite of the Japanese eel *Anguilla japonica*. After its introduction to Europe about 25 years ago, the parasite spread rapidly within the indigenous populations of the European eel *Anguilla anguilla* and subsequently the prevalence and mean intensity appeared to stabilize. Under experimental and aquaculture conditions the naïve new host appears to be more susceptible to *A. crassus* compared to the original host. Both eel species develop a immune response against *A. crassus*. The antibody response is well characterized for the European eel, but poorly characterized for the Japanese eel. It remains unclear if antibodies have any protective function against *A. crassus*. Encapsulation of larvae of *A. crassus* can be observed in naturally infected European eels. However, encapsulation of larvae following experimental infection has not been detected in European eels, but only in Japanese eels. Reinfection experiments and intraperitoneal injection of *A. crassus* homogenates failed to demonstrate the development of acquired immunity in European eels. Immunization with irradiated third stage larvae provided preliminary evidence for acquired immunity against *A. crassus* in the Japanese eel, but not in the European eel.

Introduction

Globalization of human activities opens completely new dissemination paths for parasites. Among other activities, the intercontinental and transcontinental trade of living animals always contains the possibility that parasites are transferred together with their hosts and thus cross zoogeographical borders. One of the best examples for a parasitic neozoan in Europe is the eel nematode *Anguillicola crassus* Kuwahara, Niimi & Itagaki.

All species of *Anguillicola* Yamaguti (Nematoda: Dracunculoidea) are specialized parasites of eels of the genus *Anguilla* Shaw. Until the 1980s, the geographical distribution of all five species of *Anguillicola* was restricted to the Indo-Pacific region: *A. australiensis* Johnston & Mawson from South Australia in the Australian longfin eel *Anguilla reinhardtii* Steindachner, *A. crassus* and *A. globiceps* Yamaguti from East Asia in the Japanese eel *Anguilla japonica* Teminck & Schlegel, *A. novaezelandiae* Moravec & Taraschewski from New Zealand in the shortfin eel *Anguilla australis* Richardson and probably also in the longfin eel *Anguilla dieffenbachii* Gray, and *A. papernai* Moravec & Taraschewski from the east coast of South Africa in the African longfin eel *Anguilla mossambica* Peters (Moravec & Taraschewski, 1988).

Concerning the distribution of *A. crassus* and *A. globiceps* in Asia, one should consider the great economic importance of eels in the Far East, mainly determined by the demand in Japan. Consequently, Asian eel aquaculture became a profitable industry during the past century (Tesch, 1999), causing intensive regional trade with living

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eels. Presumably eel parasites have been relocated together with their hosts, therefore it is impossible to decide if *A. crassus* and *A. globiceps* have always been coexisting species, or if their actual distribution is largely determined by human activities.

Zoogeographical restriction of *Anguillicola* to the Indo-Pacific region ended when eel aquaculture was intensified in Europe. Initially, *A. novaezelandiae* was introduced into Lake Bracciano, Italy, probably with a stock of shortfin eels imported from New Zealand in 1975 (Moravec & Taraschewski, 1988), but remained restricted to this locality. A second *Anguillicola* species introduced to Europe, *A. crassus*, proved to be a more succesful colonizer within European eel populations. It was probably introduced with eels imported from Taiwan (Køie, 1988) and first reported from Germany in 1982 (Neumann, 1985). *Anguillicola crassus* spread thoughout most of the continent within only 10 years (Moravec, 1992; Kirk, 2003). The first report of *A. crassus* in northern Africa occurred in 1994 (El Hilali *et al.*, 1996), and in 1995 it was reported from the south-eastern USA in cultured and wild American eels (Fries & Williams, 1996).

Eels become infected with *A. crassus* by ingesting intermediate hosts (copepods) or paratenic hosts (diverse fish species, amphibians, molluscs, and insect larvae) infected with the third stage larvae (L3). Development from L3 to the fourth larval stage (L4) occurs in the eel's swimbladder wall. Adult worms live in the swimbladder lumen and suck the host blood (De Charleroy *et al.*, 1990; Moravec & Konecny, 1994; Székely, 1994; Moravec, 1996; Moravec & Škoríková, 1998).

