Induction of stress by the swimbladder nematode *Anguillicola crassus* in European eels, *Anguilla anguilla*, after repeated experimental infection

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(*Received 4 August 2000; revised 23 November 2000 and 22 February 2001; accepted 22 February 2001*)

SUMMARY

The purpose of this study was to determine under laboratory conditions over a period of 311 days if infection with the nematode *Anguillicola crassus* induces stress in European eels (*Anguilla anguilla*), and stimulates the endocrine stress axis as measured by serum cortisol levels. Eels were experimentally infected with 3rd-stage larvae (L_3) in different doses to simulate natural conditions with varying infection pressures. Blood samples were drawn from the caudal vein every 2 weeks and serum cortisol concentrations were determined by radioimmunoassay (RIA). The results showed that the application of L_3 resulted in a significant increase in the cortisol levels. The period of time at which elevated cortisol values were observed was consistent with the time of larval development and the appearance of adult *A. crassus*. Thus, there is a stress response to the larval and young adult stages, but no chronic response to older adults. Therefore, it is likely that infection of eels under natural conditions especially with a high number of larvae may be a considerable stressor, in combination with different environmental factors like water temperature, pH, oxygen concentration, pollution and inter-individual relationships.

Key words: *Anguillicola crassus*, stress, cortisol, eel, *Anguilla anguilla*.

INTRODUCTION

Anguillicola crassus originating from the Far East is a natural parasite of the Japanese eel *Anguilla japonica*. It successfully spread to Europe and attained a high prevalence within the population of the indigenous European eel *Anguilla anguilla* (Taraschewski *et al*. 1987; Moravec & Taraschewski, 1988; Würtz, Knopf & Taraschewski, 1988; Sures *et al*. 1999*a*). In addition to the numerous reports on the spread and development of *A. crassus* in European eels, there is also increasing interest in the harmful effects of this parasite on its host. Following the massive eel mortality that took place in Lake Balaton, Hungary in July 1991 (Molnár, Székely & Baska, 1991) pathology and histopathology studies were conducted.

Thickening of the swimbladder wall, inflammation, infiltration of white blood cells, fibrosis and changes in the epithelial cells are the most frequent histopathological changes in infected swimbladders (Van Banning & Haenen, 1990; Molnár *et al*. 1993; Hartmann, 1994; Molnár 1994; Molnár, Szakolczai & Vetési, 1995; Würtz & Taraschewski, 2000). Additionally, infestation with *A. crassus* appears to make the fish more susceptible to secondary bacterial infections (Van Banning & Haenen, 1990). In aquaculture, heavily infected European eels show reduced growth and increased mortality (Køie, 1991). Thus, *A. crassus* is an economic concern in eel farming, and also a serious threat to the population of European eels. Eels with severe swimbladder lesions are likely to be incapable of migrating to their spawning grounds (Würtz, Taraschewski & Pelster, 1996).

Despite the pathogenicity of *A. crassus*, few studies have been conducted on the immunological response of eels (Knopf *et al*. 2000*a*, *b*). In naturally infected eels, specific antibodies against antigens from the adult nematode were detected using immuno-serological methods (Buchmann, Pederson & Glamann, 1991; Höglund & Pilström, 1994, 1995; Haenen *et al*. 1996), and some adult worm antigens were found to be subunits of a glutathione-s-transferase (Nielsen & Buchmann, 1997). Since the immune system and the neuro-endocrine system influence and condition each other (Ellis, 1981), it is important to consider the role that stress may play in the immune response to *A. crassus*. One of the most important stress hormones of fish, the corticosteroid cortisol, has been shown to increase the susceptibility of fish to fungal, bacterial and parasitic infection (Pickering, 1981; Pickering & Durston, 1983; Woo, Leatherland

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DOI: 10.1017/S003118200100823X Printed in the United Kingdom

