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Géraldine Fazio, Pierre Sasal, Gabriel Mouahid, Raymonde Lecomte-Finiger, Hélène Moné. Swim Bladder Nematodes (Anguillicoloides crassus) Disturb Silvering In European Eels (Anguilla anguilla).. Journal of Parasitology, 2012, 98 (4), pp.695-705. 10.1645/GE-2700.1 . halsde-00728919

HAL Id: halsde-00728919
https://hal.science/halsde-00728919

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J. Parasitol., 98(4), 2012, pp. 695–705
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SWIM BLADDER NEMATODES (*ANGUILLICOLOIDES CRASSUS*) DISTURB SILVERING IN EUROPEAN EELS (*ANGUILLA ANGUILLA*)

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ABSTRACT: The introduced parasite *Anguillicoloides crassus* is thought to play an important role in the decline of freshwater eel (*Anguilla* spp.) populations. These nematodes are known to negatively affect many fitness-related traits in eels. We used experimental infections to study the effect of *A. crassus* on the relative size or mass of organs, and the expression of functionally relevant genes (total of 12 parameters) that are involved in the silvering process of *Anguilla anguilla*. Our results showed that the liver mass, the hemoglobin α-chain, and androgen receptors α expression levels were significantly higher in infected eels, whereas the freshwater rod opsin expression level and the gut mass were significantly lower in infected eels. Our results suggested that infected eels were at a more advanced stage in the silvering process than uninfected counterparts of similar size. These results may be explained by 2 hypotheses. First, *A. crassus* could trigger physiological mechanisms involved in the silvering process as a side-effect of infection. Second, eels may adjust their life history traits in response to infection. The implications for eel migration and reproductive success may be either negative or positive, depending on whether the response to *A. crassus* infection results in an additional cost of the parasite or is due to the phenotypic plasticity of the host.

The European eel, *Anguilla anguilla* (L.), along with the other species of the genus, are catadromous fishes, indicating that migrations between different aquatic environments are included in their life history. After a growing phase of several years in continental waters, the yellow eels metamorphose in 5 to 6 mo into silver eels, which migrate downstream back to the Atlantic Ocean to begin their reproductive migration to the Sargasso Sea (reviewed by van Ginneken and Maes, 2005). This metamorphosis, called silvering, is a crucial step in the eel life cycle in that it sets the functional adaptations to perform a 6,000-km oceanic migration to the spawning area in high-pressure conditions. These multiple, drastic changes, which are both morpho-anatomic and physiologic, can be divided into 4 categories corresponding to the different aspects of the silvering process. First, adaptation to the marine environment involves modification of vision, i.e., enlargement of the eye area and a switch in visual pigments (Wood and Partridge, 1993; Archer et al., 1995; Zhang et al., 2000). Additionally, it includes adaptation of osmoregulation to the marine ecosystem, which is mediated, at the molecular level, by the active transport of ions and water in the secretory/absorptive epithelia of the gills, intestine, kidneys, and urinary bladder (Cutler et al., 1996; Hirose et al., 2003). Second, cessation of growth triggers the closing and further degeneration of the alimentary tract (Pankhurst and Sorensen, 1983; Tesch, 2003). Third, preparation of the spawning migration embraces morphological and physiological changes that allow long and sustained swimming at variable depth. This includes pectoral fin enlargement, fat accumulation, and increase in muscle oxygenation. Finally, there is an initiation of gonad maturation (Larsen and Dufour, 1993).

