Infection of the glass-eel swimbladder with the nematode *Anguillicola crassus*

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(*Received 11 December 1999; revised 17 January 2000; accepted 17 January 2000*)

SUMMARY

The ability of the nematode *Anguillicola crassus* to infect eel larvae (glass-eel stage) was tested. The results show that glass-eels fed on infected copepods, the natural intermediate host of the nematode, can be infected. Light microscopical examination of the infected developing swimbladder tissue revealed that the infection results in a significant thickening of the connective tissue. The basolateral labyrinth of gas gland cells is very much reduced in infected swimbladders, and the distance of gas gland cells to blood capillaries is enlarged. Critical swimming speed, defined as the speed where the larvae were no longer able to swim against the current, was similar in infected and uninfected animals. At intermediate speeds (about 60–80 % of critical swimming speed) infected eels showed a slightly higher swimming activity than control animals. Resting oxygen consumption, measured as an index of metabolic activity, within the first 2 months of infection was higher in control animals, which may be due to a reduced rate of activity in infected glass-eels. By 4–5 months after the infection, however, it was significantly higher in infected animals. This may indicate that at this stage a higher activity of the animals is required to compensate for the increase in body density, but swimming performance of infected and non-infected glass-eels was not significantly different. Oxygen consumption during swimming activity, measured in a swim tunnel at 50 % of maximal swimming speed, also was not affected. The results thus show that even glass-eels can be infected with *A. crassus*, and this probably contributes to the rapid spread of the nematode in Europe. While aerobic metabolism during swimming activity is not affected at this stage of infection, the swimbladder tissue shows severe histological changes, which most likely will impair swimbladder function.

Key words: *Anguillicola crassus*, *Anguilla anguilla*, swimbladder, glass-eel, exercise, metabolism.

INTRODUCTION

The nematode *Anguillicola crassus* Kuwahara, Niimi & Itagaki, 1974 (Kuwahara, Niimi & Itagaki, 1974), was originally described as a parasite of the Japanese eel *Anguilla japonica* (Moravec & Taraschewski, 1988). The infection rate of the Japanese eel typically is low, and the viability of this species apparently is not seriously hampered by the infection. In 1982, however, the first encounters of *A. crassus* as a parasite of the European eel in Germany were reported. Within only one decade the parasite spread over large parts of Europe (Neumann, 1985; Moravec, 1992; Evans & Matthews, 1999), and meanwhile was also discovered in the United States (Johnson *et al*. 1995). In contrast to the Japanese eel, in the European eel several tissues and especially the swimbladder tissue are significantly modified following a nematode infection and occasionally even a reduction in viability has been described (Boon *et al*. 1990*a*, *b*; Molnár, Székely & Baska, 1991; Höglund, Andersson & Härdig, 1992; Molnár *et al*. 1993; Würtz, Taraschewski & Pelster, 1996). It is not known what the impact of an infection with *A. crassus* on the spawning migration of the eel is. Although all attempts to trace the spawning migration have failed up to now, it has clearly been shown that the eel, at least occasionally, travels at a depth of several hundred meters (Fricke & Kaese, 1995; Tesch, 1995). The changes that occur during the transition from the yellow eel to the silver eel clearly indicate that the swimbladder is of major importance during this migration (Tesch, 1999).

The infection stage of the nematode for the eel is the larval stage 3 (L3), and these larvae are taken up by eating infected copepods, the intermediate host of the nematode (Haenen *et al*. 1989). In the eel the L3 leaves the gut and penetrates the swimbladder wall. Within the tissue layers of the swimbladder wall the histotrophic nematode larvae develop to larval stage 4 (L4) and to the pre-adult form, which then enters the swimbladder lumen, where the adult nematode feeds on blood from the swimbladder vasculature. Eggs of the parasite hatch within the swimbladder or soon after leaving the swimbladder via the ductus pneumaticus and gut. The infective L2 larvae are taken up by copepods (DeCharleroy *et al*. 1990).