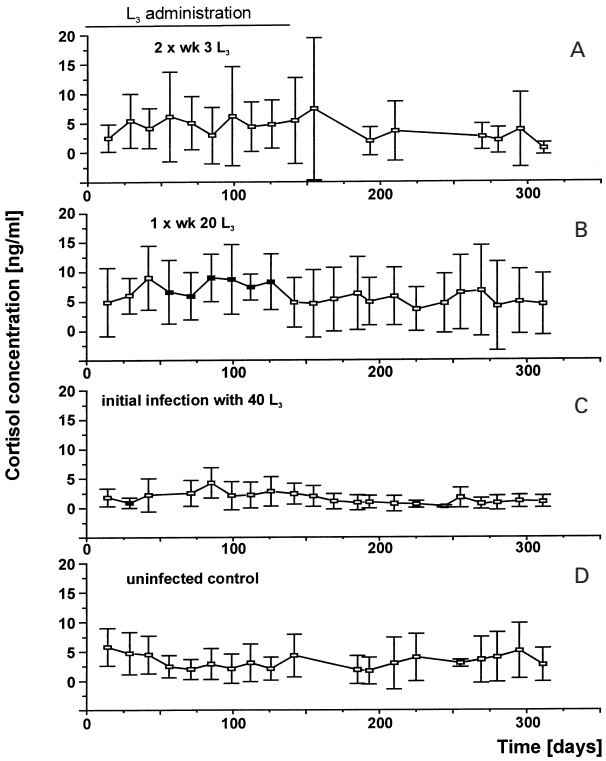

Fig. 1. Course of mean cortisol concentrations ($\bar{x} \pm$ S.D.) in eels following different treatments. (A) Eels infected twice weekly with 3 larvae of *Anguillicola crassus* for 20 weeks ($n = 8$); (B) eels infected weekly with 20 larvae of *A. crassus* for 20 weeks ($n = 8$); (C) eels initially infected with 40 larvae of *A. crassus* ($n = 6$); (D) uninfected controls ($n = 9$). Closed symbols: significant difference from controls (*U*-test, $P \leqslant 0{\cdot}05$); open symbols: no significant difference from controls.

& Lee, 1987; Pickering & Pottinger, 1989). In a recent study of wild European eels naturally infected with *A. crassus*, Kelly, Kennedy & Brown (2000) did not find any significant impact of the nematode on the cortisol levels of the host and concluded that the fish show no chronic stress response. The purpose of this study was to determine if infection with the nematode *A. crassus* induces a stress response to European eels, and stimulates the endocrine stress-axis as measured by serum cortisol levels.

MATERIALS AND METHODS

Source, maintenance and infection of eels

European eels weighing 69·0 g $\pm$ 20·9 g (mean $\pm$ S.D.) were obtained from a commercial eel farm (Limnotherm, Bergheim, Germany) known to be free of *A. crassus* (see Würtz *et al.* 1996; Knopf *et al.* 1998; Sures, Knopf & Taraschewski, 1999*b*; Würtz & Taraschewski, 2000). The absence of *A. crassus* was confirmed by necroscopy of 15 eels. All eels were kept individually in 40 l compartments, equipped with a polypropylene tube serving as hiding-place. Aerated tap water of 20 °C was supplied via a flow through system. The eels were fed twice a week with pellet food at a rate of 0·7 g per eel. Eels were allowed to acclimatize for 2 weeks prior to the experimental procedure.

Eels were experimentally infected with infective 3rd-stage larvae (L_3) of *A. crassus*. L_3 were obtained by feeding 2nd-stage larvae (L_2) collected from the swimbladder lumen of naturally infected eels to planktonic copepods, mainly comprising *Thermocyclops cf. crassus* and *Mesocyclops leuckarti* (Knopf *et al.* 1998). The 3rd-stage larvae were isolated 20 days p.i. from the intermediate hosts by the potter method described by Haenen, Wijngaarden & Borgsteede (1994) and stored in RPMI-1640 medium (Sigma, Deisenhofen, Germany) containing 0·2% Kanamycin at 4 °C until application. The L_3 were counted in a round bottom 98-well plate and suspended in approximately 100 µl of RPMI-1640 medium. This suspension was introduced into the stomachs of each eel, using a 1 ml syringe fitted with a 12 cm length of 1·5 mm diameter plastic tubing.