Based on the present knowledge of the epidemiology and pathology of *A. crassus* in the European eel, and the host's immune- and stress responses to this parasite, one must assume that *A. crassus* is a serious threat to indigenous eel populations, which are not adapted to this invasive parasite species (Sures & Knopf, 2004). The present paper compiles the present information on the susceptibility and immunity of the recently colonized European eel and the original host, the Japanese eel.

Epidemiology

The rapid spread of *A. crassus* within the European eel populations is well documented (Moravec, 1992; Kirk, 2003). The dissemination of this neozoan parasite appears to be limited only by low ambient water temperatures in Scandinavia (Höglund *et al.*, 1992b; Knopf *et al.*, 1998; Wickström *et al.*, 1998), and by high salinity in the marine environment (Dekker & van Willigen, 1989; Nielsen, 1997; Kirk *et al.*, 2000).

Long-term studies of wild European eel stocks in the River Elbe, Germany (Hartmann, 1991), the Ijsselmeer and Markermeer, the Netherlands (Haenen *et al.*, 1994), in different river catchments in Flanders, Belgium (Audenaert *et al.*, 2003), in the Rhône River delta, France (Lefebvre & Crivelli, 2004), in Neusiedler See, Austria (Schabuss *et al.*, 2005) and in the Lake Müggelsee, Germany (own data) reflect a general pattern: following the introduction of *A. crassus*, its prevalence increased rapidly within a few years. Subsequently, although values fluctuate, the prevalence and mean intensity appear to have stabilized around a specific level of 50–90% and 3–7 worms per eel, respectively (fig. 1). Whilst Lefebvre & Crivelli (2004) and Schabuss *et al.* (2005) found no trend in the mean intensity, Haenen *et al.* (1994) and Audenaert *et al.* (2003) concluded from their data that mean intensity was decreasing.

Several mechanisms may explain the possible stabilization of the infection. Pathologically altered swimbladders resulting from *A. crassus* infection might not be suitable for further infections (Van Banning & Haenen, 1990). Furthermore, there is evidence for density-dependent regulation of larval populations within the intermediate host (Ashworth *et al.*, 1996), and also within the eel host (Ashworth & Kennedy, 1999). Thus, possibly concomitant immunity plays a role in the regulation of larval development in the final host. However, to date no experimental evidence for protective immunity in the European eel is available.