Several eel species, but especially *Anguilla anguilla*, *Anguilla rostrata*, and *Anguilla japonica*, have suffered a steep decline throughout their distribution range since the early 1980s (Tseng et al., 2003; Wirth and Bernatchez, 2003; EIFAC/ICES, 2007). Explanations for these declines include climate change, freshwater habitat destruction, physical obstructions to migration, pollution, over fishing, and disease. In this context, the quality of the spawners has become a major priority in species management (EELREP, 2005). Silver eels infected with EVEX (Eel Virus European X) died after 1,000–1,500 km during simulated migration in swim tunnels (van Ginneken et al., 2005). However, doubts remain about the effect of the nematode *Anguillicoloides crassus* (formerly *Anguillicola crassus*) on the physiology of silver stages and subsequent ability to migrate to the spawning area. This parasite was accidentally introduced into Europe in the early 1980s via importation of infected eels from Taiwan and then rapidly spread through farmed and wild populations of *A. anguilla* in Europe (for synthesis, see Kirk, 2003). The hematophagous nematode causes severe histopathology in the swim bladder and impairment of gas secretion (Molnár et al., 1993; Haenen et al., 1996; Würtz et al., 1996), strongly suggesting that the function of the swim bladder as a buoyancy and hydrostatic organ is damaged as a result of heavy and/or recurrent infections. However, experimental studies on swimming performance and resistance to high pressure, which are both crucial in migrating silver stages, have given contrasting results, showing either that *A. crassus* reduces the swimming performance of their hosts significantly (Sprengel and Lüchtenberg, 1991; Palstra et al., 2007) or that it has no influence on this host trait (Vettier et al., 2003; Münderle et al., 2004). In addition, 3 of these studies were performed with yellow eels, and confirmation of the results is needed for silver eels. In point of fact, the effect of *A. crassus* on the silvering process itself has never been investigated under experimental conditions.

Proteomics and genomics are rapidly expanding disciplines that may give a new dimension to host–parasite interaction studies (Biron et al., 2005). For example, differential gene expression has been used recently in fish–parasite models to investigate the global processes underlying host susceptibility or resistance (Collins et al., 2007; Severin and El-Matbouli, 2007; Baerwald et al., 2008) and to estimate the expression of specific immune genes related to pathogen and parasite infections (Fast et al., 2006; Faliex et al., 2008; Sitjà-Bobadilla et al., 2008). In both cases, gene expression appeared to be a powerful tool for analyzing the physiological response of a host infected by a given parasite. In a previous study conducted on naturally infected eels, we analyzed, among other things, the relationships between the macroparasite community of the European eel and the expression of the freshwater and deep-sea rod opsin genes, which code for the visual pigments involved in

Received 15 November 2010; revised 25 January 2012, 9 March 2012; accepted 9 March 2012.
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DOI: 10.1645/GE-2700.1

TABLE I. Silvering's parameters investigated in this study.

Silvering's aspect	Organ	Level	Parameter*	Pattern of change	Standardized estimator†	Reference
Adaptation to the marine environment	Eyes	Morpho-anatomic	Area	Increase	I_O	Pankhurst, 1982
		Physiological	Expression of FWO gene	Decrease	None	Zhang et al., 2000
	Gills and intestine	Physiological	Expression of NKAβ1 gene	Increase	None	Kalujnaia et al., 2007
	Gills	Physiological	Expression of NKCC1a gene	Increase	None	Kalujnaia et al., 2007
Growth stop	Alimentary tract	Morpho-anatomic	Mass	Decrease	I_{GU}	Pankhurst and Sorensen, 1983
Preparation to the spawning migration	Pectoral fin	Morpho-anatomic	Length	Increase	I_F	Durif et al., 2005
	Body	Morpho-anatomic	Mass	Increase	K	Larsson et al., 1990
	Liver	Morpho-anatomic	Mass	Increase	I_H	Durif et al., 2005
	Kidney	Physiological	Expression of Hbα gene	Increase	None	Johansson et al., 1974
Sexual maturation	Testes	Physiological	Expression of ARα gene	Increase	None	Miura and Miura, 2003
		Physiological	Expression of activin B gene	Increase	None	Miura and Miura, 2003

* FWO = freshwater rod opsin; NKAβ1 = Na^+/K^+-ATPase β1; NKCC1a = $Na^+/K^+/2Cl^-$ cotransporter; Hbα = hemoglobin α-chain; ARα = androgen receptors α.
† I_O = ocular index; I_{GU} = gut index; I_F = fin index; K = Fulton's condition factor; I_H = Hepatosomatic index.

TABLE II. Sequences, amplicon sizes, annealing temperature, and extension time of the primers used for target gene amplification.