Experimental design

Eels were divided into 4 groups for the experimental infections. One group of 6 eels received only an initial dose of 40 L_3 at the beginning of the experiment. Two other groups of 8 eels were repeatedly infected with either 3 L_3 twice a week, or with 20 L_3 once a week, for 140 days to simulate different natural infection regimes with high infection pressure. Another group consisting of 8 eels was used as control and these eels were sham infected, by dosing with 100 µl of RPMI-1640 medium twice a week. Infection of the eels as well as blood sampling was finished within 4 min after the eels were caught by net, to minimize the effects of handling on the physiological parameters. For both procedures fish were not sedated which was in accordance with local animal welfare regulations. Eels were maintained under these conditions for 311 days.

Determination of cortisol

Blood samples of 150 µl were drawn from the caudal vein of unsedated eels every 2 weeks. Blood was allowed to clot for 1 h at 20 °C, centrifuged for 5 min at 2000 ***g*** and sera were collected and stored at −70 °C until analysis by a radio-immunoassay (RIA). Cortisol was extracted by adding 1000 µl of 96% ethanol to 40 µl of serum, this mixture was centrifuged for 2 min at 10000 ***g*** and the organic phase including cortisol, was carried over in open tubes. Overnight the ethanol was allowed to evaporate. The following day, 1000 µl of 5% ethanol were added to each sample to dissolve the cortisol again. The sample was divided into 3 aliquots each

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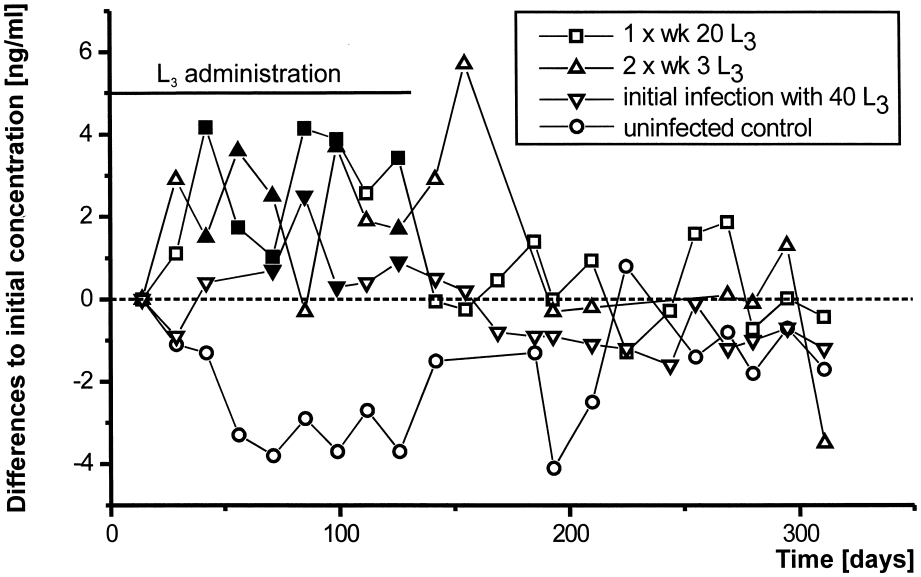

Fig. 2. Differences to basal cortisol levels in eels following different treatments. (○) Uninfected controls ($n = 8$); (△) eels infected initially with 40 larvae of *Anguillicola crassus* ($n = 6$); (▽) eels infected twice weekly with 3 larvae of *A. crassus* for 20 weeks ($n = 8$); (□) eels infected weekly with 20 larvae of *A. crassus* for 20 weeks ($n = 8$). Closed symbols: significant difference from controls (*U*-test, $P \leqslant 0{\cdot}05$); open symbols: no significant difference from controls.

containing 300 µl of extract, to which 100 µl of tritium-labelled cortisol (3000 c.p.m) and 500 µl of cortisol antiserum (dilution 1:10000 in lysocym buffer B) according to the method of Kloas, Reinecke & Hanke (1994) were added; these were then held on ice for about 3 h. To isolate the antibody–hormone complex 100 µl of dextran-activated carbon suspension were added. After centrifugation, the supernatant containing the antibody–hormone complex, was transferred to scintillation vials and filled with scintillation solution (Ultima Gold; Packard, Dreieich, Germany). A liquid scintillation counter (Tri Carb 1900 T; Packard Dreieich, Germany) was used according to the method described by Kloas *et al*. (1994).