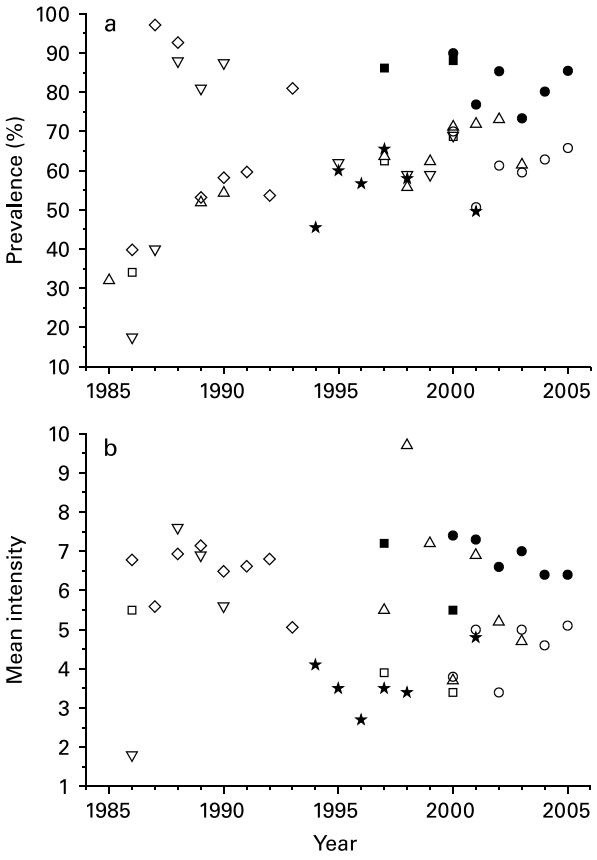

Fig. 1. Dynamics of the prevalence (a) and mean intensity (b) of *Anguillicola crassus* in European waters over two decades. Open symbols, adult worms; closed symbols, L3, L4 and adult worms. ▽, River Elbe, Germany; ◇, Ijsselmeer and Markermeer, the Netherlands; □, river catchments in Flanders, Belgium; △, Rhône River delta, France; ★, Neusiedler See, Austria; ○, Lake Müggelsee, Germany. Original data from Hartmann, 1991, Haenen *et al.*, 1994, Audenaert *et al.*, 2003, Lefebvre *et al.*, 2004, Schabuss *et al.*, 2005, and own data.

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Little is known about the epidemiology of *A. crassus* in wild eels in waters of its origin, the Far East. Egusa (1979) mentions that this parasite is widely distributed in natural waters and in eel culture ponds in Japan. One report from *A. crassus* in wild Japanese eels in China mentions a prevalence of 56.3% and an intensity of 1–11 worms (Nagasawa *et al.*, 1994). Assuming that these data are related to adult worms in the swimbladder lumen, they are similar to those found in wild European eels. This is surprising considering the different susceptibility of the two eel species observed in aquaculture (Egusa, 1979) and demonstrated under experimental conditions (Knopf & Mahnke, 2004). However, more data from wild Japanese eels are needed in order to gain a comprehensive picture allowing the comparison of the epidemiology of *A. crassus* in its original host in Asia and in its novel host in Europe.

Susceptibility of eels in aquaculture and under experimental conditions

Experience with the culture of eels in Japan indicated that the European eel is more susceptible to *A. crassus* than is the endemic Japanese eel. According to Egusa (1979), prevalence in cultured European eels is 'several tens of percent and occasionally nearly 100 percent' and infection intensities are '5 to some dozen and occasionally exceed 30'. In contrast, only 10–40% of cultured Japanese eels are infected and infection intensities are 1–3, occasionally up to 20 (Egusa, 1979; Nagasawa *et al.*, 1994).

This difference in susceptibility has recently been proved experimentally (Knopf & Mahnke, 2004). European eels and Japanese eels were infected with 30 L3 of *A. crassus* per eel and maintained under identical conditions over a period of 98 days at 23°C. Whereas no dead, encapsulated larvae were found in the European eels, almost 60% of the parasites recovered in the Japanese eels were found as dead, encapsulated larvae in the swimbladder wall. Consequently, more adult worms were found in European eels compared with Japanese eels (fig. 2). Additionally, a higher mass of adult worms indicated that the development of surviving parasites was enhanced in the European eel compared with Japanese eel (fig. 2). Finally, these traits resulted in a higher worm burden and higher reproductive success of *A. crassus* in the European eel than in the Japanese eel. The results of this study demonstrate that the European eel possesses less effective defence mechanisms against *A. crassus* than does the Japanese eel, and that these mechanisms are efficient even during a primary infection in the Japanese eel.

Humoral immune response

Infection with *A. crassus* elicits an antibody response in the European eel. Antibodies directed against *A. crassus*-antigens can be detected in naturally infected eels (Buchmann *et al.*, 1991; Höglund & Pilström, 1994, 1995; Haenen *et al.*, 1996; Nielsen & Buchmann, 1997; Knopf *et al.*, 2000b), and also in experimentally infected European eels (Knopf *et al.*, 2000a).

An infection experiment revealed that at a water temperature of 20°C, *Anguillicola*-specific antibodies are first detectable in the peripheral blood 8 weeks post-infection. However, the time course and intensity of the antibody response detected with an enzyme-linked immunosorbent assay (ELISA) using antigen preparations from the adult worm body wall and complete L3, respectively, is highly variable between individual eels, and appeared to be independent of both the number of L3 administered and the frequency of administration. Using the ELISA technique, no difference could be detected in the temporal course of the antibody response against antigens derived from adult worms and L3 (Knopf *et al.*, 2000a).

