Differences in susceptibility of the European eel (*Anguilla anguilla*) and the Japanese eel (*Anguilla japonica*) to the swim-bladder nematode *Anguillicola crassus*

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SUMMARY

The swim-bladder nematode *Anguillicola crassus* originates from the Far East where it is a parasite of the Japanese eel (*Anguilla japonica*). After *A. crassus* was introduced to Europe, it became a predominant parasite of the European eel (*Anguilla anguilla*). A study performed with experimentally infected eels (98 days, 23 °C) revealed significant differences in the susceptibility of the two eel species to this parasite. The recovery rate of 30 administered infective *A. crassus* larvae (L_3) from *A. japonica* was less than half of that from *A. anguilla* (33·2% and 13·8%, respectively). Almost 60% of the worms recovered from *A. japonica* were found as dead, encapsulated and necrotic larvae in the swimbladder wall. In contrast, no dead larvae were found in *A. anguilla*. Additionally, the development of the worms was shown to be significantly slower in *A. japonica* compared with *A. anguilla*. The lower survival rate of the worms, together with their slower development, resulted in a significantly lower adult worm burden (11 and 428 mg wet weight, respectively) and in a decreased reproductive success in *A. japonica* compared with *A. anguilla*. These results demonstrate that the original host, *A. japonica*, possesses more effective defence mechanisms against *A. crassus* than does the non-adapted host, *A. anguilla*.

Key words: *Anguilla anguilla*, *Anguilla japonica*, *Anguillicola crassus*, susceptibility, experimental infection.

INTRODUCTION

The dracunculoid nematode *Anguillicola crassus* was originally endemic to East Asia as a parasite of the Japanese eel, *Anguilla japonica* (Nagasawa, Kim & Hirose, 1994; Taraschewski *et al*. 1987). The parasite's life-cycle involves invertebrate intermediate hosts, chiefly cyclopoid copepods, which ingest the free-living 2nd-stage larvae. Within the haemocoel of the intermediate host, the larvae develop into the 3rd-stage larvae (L_3) that are infective to eel definitive hosts and a wide variety of paratenic hosts *via* predator–prey transmission (Nagasawa *et al*. 1994; Moravec & Skoríková, 1998). Further development to the 4th larval stage (L_4) occurs in the swim-bladder wall of the eel final host. After the final 4th moult, the adult worms dwell in the swim-bladder lumen and feed on their host's blood (Nagasawa *et al*. 1994).

In the early 1980s, *A. crassus* was introduced into Europe, probably with importation of eels from Taiwan into Germany. The first report of this parasite in Europe was in 1982, and in the following years, *A. crassus* proved to be a successful colonizer within the indigenous European eel (*Anguilla anguilla*) populations. Within 10 years it became a dominant parasite of *A. anguilla* over most of Europe (Moravec, 1992; Kirk, 2003), commonly with prevalences of about 80% (e.g. Sures *et al*. 1999). Meanwhile, the parasite had also been reported from North Africa and the eastern coast of North America (Kirk, 2003). The spread of the parasite over the European continent seems only to be limited in Northern Scandinavia by the prevailing low water temperature (Höglund *et al*. 1992; Knopf *et al*. 1998).

Anguillicola crassus was described as a new species by Kuwahara, Niimi & Itagaki (1974), who found the nematode in the swim-bladder of cultivated *A. japonica* and *A. anguilla* in Japan, and mentioned that 'the worms are very commonly found in the air bladder of *A. anguilla*'. Egusa (1979) reported only negligible pathological effects of *A. crassus* on *A. japonica* whereas this parasite caused morbidity among *A. anguilla* cultivated in Japan. According to Egusa (1979), only 10–40% of cultured *A. japonica*, but 'several tens of percent and occasionally nearly 100 percent' of cultured *A. anguilla* were infected with *A. crassus*. Additionally, he stated that 'the number of nematodes per eel is usually one to three, but occasionally attains to about 20' in *A. japonica*, but '5 to some dozen and occasionally exceed 30' in *A. anguilla*. However, to our knowledge, the differences in susceptibility reported by Egusa (1979) have never been investigated experimentally. It was the aim of the present study to evaluate *via* experimental

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infections differences in the susceptibility to *A. crassus* between the original host *A. japonica* and the new host *A. anguilla*.