It is generally believed that adult eels have a predatory life-style, although a parasitological study indicated that planktonic animals are ingested by adult eels (Kennedy *et al*. 1992). This, however, does

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https://doi.org/10.1017/S003118209900606X Published online by Cambridge University Press

not exclude the possibility of accidental swallowing of planktonic copepods while feeding on larger prey, or the possibility that infected smaller fish or invertebrates were eaten. Meanwhile, larger crustacea, snails and even small fish and amphibians have been identified as paratenic hosts (Moravec & Konecny, 1994; Székely, 1994; Moravec, 1996; Székely, 1996). The larval form of the eel in the glass-eel stage certainly feeds on copepods. Glass-eels enter the European rivers and streams after a journey of about 3 years from the Sargasso Sea. If the glass-eels could already be infected by *A. crassus*, the eel could be infected at any developmental stage of its life-cycle in freshwater. This certainly would enhance the probability for an infection, and therefore contribute to the rapid spreading of the nematode in the various eel populations. The present study therefore was undertaken in order to test the possibility that glass-eels feeding on copepods can be infected by the nematode *A. crassus*.

MATERIALS AND METHODS

Glass-eels (*Anguilla anguilla*) entering the river Severn in Great Britain were caught during March 1998 and immediately transported to Innsbruck by plane. Glass-eels were kept in a fresh water aquarium at a temperature of 20 °C and fed *Artemia* once a day.

Infection with Anguillicola crassus

Infectious L2 larvae were dissected from the swimbladder of eels caught in the river Rhine near Karlsruhe (Germany). Previous studies revealed that more than 80 % of the eel population of this area is infected with *A. crassus* (Würtz, Knopf & Taraschewski, 1998). Copepods of the species *Cyclops abyssorum* (Sars) were caught with a plankton net in Lake Knielinger near Karlsruhe (Germany) and immediately transferred to Innsbruck. Copepods were kept in a freshwater aquarium and fed with yeast. To infect the copepods they were fed with L2 larvae of *A. crassus* for 2 consecutive days. Success of the copepod infection was checked by light microscopical inspection. The nematode was allowed to develop in the copepods for 3 weeks. After 3 weeks the infected copepods were fed to the glass-eels on 4 consecutive days, and infection was checked by light microscopical inspection of the glass-eels.

Histology

For light histological examination, glass-eels and juvenile eels up to 5 months after infection with *A. crassus* were anaesthetized with 0·1 g/l MS222 and fixed in Bouin's fluid for 1 week or longer. Animals were cut into pieces containing the swimbladder, dehydrated in a standard ethanol series and embedded into paraffin wax. The specimen were cut serially (10 μm) with an Autocut (Reichert-Jung, Austria) and stained with Heidenhain's Azan technique.

Respirometry

Resting oxygen consumption was measured with an intermittent flow respirometer according to the principle described by Forstner (1983) and modified to a 3-chamber system as outlined by Malle (1996). The system consisted of 3 respirometer chambers of 30 ml each and an electrode chamber. Water flow through the chambers was created by 2 water pumps, and multi-path solenoid valves directed the water to the different chambers. The water pumps as well as the multi-path solenoid valves were controlled by a personal computer using a custom made software package (Cyclobios, Innsbruck, Austria). The respirometer chambers were darkened and the arrangement of the solenoid valves and of the electrical pumps allowed all changes in water circulation to be performed without disturbing the animals. The decline in oxygen partial pressure within each of the 3 chambers was measured with an oxygen electrode (YSI) for a period of 15 min once every hour. During this period the first water pump connected the respirometer chamber to the oxygen electrode in a closed system. The decline in water P_{O_2} during the measuring period never exceeded 2–3 kPa. Over the remaining 45 min a slow water current created by the second pump assured full oxygenation of the respirometer chamber. The data were acquired and stored on a personal computer using a custom made software package (DATGRAPH, Cyclobios, Innsbruck, Austria). For each animal data were collected over a period of 24 h. One h before and 1 h after these measurements background respiration (bacterial respiration) of the system was measured. The respirometer was operated in a temperature controlled room at $20 \pm 0{\cdot}5$ °C.

Swimming respirometry

To measure the oxygen consumption during swimming activity a swimming respirometer modified after Brett (1964) was used. Water flow was created with an adjustable pump (Westabo, Typ U2814), and oxygen partial pressure was measured with an oxygen electrode (YSI). The respirometer was submerged in a water bath at a temperature of $20 \pm 0{\cdot}5$ °C. Control of water flow and data acquisition were achieved with a personal computer and custom-made software. Glass-eels are negatively phototactic. In order to enhance the swimming activity of the glass-eels the rear part of the respirometer with the grid at the end was especially illuminated with a 150 Watt lamp.