Several attempts were performed with sera from naturally infected Europen eels to characterize specific antibodies directed against *A. crassus* antigens (Buchmann *et al.*, 1991; Höglund & Pilström, 1995; Haenen *et al.*, 1996; Nielsen & Buchmann, 1997; Knopf *et al.*, 2000b). Although the results obtained by the Western blot technique vary in detail, it is clear that the European eel produces a variety of antibodies which are directed against *A. crassus* antigens with molecular weights ranging from 14 to 200 kDa. However, from the work with sera from naturally infected eels with an

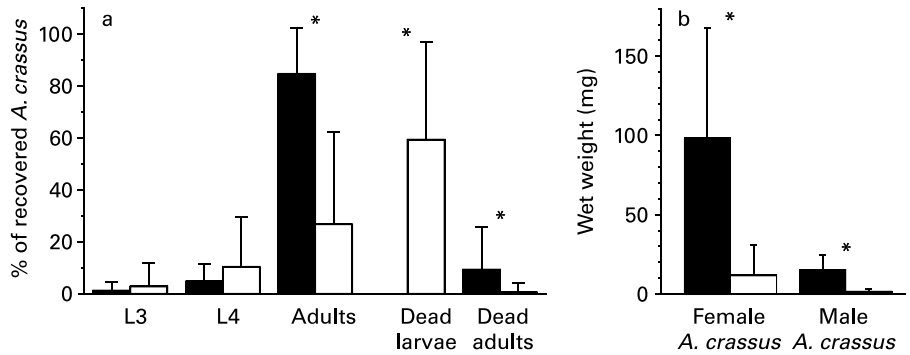

Fig. 2. Composition of the *Anguillicola crassus* infrapopulation (a) and wet weight (b) of individual adult worms in experimentally infected European (■) and Japanese eels (□). Percentages are calculated from the number of recovered worms; asterisks indicate significant differences, $P < 0.005$. Original data from Knopf & Mahnke, 2004.

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unknown parasitological background, it remains an open question which of these antibodies were produced following infection with *A. crassus* and which are cross reacting antibodies.

Western blot analysis performed with sera from experimentally infected European eels revealed a strong and consistent response to antigens with a molecular weight of about 100 kDa located in the body wall, especially in the gelatinous outer cuticle of the adult worm (Knopf *et al.*, 2000b; fig. 3). The predominant reaction of antibodies to cuticular antigens is in agreement with the finding that antigen preparations from the cuticle are useful in an ELISA measuring the antibody response to *A. crassus* in the European eel (Höglund & Pilström, 1995; Nielsen & Buchmann, 1997). Based on the blurred character of the conspicuous band obtained with the outer cuticle antigen preparation, Knopf *et al.* (2000b) suggested that the antigens might be lipophilic glycoproteins that are secreted by the adult worms and elicit the host's immune response after they passed through the swimbladder epithelium. Nielsen & Buchmann (1997) suggested that gluthathione-s-transferase (GST) is an important antigen of *A. crassus*. Using rabbit anti-bovine-GST they detected antigens located in the cuticle, gonads and excretions/secretions of adult *A. crassus* with molecular weights expected for GST and its subunits (approximately 45 kDa and 25 kDa). However, these results are not fully conclusive because some additional bands detected by the anti-GST were not consistent with the molecular weight of GST and its subunits.