MATERIALS AND METHODS

Source of eels

Anguilla anguilla weighing about 95 g were obtained from an eel farm free of *Anguillicola crassus* (Domäne Voldagsen, Einbeck, Germany). Some 40 eels were checked to confirm the absence of the parasite. *Anguilla japonica* were imported as glass-eels from Japan and raised in a recirculation system free of *A. crassus* to a weight of about 95 g. Since feeding by metamorphosed young eels begins only at the end of the glass-eel stage and at the beginning of subepidermal pigmentation (Tesch, 1999), an infection of these eels with *A. crassus* was very improbable.

Third-stage larvae of Anguillicola crassus *and infection*

Infective 3rd-stage larvae (L_3) of *A. crassus* were produced and given to the eels *via* intubation as previously described by Knopf *et al.* (1998). Briefly, 2nd-stage larvae (L_2) were collected from the swim-bladder lumen of naturally infected eels originating from Lake Müggelsee, Berlin. Wild-caught planktonic copepods were used as intermediate hosts. After 14 days at 20 °C, L_3 were isolated from the copepods according to the method of Haenen, van Wijngaarden & Borgsteede (1994). For the experimental infection, groups of 30 L_3 were suspended in approximately 100 μl of RPMI-1640 medium (Sigma, Taufkirchen, Germany) in the cavities of a round-bottomed microtitre plate. This larval suspension was orally administered to the eels by means of a stomach tube (1·5 mm diameter). Subsequently, the cavities of the microtitre plate were checked for remaining L_3, in order to determine the exact dose of larvae that had been successfully administered to each eel. Each eel received at least 25 L_3.

Experimental design

The eels were divided into 2 groups of *A. anguilla* and 2 groups of *A. japonica*, each containing 50 eels with a mean body weight of about 95 g, and maintained in 400 l tanks that are part of an experimental recirculation system. After 3 weeks of acclimatization, 1 group of each eel species was experimentally infected with 30 L_3 of *A. crassus* per eel. The other groups of both eel species were sham-infected with the medium, and served as controls. All groups were fed with eel pellet food (European Eel Grower 28, Trouw Nutritor, Putten, Netherlands) at a daily rate of 1·5% of the body weight of the eels. Water temperature was adjusted to 23 °C.

At day 98 post-infection (p.i.) eels were killed by decapitation and the swim-bladder was examined, and living and dead/encapsulated larvae and adults of *A. crassus* in the swim-bladder wall or swim-bladder lumen, were enumerated. Larvae with a body length exceeding 1·5 mm were counted as L_4 (Knopf *et al.* 1998). Male and female adult worms were individually weighed. Nematodes showing no reaction to mechanical stimulation were considered dead. The presence of *A. crassus* eggs in the swim-bladder lumen was evidence of reproduction of the nematodes.

Statistical analysis

Differences in eel mortality, and percentage of eels containing eggs of *A. crassus* in the swim-bladder, between experimental groups were analysed with the χ^2-test. Differences in worm numbers and worm percentages between two experimental groups were analysed using the Mann–Whitney *U*-test. Statistical analysis of sequentially measured values within a group were performed with the Kruskal–Wallis test. Significance was accepted when $P<0{\cdot}05$. Statistical analyses were performed with SPSS 9.0 (SPSS Inc., Chicago, USA).

RESULTS

An ongoing mortality occurred within both groups of *A. anguilla* from the 4th week post-infection until the end of the experiment. At 98 days p.i., mortality was 52% for the group infected with *A. crassus*, and 40% for the uninfected control, showing no significant difference. Most likely, this mortality was caused by a heavy infection with the gill monogenean *Pseudodactylogurus bini* which was introduced into the recirculation system together with the eels. Within both groups of *A. japonica*, all eels survived until the end of the experiment.

Of the experimentally infected *A. japonica*, 10% did not contain larval or adult worms (if not specified, in the text that follows, 'worms' refers to both larvae and adults), 32% had only dead worms, and 58% had living worms. Of the experimentally infected *A. anguilla*, all specimens had living worms. The recovery of administered *A. crassus*, whether as living or dead worms, varied significantly between eel species, being 13·8% in *A. japonica* and 33·2% in *A. anguilla* (Table 1). Dead, encapsulated and necrotic larvae of *A. crassus* in the swim-bladder wall were seen only in *A. japonica*, where they constituted almost 60% of the number of recovered parasites (Table 1). The percentage of living L_3 and L_4, related to the total number of recovered worms, did not differ significantly between the 2 eel species (Table 1). However, the percentage of living L_4 related to the number of living worms, was significantly higher in *A. japonica* than in *A. anguilla* (Table 2).