In a first series of experiments the maximal swimming speed was determined. In each run 5

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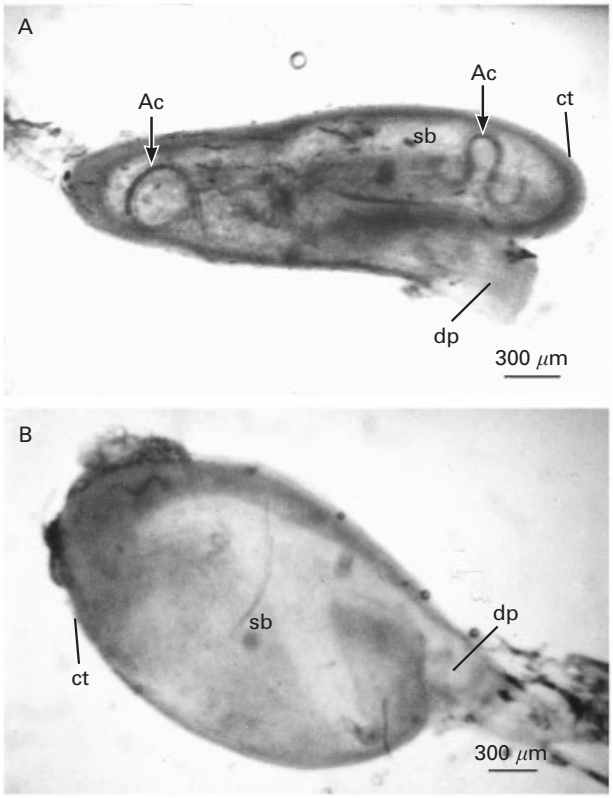

Fig. 1. Infection of a glass-eel swimbladder (sb) with *Anguillicola crassus*. (A) Swimbladder of a glass-eel about 1 week after infection with the nematode *A. crassus*; (B) swimbladder about 6–8 weeks after infection with the nematode. Ac, *A. crassus*; dp, ductus pneumaticus; ct, connective tissue; sb, swimbladder

glass-eels were introduced into the respirometer chamber at an initial water velocity of 6 cm sec^{-1}, which translates to about 1 body length per sec (1 bl sec^{-1}). This speed was maintained for 30 min and thereafter the flow was reduced to 2 cm sec^{-1} (0·33 bl sec^{-1}) for 90 min. This recovery period was followed by a new 30 min test period, in which the water velocity was increased by 1 cm sec^{-1} to 7 cm sec^{-1}. The test period was again followed by a recovery period with a water velocity of 2 cm sec^{-1} for 90 min. In this way the water velocity was increased stepwise in increments of 1 cm sec^{-1}. The maximal swimming speed was determined as the water velocity at which the animals did not swim for more than 60 sec, and at this point the water velocity was reduced to 2 cm sec^{-1} (0·33 bl sec^{-1}). Due to the ever-changing flow conditions reliable measurements of oxygen consumption were not possible in this series.

In a second series, performed 5 months after artificial infection of the animals, infected and non-infected glass-eels were exposed to a water velocity of 7·5 cm sec^{-1} (1·25 bl sec^{-1}), which translates to 50 % of the maximal swimming speed. Water velocity was kept at this speed for 60 min and oxygen consumption was measured continuously. Over the following 60 min the water velocity was reduced to 2 cm sec^{-1} (0·33 bl sec^{-1}), before resuming the next measuring cycle at a velocity of 7·5 cm sec^{-1} (1·25 bl sec^{-1}). This protocol was repeated for 24 h. One h before and 1 h after these measurements background respiration (bacterial respiration) of the system was measured.

Statistical analysis

Data are presented as mean ± S.E.M. Statistical differences between infected and control animals were tested using Student's *t*-test and analysis of variance (software package Statistica). Significance was accepted for $P < 0{\cdot}05$.

RESULTS

Artificial infection of glass-eels with the nematode *A. crassus* was successfully performed by feeding glass-eels with infected copepods. The infection rate was almost 100 %. Light microscopical examination of the swimbladder revealed the presence of *A. crassus* in the swimbladder wall (Fig. 1 A). Within 6 to 8 weeks after infection a proliferation of connective tissue was observed (Fig. 1 B).

Histological examination of the tissue layers revealed a thick lamina propria and largely undifferentiated epithelial cells in non-infected animals. A comparison of an uninfected and an infected swimbladder demonstrated that an infected swimbladder shows a significant proliferation of connective tissue and of the lamina propria. The lumen of the infected swimbladder was severely reduced (Fig. 2 A, B; Table 1). Examination of the various steps of an infection elucidated that the larval stage 3 of the nematode migrates through the various layers of swimbladder tissue. In the early stage of an infection encapsulated larvae were often observed within the connective tissue (Fig. 2 C). In heavily infected swimbladders at a late stage even a rupture of nematodes was observed. As a result almost any structural information on the swimbladder tissue was obscured. The swimbladder lumen was filled with a dark fluid containing parts of nematodes, nematode eggs with developing larvae and decaying swimbladder tissue (Fig. 2 D).