To date it was not possible to demonstrate a stage-specific antibody response against the invasive L3

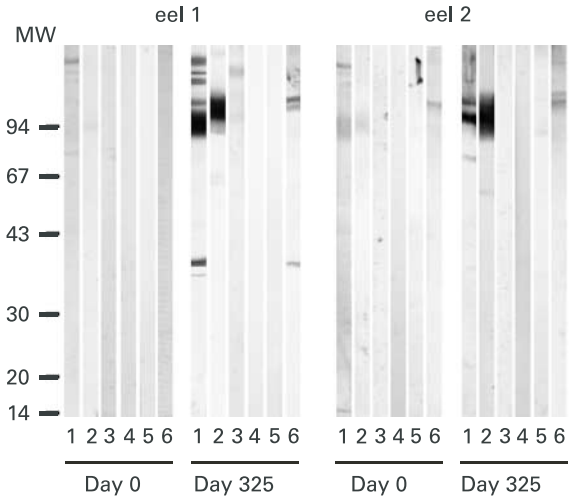

Fig. 3. Pattern of the antibody response of *Anguilla anguilla* (eel 1 and eel 2) after repeated experimental infection with *Anguillicola crassus* (three L3 twice a week over 140 days and 25 L3 at day 185). Western blots were performed with sera taken before infection and at day 325 using crude antigen extracts from the body wall (1), outer cuticle (2), intestinal wall (3), intestinal contents (4) and female reproductive system (5) of adult worms, and a somatic L3 antigen (6). Molecular weight (MW) in kilodaltons. Modified from Knopf *et al.*, 2000a.

(Haenen *et al.*, 1996; Knopf *et al.*, 2000a). This is surprising, because in contrast to adult worms lying in the swimbladder lumen and sucking blood, larvae migrate in the tissue and thus are in direct contact with the host's immune system. From the present information available from the European eel, Knopf *et al.* (2000a) concluded that adult *A. crassus* rather than invading L3 might elicit the antibody response. If one calculates about 50 days at 20°C for the development of *A. crassus* from the infection of the eel until first worms appear in the swimbladder lumen, and about one week for the induction of a primary antibody response, this hypothesis is consistent with the late appearance of *Anguillicola*-specific antibodies in the peripheral blood of European eels after experimental infection (Knopf *et al.*, 2000a).

Little is known about the antibody-response of Japanese eels to *A. crassus*. Ushikoshi *et al.* (1999) detected antibodies directed against cuticular antigen preparations from adult *A. crassus* by means of an ELISA. Nielsen (1999) compared the antibody-response of Japanese eel with that of the European eel following intraperitoneal injection of excretory/secretory (ES) and outer cuticle antigen preparations from adult *A. crassus*. Using an ELISA performed with polyclonal antibodies raised against immunoglobulins from the European eel, he revealed a higher antibody response to ES-antigen preparation in the Japanese eel compared with the European eel. This difference was not found for the outer cuticle-antigen preparation. From this result Nielsen (1999) concluded that differences in the antibody response might contribute to the different susceptibility of both eel species to *A. crassus*. However, it must be considered that antigen injection can hardly be compared with the qualitative and quantitative dynamics in the release of antigens during infection.

Furthermore, it remains to be seen if antibodies have any protective function against *A. crassus* (Knopf *et al.*, 2000a). It is unlikely that antibodies alone are able to kill the nematode. However, they might opsonize the surface of the parasite, and thus increase the efficacy of cell-mediated effector mechanisms, as experimentally shown for the killing of the eyefluke *Diplostomum spathaceum* in rainbow trout (Whyte *et al.*, 1989).

Cellular immune response

When migrating from the intestine to the swimbladder wall, *A. crassus* larvae damage tissues of the eel and induce inflammatory responses within 5 h post-infection in European eels (Haenen *et al.*, 1989). Lefebvre *et al.* (2004) found that splenic enlargement in wild European eels correlated with the number of *A. crassus* in the swimbladder lumen and with the severity of swimbladder damage, suggesting an increased synthesis of immune cells. From haematological studies performed with naturally infected and experimentally infected European eels, it appeared that *A. crassus* infection causes an increasing granulocyte number (Boon *et al.*, 1990b; Höglund *et al.*, 1992a; Van der Heijden *et al.*, 1996), giving evidence for cellular immune reactions. However, this result could not be confirmed by Boon *et al.* (1990a) and Palíková & Navrátil (2001). If and how *A. crassus* infection

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modulates the number of lymphocytes in the peripheral blood of the European eel is not clear from the literature. Höglund *et al.* (1992a) found a reduced number of lymphocytes in the peripheral blood of naturally infected eels, whereas Van der Heijden *et al.* (1996) found an increased number of B-lymphocytes and lymphoid cells in general as a result of experimental infection.