Target gene	Accession no.	Target organ	Primer*	Sequence	Amplicon size (bp)	Annealing temp. (°C)	Extension time (sec)
FWO	AJ249202	Eyes	FWO forward	5′-CATCTCATTCCTGGTC-3′	319	60	17
			FWO reverse	5′-TCGTGGTAATATGCCGTG-3′			
NKAβ1	AJ239317	Gills and intestine	NKAβ1 forward	5′-CACAATGAACACTGAGCTGC-3′	157	62	8
			NKAβ1 reverse	5′-GAAATGGGGGAGAGAGAAAG-3′			
NKCC1a	AJ486858	Gills	NKCC1a forward	5′-CACTGGGATTGTGTATCT-3′	313	60	16
			NKCC1a reverse	5′-GATGTTGTCTTTGCATAAAGC-3′			
Hbα	EU018411	Kidney	Hbα forward	5′-CAACTCTCCCGAGGTC-3′	209	64	11
			Hbα reverse	5′-CGGGTGTGAAATCATTGG-3′			
ARα	AB023960	Testes	ARα forward	5′-CTACCGGGCTCTAGCG-3′	302	60	15
			ARα reverse	5′-AGTCCGACCCCAGTAT-3′			
Activin B	AB025356	Testes	Activin B forward	5′-CGACTGGATCATCGCC-3′	312	64	16
			Activin B reverse	5′-TTGAGAGTTCGAGCGT-3′			
β-actin	AB074846	All	β-actin forward	5′-CGGAATCCACGAGACC-3′	205	65	12
			β-actin reverse	5′-TCCAGACGGAGTATTTGC-3′			

* FWO = freshwater rod opsin; NKAβ1 = Na^+/K^+-ATPase β1; NKCC1a = $Na^+/K^+/2Cl^-$ cotransporter; Hbα = hemoglobin α-chain; ARα = androgen receptors α.

TABLE III. Biological characteristics and estimators of silvering (mean ± SD (min–max)) in male eels (N = 54) at times of infection and dissection. *t*-tests for dependent samples were performed between dates.

Silvering's aspect	Biological characteristics and silvering's estimators†	Infection time*	Dissection time	Paired *t*-test
	M_T (g)	99.6 ± 21.7 (58.1–150.9)	85.7 ± 19.3 (48.2–131.7)	$t = 18.41$, $P < 0.0001$
	L_T (mm)	392 ± 26 (343–449)	390 ± 25 (341–444)	$t = 5.99$, $P < 0.0001$
Adaptation to the marine environment	I_O	7.4 ± 1.1 (5.5–9.6)	11.9 ± 1.5 (7.1–14.5)	$t = -28.12$, $P < 0$ .001
Growth stop	I_{GU}	N.A.	1.038 ± 0.476 (0.529–3.196)	
Preparation to the spawning migration	I_F	4.8 ± 0.3 (4.0–5.6)	5.2 ± 0.3 (4.5–6.1)	$t = -15.01$, $P < 0.0001$
	K (g/cm^3)	0.163 ± 0.015 (0.128–0.201)	0.143 ± 0.013 (0.116–0.171)	$t = 16.49$, $P < 0.0001$
	I_H	N.A.	1.024 ± 0.208 (0.550–1.775)	

* N.A. = data not available at time of infection.
† M_T = total mass; L_T = total length; I_O = ocular index; I_{GU} = gut index; I_F = fin index; K = Fulton's condition factor; I_H = Hepatosomatic index.

vision in freshwater and marine environments, respectively (Fazio, Moné, Lecomte-Finiger, and Sasal, 2008). We demonstrated first the absence of a relationship between the abundance of the nematode and the expression level of the freshwater rod opsin gene and, second, a significant positive relationship between the abundance of the nematode and the expression level of the deep-sea rod opsin gene. We then hypothesized that *A. crassus* may have an effect on the fish's silvering and migratory processes, being aware that an experimental approach was required for confirmation.

In the present study, we used experimental infections to examine the effect of the nematode on the morpho-anatomic and physiological transformations of the silvering process. We analyzed the impact of *A. crassus* infection on 12 parameters related to the different aspects of the silvering process, e.g., adaptation to marine environment, growth cessation, oceanic migration, and initiation of sexual maturation. We should emphasize that it was not our intention to study the parasite effects on the whole complexity of the silvering process, but rather experimentally determine whether there was an influence of the nematode on the relative size or mass of some relevant organs and the expression of some fundamental genes.