Oxygen consumption was measured as an index of total metabolic activity of glass-eels. Even in long-term measurements over a period of more than 24 h resting oxygen consumption of infected and non-infected glass-eels usually varied between 1 and 4 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Fig. 3 A). This scatter was similar in both groups. Due to the small body mass range of the animals in both groups no correlation between body mass and oxygen consumption was observed, and so an overall mean value could be calculated from the data. Within the first 2 months after infection, non-infected glass-eels on average had an oxygen consumption of $2{\cdot}76 \pm 0{\cdot}93$ $\mu mol \cdot g^{-1} \cdot h^{-1}$ ($n = 27$) compared to $2{\cdot}14 \pm 1{\cdot}0$ $\mu mol \cdot g^{-1} \cdot h^{-1}$

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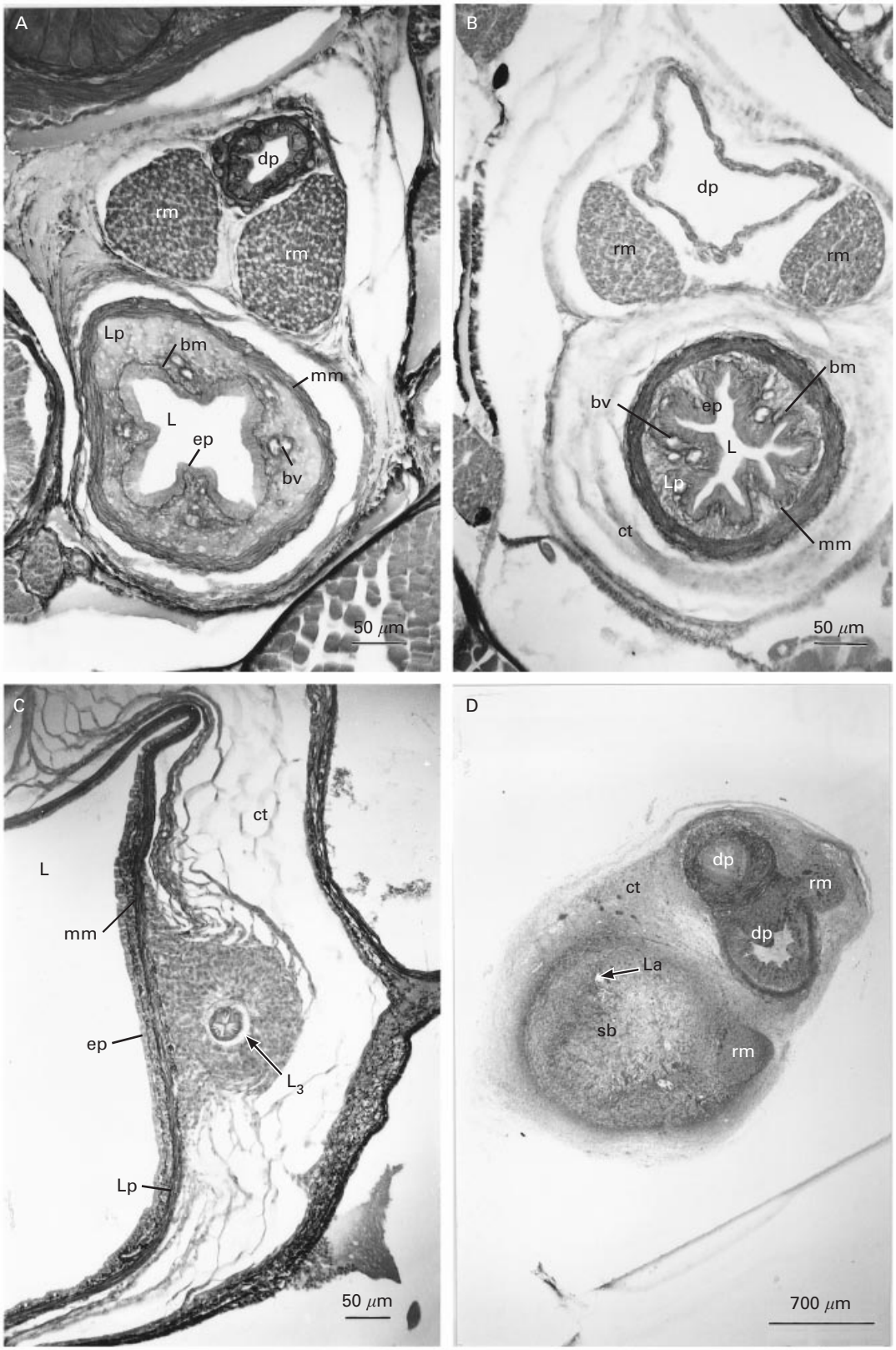