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Table 1. Fate of 30 L_3 *Anguillicola crassus* administered to individual *Anguilla japonica* and *Anguilla anguilla* at day 98 p.i. (23 °C)

(Expressed as % recovery; % living L_3, L_4, dead larvae, living and dead adults are calculated from the number of recovered worms (whether dead or alive). Numbers of eels are given in parentheses. Asterisks indicate significant differences between the 2 eel species (N.S. = not significant; *** $P<0{\cdot}005$).)

	A. japonica Mean ± S.D.	*A. anguilla* Mean ± S.D.	Significance
% Recovery	13·8 ± 12·5 (50 eels)	33·2 ± 12·8 (24 eels)	***
% Living L_3	2·8 ± 9·2 (45 eels)	1·2 ± 3·2 (24 eels)	N.S.
% Living L_4	10·4 ± 19·3 (45 eels)	4·8 ± 6·6 (24 eels)	N.S.
% Dead larvae	59·4 ± 37·8 (45 eels)	0·0 (24 eels)	***
% Living adults	26·8 ± 35·5 (45 eels)	84·8 ± 17·8 (24 eels)	***
% Dead adults	0·6 ± 3·7 (45 eels)	9·3 ± 16·3 (24 eels)	***

Table 2. Composition of the *Anguillicola crassus* infrapopulation in experimentally infected *Anguilla japonica* and *Anguilla anguilla* at day 98 p.i. (23 °C)

(Calculated from the numbers of living worms; wet weight of individual male and female *A. crassus*, wet weight of all adult worms per swim-bladder, and percentage of eels containing eggs/L2 in the swim-bladder lumen. Sample sizes are given in parentheses. Asterisks indicate significant differences between the 2 eel species (N.S. = not significant; * $P<0{\cdot}05$; *** $P<0{\cdot}005$).)

	A. japonica Mean ± S.D.	*A. anguilla* Mean ± S.D.	Significance
% L_3	9·4 ± 24·6 (29 eels)	1·2 ± 3·3 (24 eels)	N.S.
% L_4	30·8 ± 38·4 (29 eels)	5·5 ± 7·9 (24 eels)	*
% Adults	59·8 ± 42·2 (29 eels)	93·3 ± 8·1 (24 eels)	*
Wet weight of individual males (mg)	1·5 ± 1·4 (26 worms)	15·1 ± 9·4 (107 worms)	***
Wet weight of individual females (mg)	11·8 ± 19·3 (17 worms)	98·3 ± 69·5 (88 worms)	***
Wet weight of all adults per eel (mg)	10·9 ± 19·2 (22 eels)	427·9 ± 276·2 (24 eels)	***
% Eels containing L_2/eggs	2 (50 eels)	88 (24 eels)	***

Whereas only 27% of the recovered worms became adult in *A. japonica*, 94% of the worms became adult in *A. anguilla* during the 98-day experiment (Table 1). Only approximately 1% of the adult worms in *A. japonica* were dead, but about 9% of the adult worms in *A. anguilla* were dead (Table 1). Excluding dead parasites from the calculation, the percentages of adult worms in *A. japonica* and *A. anguilla* were 60% and 93%, respectively (Table 2). The different recovery rates and percentages of adult worms in the 2 eel species correspond to significantly different numbers of adult worms per eel (1·5 ± 1·9 in *A. japonica* and 8·8 ± 3·6 in *A. anguilla*).

The adult *A. crassus* found in the 2 eel species differed significantly in their wet weight. Male and female worms in *A. japonica* were about 10-fold lighter compared to the specimens found in *A. anguilla* (Table 2). The different numbers and wet weight of adult *A. crassus* resulted in an approximately 40-fold lower worm burden (wet weight of adult *A. crassus* per eel) in *A. japonica* than in *A. anguilla* (Table 2). The reproduction of *A. crassus*, indicated by the presence of eggs containing the 2nd stage larvae (L_2), was observed in only 2% of *A. japonica*, but in 88% of the surviving infected *A. anguilla* (Table 2).