MATERIALS AND METHODS

Collection and husbandry of eels

European eels *Anguilla anguilla* (Linnaeus, 1758) were caught by a professional fisherman in July 2005 in Palavasian lagoons (43.54°N, 03.92°E, Hérault, France) where prevalences and intensities by *A. crassus* were the lowest so far recorded in the Lion Gulf (Fazio, Sasal, et al., 2008). Fifty-four eels about to begin the process of silvering were selected. They exhibited ocular hypertrophy (ocular index: I_O), a differentiated lateral line, and a contrasting color (silver) with a total body length (L_T) less than 450 mm, in order to ensure we were working only with males (Acou et al., 2005; Durif et al., 2005). They were brought to the laboratory in oxygenated lagoon water and transferred into ten 100-L tanks filled with artificial salt water (37 g/L). They all received a mebendazole (Sigma Chemical Co., St. Louis, Missouri) treatment (1 mg/L for 24 hr) for monogeneans (Buchmann, 1993).

TABLE IV. Results of experimental infections with *Anguillicoloides crassus*.

No. of eels that received an infective dose of third-stage larvae	37
Proportion of infected eels (%)	100
No. (mean ± SD (min–max)) of recovered parasites per eel	
Third-stage larvae	0
Fourth-stage larvae	0.2 ± 0.4 (0–1)
Adults	7.2 ± 5.2 (0–19)
All	7.4 ± 5.4 (1–19)
No. of eels with second-stage larvae in the swim bladder	29
Biomass (mean ± SD (min–max)) of adult worms per eel (mg)	459 ± 386 (1–1,725)

Cultivation of third-stage (L_3) larvae of *A. crassus*

L_3 larvae of *A. crassus* were cultivated following a modified version of the procedure described by De Charleroy et al. (1990) and Haenen et al. (1994). Second-stage (L_2) larvae and eggs containing L_2 larvae of *A. crassus* were isolated from naturally infected eels caught in Mediterranean lagoons. L_2 and eggs were suspended into fresh water and incubated for 2 or 3 days at 20 C until hatching. Copepods (*Cyclops* spp.) collected at the Villeneuve-de-la-Raho Lake (42.63°N, 2.90°E, Pyrénées-Orientales, France) were fed L_2 larvae (~100 L_2 larvae per 10 copepods). Subsequently, copepods were maintained at 24 C in oxygenated water and fed once a day with *Paramecium* sp. The presence of a brace-shaped sclerified structure at the anterior end of the larvae, called the "buccal ornamentation" (Blanc et al., 1992), confirmed the presence of L_3 larvae in a few, randomly chosen copepods using a compound microscope. At this point, L_3 larvae were recovered from the copepods with a tissue grinder in physiological serum (8.5‰) and counted using a binocular microscope.

Eel infection protocol, 11-ketotestosterone treatment, and rearing conditions

The experimental infections of *A. anguilla* were performed after 1 wk of acclimatization in the tanks. Eels were anesthetized in 0.1 ml/L Eugenol (Merck Schuchardt OHG, Hohenbrunn, Germany) and then weighed (total mass, M_T, to the nearest 0.1 g) and measured (total length, L_T, in millimeters). The Fulton's condition factor at the time of infection (K) was calculated as $K = (M_T/L_T^3) \times 100$, with weight in grams and length in centimeters (Bolger and Connolly, 1989). The following measurements were made to the nearest 0.1 mm on the left side of eels: pectoral fin length (L_F), and horizontal (D_h) and vertical (D_v) eye diameters. The fin index (I_F), the ocular index (I_O) (Pankhurst, 1982), and the eye area (A_E) were calculated as $I_F = 100 \times L_F/L_T$, $I_O = ([(D_h + D_v)/4]^2 \times \pi/L_T) \times 100$, and $A_E = \pi \times D_h \times D_v/4$. The experimental infections were carried out as described in Fazio, Moné, Mouahid, and Sasal (2008). Briefly, 50 L_3 larvae were intubated into the stomachs of 37 eels, using syringes with a blunt cannula filled with physiological serum. Another 17 were not exposed to parasites and designated as controls. This dose was chosen based on our previous experience (Fazio, Moné, Mouahid, and Sasal, 2008), in order to obtain a range of parasite numbers that were consistent with those occurring in the field.

Silvering is a flexible process that may be temporarily arrested if chances of successful migration are compromised (Svedäng and Wickström, 1997).