Statistical analysis

Mean concentrations (ng/ml serum) of cortisol ($\bar{x} \pm$ S.D.) are presented in Fig. 1. Cortisol concentrations between all groups were analysed for each sampling time with the Kruskal–Wallis test. The Mann–Whitney *U*-test was used to test for significant differences between each group of infected eels and the control if significant differences between all groups were confirmed by the Kruskal–Wallis test. Statistically significant differences between successive cortisol concentrations within each experimental group were evaluated with the Wilcoxon test. Significance was accepted when $P < 0{\cdot}05$.

After initial values (14 days after initial infection) were tested for homogeneity, variations between individual cortisol concentrations at day 14 were compensated by subtraction of each value during the study by the initial value and plotted in Fig. 2.

RESULTS

Mean initial cortisol concentrations for each treatment ranged between 1·8 ng/ml and 5·8 ng/ml (Fig. 1). Although there was considerable variation in mean cortisol concentrations, statistical analysis revealed significant differences. Whereas initial concentrations at day 14 were not different between all groups, cortisol levels in the eels repeatedly infected with 20 L_3 were significantly higher compared to the controls from day 56 to day 126 (Fig. 1). Additionally, the eels infected on 1 occasion only had significantly lower cortisol concentrations at day 29 compared to the uninfected controls (Fig. 1). Comparing the controls and the eels infected repeatedly twice weekly, no significant differences were found.

Statistical analysis of successive data within each treatment group revealed significant changes of cortisol concentrations. Eels infected twice weekly with 3 L_3 showed a significant increase in the mean cortisol concentration from $2{\cdot}5 \pm 2{\cdot}3$ ng/ml to $5{\cdot}4 \pm 4{\cdot}6$ ng/ml between days 14 and 29. The mean cortisol levels in the eels repeatedly infected with 20 L_3 showed a significant elevation from $6{\cdot}0 \pm 3{\cdot}0$ ng/ml to $9{\cdot}0 \pm 5{\cdot}9$ ng/ml between days 29 and 42. Between days 71 and 85 the cortisol levels of the initially infected eels were significantly elevated from $2{\cdot}5 \pm 2{\cdot}2$ ng/ml to $4{\cdot}3 \pm 2{\cdot}6$ ng/ml. The cortisol levels of the control eels which were not inoculated with larvae did not show any significant change over the experimental period.

Calculation of the differences of each value during the study and the initial value for each individual fish (Fig. 2) visualizes the increased release of cortisol in infected eels at the beginning of the experiment (day

https://doi.org/10.1017/S003118200100823X Published online by Cambridge University Press

14 to approx. day 150). In contrast, values of the control eels decreased. Subsequently cortisol values returned to the base level in all groups (Fig. 2).

DISCUSSION

The dominant role of plasma corticosteroids, mainly cortisol, as primary messengers of a stress response in teleostean fish is generally recognized (Barton & Iwama, 1991; Wendelaar Bonga, 1997, Kloas, 1999). A mean basal level for cortisol in eels of 3·9 ng/ml in the present study falls in the range of 2 to 5 ng/ml mentioned in a review by Barton & Iwama (1991). Elevation of plasma cortisol was recorded for each experimentally infected group of eels. However, the mean cortisol concentrations were only slightly elevated considering that an acute stress response in European eel following emersion and handling resulted in a post-stress cortisol concentration of 108 ng/ml (Gilham & Baker, 1985). In salmonid fish an acute stress even leads to a temporary increase of cortisol concentrations from a pre-stress level of less than 5 ng/ml to 40–200 ng/ml (Pickering & Pottinger, 1989). However, chronic stress resulted in an increase of plasma cortisol levels to approximately 10 ng/ml (Pickering & Pottinger, 1989). This is concurrent with our results, where mean cortisol concentrations increased from 4·9 to 9·0 ng/ml in the eels infected with most worms (repeatedly with 20 L_3). Although the stress response of eels due to infection with *A. crassus* is hardly perceptible regarding the mean cortisol concentrations, calculation of the differences with basal cortisol levels illustrates a positive stress response of infected eels. Decreasing cortisol levels in the controls during the same time can be explained by adaptation of eels to the experimental conditions.