In histological sections obtained from experimentally infected European eels Haenen *et al.* (1989) identified 'numerous mononuclear phagocytes' around some of the larvae located in the submucosa of the digestive tract of experimentally infected eels, but later on no inflammatory reactions around the larvae in the swimbladder.

Although encapsulated larvae have never been found in experimentally infected European eels (Haenen *et al.*, 1989; Knopf & Mahnke, 2004), encapsulation of L3 and L4 can be observed in wild-caught, naturally infected European eels (Molnár, 1994; Audenaert *et al.*, 2003), where nodules containing larvae are much more common in the swimbladder wall than in the intestinal wall (Molnár, 1994). The tissue reaction against L3 and L4, normally located in the subserosa of the swimbladder, was described in detail by Molnár (1994), who showed from histological examinations that initially epithelioid macrophages aggregate around the larvae. Granulation tissue formed by these epithelioid cells might restrict the movement of larvae, without killing them. Subsequent steps were thickening of the epithelioid layer, elongation of the external epithelioid cells, formation of a connective tissue capsule and necrosis of the epithelioid cells in the centre of the nodule. Foreign-body giant cells and Langerhans-type giant cells are detectable among the epithelioid cells surrounding the parasite (Molnár *et al.*, 1993; Molnár, 1994). Molnár (1994) assumes that finally the surrounded larva is killed by phagocytes, the effect of cytokines, and impaired feeding of the parasite.

Occasionally, probably through injuries caused by the adult worms, second stage larvae (L2) that hatch from eggs released by adult worms aberrantly penetrate into the tissues of the swimbladder wall where they die. Intensive formation of granulation tissue containing mononuclear cells, macrophages and giant cells can be observed around such abnormally located L2 in the serosa and subserosa (Molnár *et al.*, 1993, 1995). Abnormally located adult worms in the swimbladder wall, typically in the subserosa, are surrounded by a thin connective tissue capsule and melanomacrophages, involved in the removal of tissue debris and the necrotic parasite. Since, in such cases melanomacrophages also accumulated around normally located L3 and L4, Molnár *et al.* (1995) suggested that these larvae are also affected by the host response induced by the abnormally located adult worms.

The characteristic presence of eosinophilic granulocytes in response to nematode larvae, common in mammalian hosts, appears to be uncommon in eels; eosinophilic cells within the inflammatory tissue around the migrating larvae were not identified by Haenen *et al.* (1989) and Molnár (1994). However, Molnár *et al.* (1993) found eosinophilic granulocytes within the granulation tissue surrounding larvae located in the submucosa of the swimbladder, on the peritoneum, and in the intestinal wall of a single heavily infected wild European eel. Würtz & Taraschewski (2000) identified granulocytes and macrophages around *A. crassus* larvae in the swimbladder wall by transmission electron microscopy, but these cells did not appear to attack the L3 and L4. Thus, the authors assumed that these cells only remove cell debris created by the larvae, which themselves are immunologically camouflaged. On the other hand, using an *in vitro* chemotaxis assay, Knopf (1999) demonstrated that the presence of L3 lead to increasing migration activity of phagocytes from infected eels compared to uninfected controls. However, *in vitro* these cells did not kill the larvae.

It is surprising that in the same swimbladder wall some larvae elicit an intense host reaction, whilst others are present undisturbed by any cellular host response (Molnár *et al.*, 1993; Molnár, 1994). Molnár (1994) suggested that the diversity in the host response might be due to activated defence mechanisms after repeated invasions by a large number of larvae. This could explain differences between individual eels, but it does not explain the different host reaction to individual larvae located in one and the same swimbladder. It remains open for further research if the presence of encapsulated larvae in the European eel is due to protective immune mechanisms, or whether only dead or less viable larvae are isolated by the host via encapsulation.