Fig. 2. Light microscopical comparison of an uninfected and an infected swimbladder. (A) Uninfected swimbladder; (B) infected swimbladder which shows proliferated connective tissue (ct). The lumen (L) of the swimbladder is largely reduced. (C) Encapsuled larvae (c) within the connective tissue. (D) Heavily infected swimbladder. The swimbladder lumen was completely filled with a dark fluid containing parts of nematodes, nematode eggs, and decaying swimbladder tissue including blood cells. bm, Basement membrane; bv, blood vessel; dp, ductus pneumaticus; ep, epithelium; mm, lamina muscularis mucosae; La, *Anguillicola* larvae; Lp, lamina propria mucosae; rm, rete mirabile.

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Table 1. Morphometric data of the swimbladder, obtained from exact cross-sections located at 20% of total swimbladder length measured from the distal pole in larvae sampled in early April (early larvae, body mass 0·2–0·3 g) and in infected and non-infected older larvae, sampled 5 months later (September, body mass 0·6–1·2 g)

(Mean ± S.E.; $n = 5$; *, significantly different from early larvae; †, significantly different between infected and non-infected glass-eels.)

	Early larvae	Non-infected	Infected
Sb length (μm)	1260 ± 88	9104 ± 906*	2886 ± 780*†
Area of the lamina propria (μm^2)	6228 ± 1114	17176 ± 3463*	5818 ± 1111†
Area of the lamina submucosa (μm^2)	4732 ± 858	177485 ± 51264*	100848 ± 41068*
Thickness of the epithelium (μm)	11·2 ± 1·1	2·5 ± 0·4*	6·4 ± 1·9†
Epithelial girth (μm)	228 ± 45	2920 ± 372*	1023 ± 268*†
Cross-sectional area of sb lumen (μm^2)	835 ± 207	316092 ± 89550*	32136 ± 15298*†

($n = 29$) in infected glass-eels ($P < 0·05$). By 4–5 months after infection, resting oxygen consumption (Fig. 5B) of non-infected glass-eels was $2·29 \pm 0·74$ $\mu mol \cdot g^{-1} \cdot h^{-1}$ ($n = 26$) compared to $2·94 \pm 1·29$ $\mu mol \cdot g^{-1} \cdot h^{-1}$ ($n = 27$) in infected glass-eels ($P < 0·05$).

In the swimming respirometer glass-eels showed discontinuous swimming behaviour. They swam against the current for some time, then drifted back, swam again, and occasionally even rested for a few seconds at the grid at the end of the swim tunnel. The time spent at the grid at the end of the swim tunnel increased with increasing water velocity. The maximum swimming speed was determined as 15 cm sec^{-1} for infected as well as for non-infected eels. Another important parameter emerging from these experiments was the relative activity of the glass-eels within the respirometer chamber. While at intermediate water velocity infected eels appeared to spend more time swimming than the control eels, near maximum swimming speed there was no difference between the two groups ($P > 0·05$; Fig. 4).

In the swimming respirometer oxygen uptake was measured at the resting water velocity of 0·33 bl sec^{-1} and at an intermediate water velocity of 1·2 bl sec^{-1} (Fig. 5). At resting water velocity both groups had a very similar oxygen consumption rate, and at a water velocity of 7·5 cm sec^{-1} (about 1·2 bl sec^{-1}) oxygen consumption of infected and non-infected glass-eels was significantly higher than at the resting velocity. A comparison of the oxygen uptake of non-infected glass-eels and infected animals at the higher water velocity showed with $8·11 \pm 3·80$ $\mu mol \cdot g^{-1} \cdot h^{-1}$ compared to $6·46 \pm 4·24$ $\mu mol \cdot g^{-1} \cdot h^{-1}$ slightly higher values for the control animals ($n = 11$), but this difference was not significant ($P > 0·05$). At maximum swimming speed the formation of air bubbles within the respirometer chamber could not be avoided, so that a determination of oxygen uptake was not possible.