Since 50 to 80 days are necessary for *A. crassus* to develop from the L_3 stage into adult worms at a water temperature of about 20 °C (De Charleroy *et al.* 1990; Knopf *et al.* 1998; Sures *et al.* 1999*b*), the time of significant elevations of cortisol levels within each of the 3 experimentally infected groups following day 14, day 29 and day 71, respectively, shows that definitely L_3, but probably also young adults of *A. crassus* elicit a stress response in the eels. The intensity of cortisol release appeared dependent on the dose of infection. Eels which were infected with most worms (repeatedly with 20 L_3) showed the most obvious reaction, characterized by an early and significant elevation of the cortisol levels and significantly higher values compared to the control eels over 70 days. Thus, our data show that an infection of eels with larvae results in a slight, but significant increase of the cortisol levels. However, the stress response seems to be limited to the time of larval development and the first weeks of adult *A. crassus*. No elevated cortisol levels were detected in the present study when the adult worms were older than approximately 60 days. This agrees with the results of Kelly *et al.* (2000) who did not find any stress response of eels due to *A. crassus*. The authors sampled naturally infected eels from the field, maintained them under laboratory conditions and compared their cortisol levels with those of uninfected wild control eels treated in the same manner. No correlations were found between load of adult *A. crassus* in the lumen, or of larvae in the wall of the swimbladder and any of the physiological parameters investigated, including cortisol. But in contrast to our experimental infection, Kelly *et al.* (2000) did not know the age of the worms and the time of infection, and concluded that there is little evidence of an adverse effect of chronic *A. crassus* infections on the physiological status of wild European eels. However, Kelly *et al.* (2000) suggested that the eels had already recovered from the stress resulting from an acute infection at the time their blood was analysed for cortisol concentrations. This suggestion is supported by our results, which show a stress response to the larval and young adult stages, but no chronic response to older adults.

Compared to naturally infected eels, the use of uninfected eels which were experimentally inoculated is advantageous for determining the impact of an infection on the stress status since the development of the infection is known. However, the experimentally inoculated eels may have been less stressed than wild eels due to the comparatively benign laboratory conditions. For example no inter-individual stress could occur as all eels were kept in separate compartments. In their natural environment, eels are confronted with a wide variety of factors that influence their physiological homoeostasis or even threaten their life. Consequently, mean cortisol concentrations determined by Kelly *et al.* (2000) were slightly higher than the concentrations determined in the present study.

Barton & Iwama (1991) emphasized that 'the cumulative effect of stress may … manifest itself at the fish population level and may be outwardly apparent as significant mortality'. In salmonids, even a slight but chronic elevation of plasma cortisol from 1–2 ng/ml to only 10 ng/ml is sufficient to depress disease resistance (Pickering & Pottinger, 1989). On the other hand studies on the combined effects of simultaneous parasite infections and pollution, revealed a higher susceptibility of parasitized hosts to environmental pollutants (reviewed by MacKenzie *et al.* 1995; Lafferty, 1997; Sures, Siddall & Taraschewski, 1999*c*). Thus it is likely that under natural conditions infection of eels with a high number of *A. crassus* larvae may be a considerable stressor in combination with environmental factors such as water temperature, pH, oxygen concentration, pollution and inter-individual relationships. Experimental work confirmed that *A. crassus* infection impairs the resistance of eels to

https://doi.org/10.1017/S003118200100823X Published online by Cambridge University Press

oxygen deprivation and high temperature (Molnár, 1993). This became evident during the mass mortality of eels in Lake Balaton, Hungary, in summer 1991 (Molnár *et al*. 1991) which probably was caused by the coincidence of high water temperature, low oxygen content, a high prevalence of *A. crassus* and secondary bacterial infections (Molnár, 1993; Békési, Hornok & Székely, 1997).

Due to the insufficient knowledge on possible antagonistic or synergistic effects of parasitic infections and environmental factors on the physiological status of the hosts further laboratory studies are necessary. The host–parasite combination European eel–*A. crassus* seems to be a useful model to investigate these factors.

Thanks are due to Dr Sarah Poynton for revision of the English.

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