Little is known about the cellular immune response of the Japanese eel due to infection with *A. crassus*. However, encapsulation and killing of 60% of larvae experimentally administered to Japanese eels, but no comparable reaction in similarly treated European eels, strongly indicates that cellular effector mechanisms work much more effectively in the Japanese eel compared to the European eel (Knopf & Mahnke, 2004).

Immunopathology

For the European eel, pathological changes of the swimbladder wall due to infection with *A. crassus* are well described (Van Banning & Haenen, 1990; Molnár *et al.*, 1993, 1995; Molnár, 1994; Haenen *et al.*, 1996; Nimeth *et al.*, 2000; Würtz & Taraschewski, 2000). The most characteristic change is thickening of swimbladder tissues, impairing function of this hydrostatic organ (Würtz *et al.*, 1996; Nimeth *et al.*, 2000). Differences in the pathogenicity of *A. crassus* in the European and Japanese eels became obvious in the culture of both eel species in Japan: pathological changes in the swimbladder wall due to heavy infections with *A. crassus* are reported for farmed European eels, but the parasite causes 'almost no serious damage' to farmed Japanese eels (Egusa, 1979; Nagasawa *et al.*, 1994).

Whereas Molnár *et al.* (1993) suggested the immune response to repeated larval infection as the main factor for the swimbladder lesions in the European eel, Van Banning & Haenen (1990) assumed that irritation and inflammation caused by L2 aberrantly located in the swimbladder wall is most harmful to the host. In any case, several pathological changes such as infiltration of inflammatory cells and dilated blood vessels are immunopathological effects. It remains an open question to what extent the pronounced pathomorphological changes of the swimbladder wall result from immuno-

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logical processes, or from toxic products released from the worms or injuries caused by migrating larvae or blood-sucking adult worms (Molnár *et al.*, 1993).

Reinfection and immunization studies

Reinfection experiments were performed with European eels with the aim of demonstrating acquired immunity to *A. crassus*. Haenen *et al.* (1996) used doses of 0–40 L3 for the primary infection and reinfected eels with 20 L3 on day 56. Examination of eels on day 112 revealed no indication for acquired immunity resulting from the primary infection. In order to simulate natural conditions, Knopf (1999) applied repeated infections with three L3 twice a week over a period of 140 days, followed by a final administration of 25 L3 on day 185. Subsequently, 150 and 180 days after the last infection, eels were treated with levamisole in order to kill existing worms. After a further 35 days, eels were challenged with 25 L3 and after 139 days finally examined for *A. crassus*. Although the initial repeated infection elicited a clear antibody response (Knopf *et al.*, 2000a), it did not affect the outcome of the challenge infection compared to controls receiving a primary infection with 25 L3.

In European eels, the intraperitoneal injection of homogenized L3 or adult *A. crassus* induced a very uniform antibody response within one week and peaking on days 23 and 33, respectively. However, subsequent challenge infection with 20 L3 on day 84 and examination of swimbladders 120 days p.i. revealed no evidence of protective immunity. The antibody response elicited by the challenge infection was not enhanced or increased as expected for a typical secondary antibody response, but it was similar to that following a primary infection (Knopf, 1999).

Promising results have recently been obtained from a vaccination experiment using irradiated L3 (K. Knopf & R. Lucius, unpublished). Both European and Japanese eels were immunized with 40 irradiated L3 of *A. crassus* and one month later challenged with 40 normal L3. The vaccination resulted in a significant reduction in the number of adult worms developing from the challenge infection in the Japanese eel compared with non-vaccinated controls. In contrast, the number of adult worms in vaccinated European eels did not differ from that in non-vaccinated controls. These preliminary results suggest that acquired immunity to *A. crassus* occurs in the Japanese eel.

In conclusion, experimental evidence for protective immunity in the European eel is still unavailable. Obviously, defence mechanisms against *A. crassus* are less effective in the naïve European eel compared to the original host, the Japanese eel. This difference in immunity might determine differences in the susceptibility of the two eel species to this parasite.

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