DISCUSSION

While initially copepods were considered the main intermediate host of *Anguillicola crassus*, recent studies revealed that quite a number of larger animals may also serve as paratenic host. Székely (1994, 1996) reported that several species of fish may serve as paratenic host. Furthermore, several crustacea including gammarids, ostracods, the crayfish *Orconectes limosus* and also several snails can probably be paratenic hosts for the nematode (Kennedy & Fitch, 1990; Thomas & Ollevier, 1992; Moravec & Konecny, 1994; Pazooki & Székely, 1994; Székely, 1995; Moravec, 1996). This study clearly demonstrates that glass-eels entering the European freshwater system can readily be infected with *A. crassus* by feeding on copepods, the intermediate host of the nematode, and copepods are the common food of glass-eels. Thus, the rapid spread of the nematode within the European eel population at least in part can be attributed to the fact that the infectious L3 can use a variety of animals as paratenic host in order to finally reach the eel, and that any developmental stage of the eel in freshwater can be infected.

Histological examination of infected glass-eel swimbladders shows severe damage of the tissue. Already Egusa (1979) reported that the swimbladder of the adult European eel, farmed in Japan, was much more affected by an infection with *A. crassus* than the swimbladder of the Japanese eel. Typical modifications of the swimbladder following an infection include: a proliferation of the epithelial cells forming a pseudostratified layer, hypertrophy of the connective tissue with an increase in collagen

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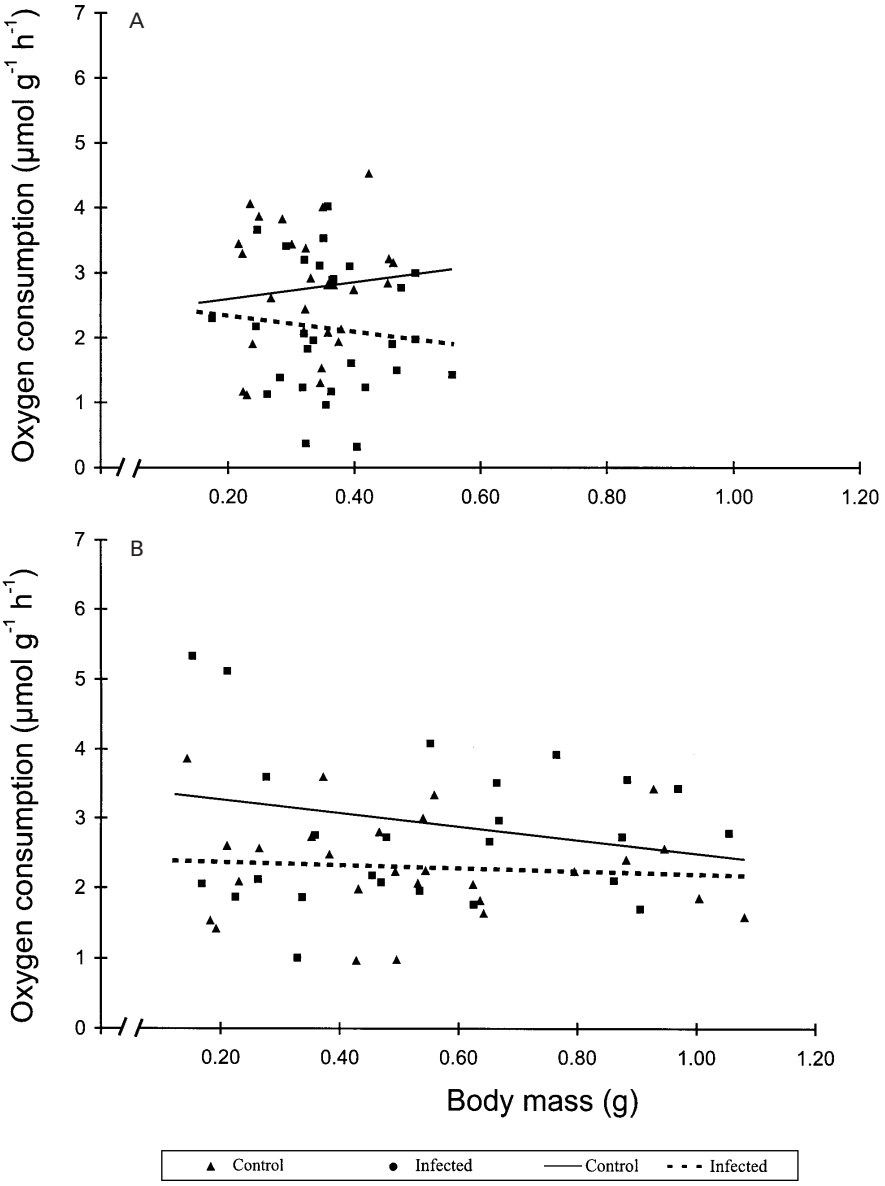