DISCUSSION

The findings of the present experimental study strongly indicate that the defence mechanisms of *A. japonica* against *A. crassus* are more effective than those of *A. anguilla*, even during the first infection with this parasite. The recovery of *A. crassus* from *A. japonica* (13·8%) was significantly lower than that in *A. anguilla* (33·2%), and almost 60% of the worms found in *A. japonica* were encapsulated dead larvae in the swim-bladder wall, whereas, no encapsulated larvae were found in *A. anguilla*. Neither encapsulated larvae nor severe pathological changes of the swim-bladder wall have previously been reported for *A. anguilla* experimentally infected with *A. crassus* (e.g. Haenen *et al.* 1989). However, encapsulated and destroyed L_3 and L_4 were found in the swim-bladder wall of wild *A. anguilla* by Molnár *et al.* (1993) and Molnár (1994). Molnár (1994)

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described that, commonly, even in the same swimbladder, some larvae elicited a cellular host reaction, and others did not. He suggested that a successful cellular host response might develop after repeated invasions by larvae, but until now we have no experimental evidence for the development of a protective immunity against *A. crassus* in *A. anguilla*. Although it was possible to characterize the antibody response against *A. crassus* in *A. anguilla* (Knopf *et al.* 2000*a*), it appears that this is elicited only by the adult worms, and it remains unclear if the antibodies have any protective function (Knopf *et al.* 2000*a*).

It remains unclear if, and how, the presence of encapsulated larvae of *A. crassus* is due to protective immune mechanisms, or whether only less active or dead larvae are merely isolated by the host *via* encapsulation. Whyte, Chappell & Secombes (1989) obtained experimental evidence from rainbow trout that killing of the eye-fluke *Diplostomum spathaceum* by activated macrophages, was increased if the target flukes were opsonized with antibodies; and for mammals there are indications that antibody-dependent cell-mediated effector mechanisms are involved in the killing of several pathogenic helminth species (Butterworth, 1984). Assuming that this mechanism might also be effective against *A. crassus*, could explain the low killing capacity of *A. anguilla*, because no larval-specific antibody response could be demonstrated in this eel species (Knopf *et al.* 2000*a*). Würtz & Taraschewski (2000) did not find signs that phagocytes of *A. anguilla* attached to the larvae of *A. crassus* migrating in the host's tissue, and thus concluded that the phagocytic granulocytes and macrophages found around, but not attached to, the larvae probably only remove cell debris caused by their destructive activity.

However, to date, our knowledge about the immune response of eel species to *A. crassus* is limited, because experimental studies on the immune response have only focused on the antibody response of the host (cellular aspects have yet to be evaluated), and most studies concern *A. anguilla*. Although in *A. anguilla* no antibody response has previously been detected against the invasive L_3, a clear antibody response was detected against the outer cuticle of the adult worms (Knopf *et al.* 2000*a*). Cuticular antigen preparations from adult worms have been shown to possess a high specificity for immunoserological tests to investigate the immune response of *A. anguilla* (Höglund & Pilström, 1995; Knopf *et al.* 2000*b*; Sures & Knopf, 2004), and for *A. japonica* (Ushikoshi *et al.* 1999). Nielsen (1999) used an enzyme-linked immunosorbent assay (ELISA), performed with polyclonal antibodies raised against immunoglobulins from *A. anguilla*, to compare the antibody response of *A. japonica* and *A. anguilla* to intraperitoneally injected excretory/secretory (ES) and outer cuticle antigen preparations from adult *A. crassus*. The experiment revealed a higher antibody response of *A. japonica* against ES-antigens from *A. crassus* compared to that of *A. anguilla* against *A. crassus*. Consequently, Nielsen (1999) concluded that differences in intensity of the antibody response might partly explain the assumed different susceptibility of the 2 eel species to infections with *A. crassus* (see Introduction section).

The higher percentage of larval stages found in *A. japonica* than in *A. anguilla* indicates that the development of *A. crassus* is significantly slower in the former eel species. This difference in the duration of development of *A. crassus* in the 2 eel species was also manifest in the approximately 10 times lower wet weight of the few adult worms that were found in *A. japonica* compared to those found in *A. anguilla*. Finally, although the same number of infective larvae were administered to both species of eels, the lower number, and smaller size of adult worms resulted in a lower worm burden and reproductive success of *A. crassus* in *A. japonica* compared to *A. anguilla*.