Fig. 3. (A) Oxygen consumption in relation to body weight in control animals and in glass-eels infected with the nematode *Anguillicola crassus* measured 1–2 months after infection. Regression lines: Uninfected: $y = 1{\cdot}29x + 2{\cdot}34$ ($n = 27$); infected: $y = -1{\cdot}24x + 2.59$ ($n = 29$). (B). Oxygen consumption in relation to body weight in control animals and in glass-eels infected with the nematode *A. crassus* measured 4–5 months (B) after infection. Each data point gives the average rate of oxygen uptake of 1 glass-eel measured over a period of 24 h in an intermittent flow respirometer system. Regression lines: Uninfected: $y = -0{\cdot}22x + 2{\cdot}41$ ($n = 26$); infected: $y = -0{\cdot}98x + 3{\cdot}46$ ($n = 27$).

content, accumulation of macrophages, necrotic areas and oedema (Molnár *et al*. 1993; Molnár, Szakolczai & Vetési, 1995; Würtz, 1996). The proliferation of epithelial cells, which are the gas gland cells in the eel, coincides with a reduction of the basolateral labyrinth (Würtz, 1996). These membrane foldings represent a structural prerequisite for the swimbladder function (Pelster, 1997). Transport mechanisms in these membranes are essential for the release of acidic metabolites from gas gland cells into the blood stream, in order to reduce the effective gas transport capacity of the blood (Pelster, 1995). It is not surprising, therefore, that an infection with the nematode *A. crassus* results in a significantly reduced oxygen as well as CO_2 content of the swimbladder (Würtz *et al*. 1996). This demonstrates a significantly reduced gas secreting activity of the swimbladder, and thus indicates an impaired swimbladder function (Piiper, Canfield & Rahn, 1962; Pelster, 1997). Our present results demonstrate that even in the glass-eel, in which the histological differentiation of the swimbladder tissue is not yet completed (Zwerger *et al*. 1999), a severe proliferation of connective tissue and of the lamina propria largely reduce the swimbladder lumen. The histological alterations of the infected glass-eel swimbladder thus are similar to the changes described for the adult swimbladder tissue. Even worse, in a swimbladder containing ruptured nematodes the different layers of swimbladder tissue

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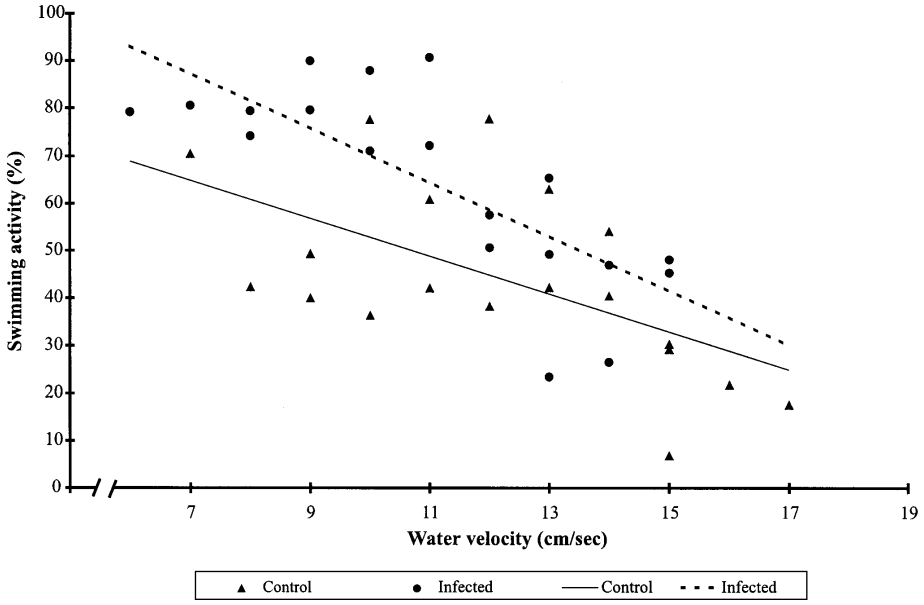

Fig. 4. Swimming activity in relation to the water velocity in control animals (non-infected) and in glass-eels infected with *Anguillicola crassus*. Experiments were performed 5 months after infection of the glass-eels. Regression lines: Uninfected: $y = -3{\cdot}99x + 92{\cdot}8$ ($n = 19$); infected: $y = -5{\cdot}74x + 127{\cdot}5$ ($n = 19$).

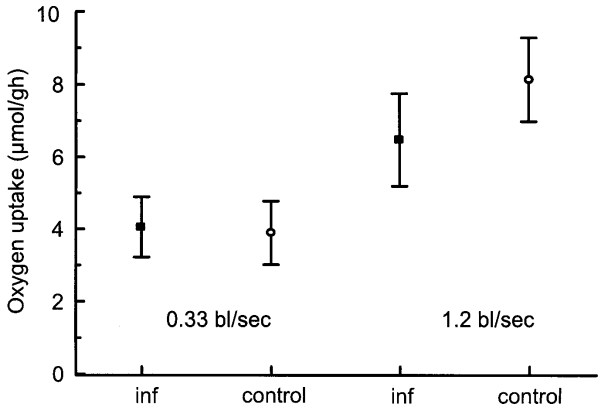

Fig. 5. Oxygen uptake of glass-eels measured in the swimming respirometer 5 months after infection at a resting water velocity of 0·33 bl sec^{-1} and at an intermediate water velocity of 1·2 bl sec^{-1}. Compared to resting water velocity oxygen consumption was significantly elevated at intermediate water velocities ($P < 0{\cdot}05$), but no difference was observed between infected and non-infected glass-eels.

completely disappear and decaying tissue becomes visible. Almost no gas is present in such a swimbladder. Based on the impairment of swimbladder function known from infected adult eels, we conclude that the infected glass-eel swimbladder can hardly function as a hydrostatic organ, and that the proper differentiation of the swimbladder tissue, which occurs only after the glass-eels enter the freshwater system (Zwerger *et al*. 1999) cannot occur with an infection with the nematode *A. crassus*, or is at least severely impaired.

Although the histological examination revealed severe damage to the swimbladder tissue, we did not observe an increase in mortality in infected glass-eels. A reduced stress resistance of infected eels and even an increased mortality has been observed in adult eels (Van Willigen & Dekker, 1989). The initial spread of the nematode indeed caused a significant mortality in the eel population in Lake Balaton (Molnár *et al*. 1991). This may indicate that the physiological performance of the developing glass-eel is not as much affected by the parasite as the performance of the adult eels.

This conclusion is supported by our metabolic studies and by the swim tunnel experiments. Oxygen consumption neither at rest nor during swimming activity revealed a clear-cut difference between infected and non-infected animals. Oxygen consumption of infected eels was slightly, but significantly lower at about 2 months after infection, but 4–5 months after infection it was significantly higher. These differences and especially the changes occurring between 2 and 4–5 months are not easily explained, especially if the scatter of the data is taken into account. One may speculate that the infection in an early state of infection results in a reduced activity of the glass-eels, and thus a reduced rate of oxygen consumption. In a later state of infection, a higher activity of the animals is required to compensate for the increase in body density, but swimming performance of infected and non-infected glass-eels was not significantly different. In spite of the fact that the glass-eels were totally undisturbed in the remote controlled respirometer for more than 24 h the variability in oxygen consumption was remarkable. It may well be that this obscured a possible effect of the parasite. On the other hand, if the normal variability of the metabolic activity is so high, a minor effect induced by the parasite can hardly be of major physiological significance. We therefore con-

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clude that the metabolic activity of glass-eels is not severely affected by the infection with *A. crassus*. In contrast, in adult eels infected with more than 10 nematodes an 18·6% reduction in maximum swimming speed was observed (Sprengel & Lüchtenberg, 1991).

While the infection of adult eels with the nematode *A. crassus* by feeding on various intermediate and paratenic hosts is well established, the results of the present study clearly show that glass-eels can be infected by feeding on infected copepods, and this may significantly contribute to the rapid spread of the nematode all over Europe. The infection significantly alters the histology of the swimbladder tissue and most likely impairs the ability of the swimbladder to act as a hydrostatic organ. Metabolic activity, as measured by total oxygen consumption, and also swimming activity are not immediately modified by the nematode infection. Based on the severe damage to the swimbladder tissue we conclude that the infection in the long run will reduce the viability of the eels. This may contribute to the significantly reduced catches of eels within recent years.

The study was financially supported by the Jubiläumsfonds der Oesterreichischen Nationalbank (OENB, Nr. 6973).

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