The more effective defence mechanisms in *A. japonica* than in *A. anguilla*, coupled with the slower development and the slower reproduction of *A. crassus*, can explain the lower prevalence and intensity of this parasite in *A. japonica* compared to *A. anguilla* under aquaculture conditions in Japan (Egusa, 1979). Interestingly, the mean numbers of adult *A. crassus* found in the present study after a single experimentally administered dose of 30 L_3 ($1{\cdot}5 \pm 1{\cdot}9$ in *A. japonica*, $8{\cdot}8 \pm 3{\cdot}6$ in *A. anguilla*), are similar to the numbers mentioned by Egusa (1979) to be usual for naturally infected eels in Japanese aquaculture (1–3 in *A. japonica*, 5 and more in *A. anguilla*). Evidence for density-dependent constraints on the infrapopulation growth of *A. crassus* in *A. anguilla* has been found by Ashworth & Kennedy (1999). They examined naturally infected eels, either immediately post-capture or after maintenance under zero-reinfection conditions in the laboratory, and concluded that an increasing intensity of adult worms leads to a decrease in larval development and maturation of females. A small percentage of dead adult worms (9·3%), as found in our experimentally infected *A. anguilla*, has previously been observed in both experimental studies (Knopf *et al.* 1998) and also in field studies (Kennedy & Fitch, 1990).

From an evolutionary point of view, it appears reasonable that the original host *A. japonica*, has better developed defence mechanisms against *A. crassus* than does the non-adapted host *A. anguilla*. One might assume that during co-evolution, development of both the host's immune system and the parasite, to a condition of mutual tolerance, resulted in a balanced host–parasite system without significant harm to the host. This assumption is supported by the statement of Egusa (1979) that *A. crassus* causes almost 'no serious damage' to *A. japonica*. In contrast, the immune response in the non-adapted *A. anguilla* appears to be less effective, and severe

pathological changes of the swim-bladder wall in eels living under natural conditions are attributed to *A. crassus* (van Banning & Haenen, 1990; Molnár *et al.* 1993; Molnár, Szakolczai & Vetési, 1995; Würtz & Taraschewski, 2000). Several of the observed inflammatory changes, such as dilated blood vessels and infiltration of inflammatory cells, are immunopathological effects, and the question arises if the immune response of *A. anguilla* against *A. crassus* is more harmful than protective.

The 33·2% recovery of administered *A. crassus* in *A. anguilla* at day 98 p.i. was comparable to previous experimental studies with this host–parasite system. Published recovery rates after a single infection with different numbers of L_3 and different durations of the experiment range from 10% to 48%. De Charleroy *et al.* (1990) infected eels with 8 larvae, maintained them for 4 weeks at 16·5 °C, and then recovered 38·2% of the worms. Höglund & Thomas (1992) infected eels with 40 L_3 isolated from paratenic fish hosts (black goby, *Gobius niger*), and recovered 22% of the worms after 8 weeks at 20 °C. Haenen *et al.* (1996) performed single infections with 1–40 L_3 and a water temperature between 18 and 20 °C for 8 weeks. This resulted in recovery rates ranging from 10 to 20%. Knopf *et al.* (1998) recovered 35 and 48% of 20 L_3 in 2 experiments in which eels where maintained for 17 weeks at 19 °C. Furthermore, the percentages of the developmental stages (L_3, L_4 and adult), the wet weight of male and female adult worms and the reproductive success (measured as percentage of eels in which the parasites have reproduced) of *A. crassus* in *A. anguilla* in the present experiment were similar to the results obtained in these previous experiments (Knopf *et al.* 1998). This shows, that experimental infections with *A. crassus* performed under similar conditions produce reproducible results, even if differences between individual eels result in a high variability of the data.

Further insights into the immune response of eels against *A. crassus*, might be obtained by comparative studies with *A. crassus* and the closely related eel species *A. japonica* and *A. anguilla*. The obvious difference between the 2 eel species in their ability to kill the larvae of *A. crassus* offers a promising possibility of investigating the underlying immunological principles. Additional opportunities to extend this approach are possible since the American eel *Anguilla rostrata* is also susceptible to *A. crassus* (Kirk, 2003), and *A. anguilla* is also a suitable host for 2 other *Anguillicola* species, the South African *Anguillicola papernai* (unpublished data), and *Anguillicola novaezelandiae*, that was introduced from New Zealand to Italy (Moravec & Taraschewski, 1988).

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