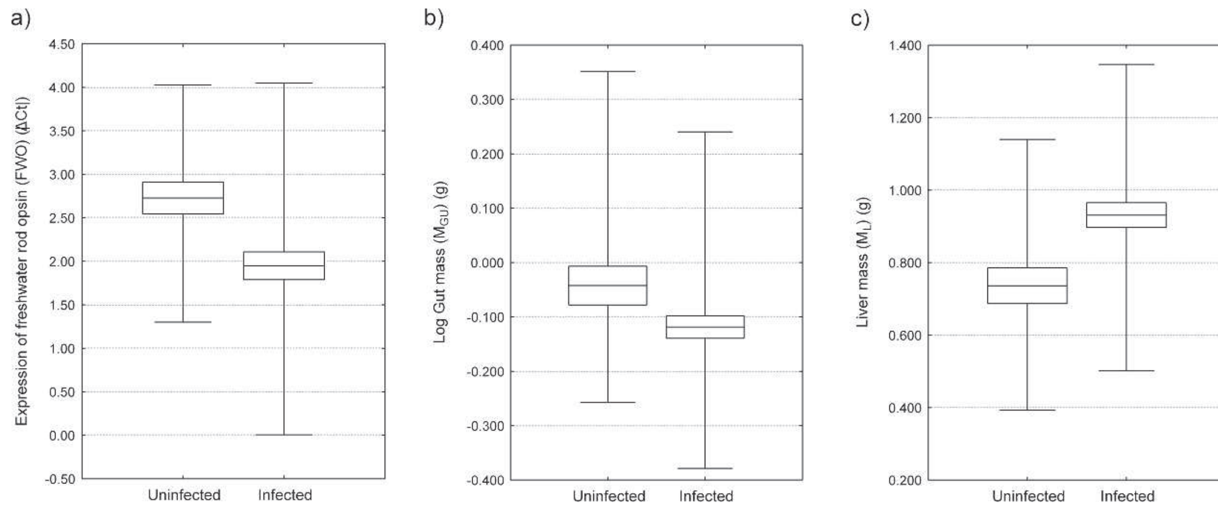

FIGURE 1. Significant effect of *Anguillicoloides crassus* infection state on (**a**) the expression of the freshwater rod opsin (FWO) gene, (**b**) the gut mass (M_{GU}), and (**c**) the liver mass (M_L) in male eels. The solid line represents the mean, the box stands for ± standard error of the mean, and the whiskers for the minimum and maximum values of the sample. $|\Delta Ct|$ = absolute value of relative level of cycle threshold.

Durif et al. (2009) even showed that silver eels held captive in tanks beyond their normal migration period may regress to yellow eels. To minimize the risk of silvering arrestment or reversion, we used 11-ketotestosterone (11-KT) to artificially maintain the silvering process in a sub-sample of male eels; the remaining individuals were given a chance to naturally pursue their metamorphosis. This steroid hormone, which is a potent androgen in fish (Fostier et al., 1983), induces spermatogenesis in vitro in male Japanese eels, *A. japonica* (T. Miura et al., 1991; C. Miura et al., 1996). To our knowledge, there are no available data on the effect of this steroid on the whole silvering processes in male European eels in vivo. However, 11-KT treatment induced silvering-related changes in short-finned female eels (*A. australis*) in vivo (Rohr et al., 2001). Seventeen of the 37 infected eels and 7 of the 17 uninfected eels received several injections of 11-KT (Sigma); 2 μg of 11-KT per 1 g of eels, homogenized in about 0.5 ml of physiological serum (6‰), were weekly injected in the body cavity (S. Dufour, pers. comm.). This treatment began 1 wk after the experimental infections and continued for 5 wk.

Eels were starved during all the experimental procedures. All the tanks were in a single room where the photoperiod was 12/12 hr, and the temperature was between 20 and 25 C. Each experimental tank was equipped with a recirculation system (320–760 L/hr) with a mechanical-biological filter (model 2042020, Eheim, Deizisau, Germany); water was aerated using air stones. Feces were siphoned out twice each week, and tank water was entirely renewed weekly with water that previously passed through an ultraviolet lamp to prevent bacterial contamination.

Parasite recovery and tissue conservation

Five months post-infection, the eels were anesthetized, weighed, and measured. The following measurements were again made on the left side of eels, i.e., pectoral fin length (L_F), and horizontal (D_h) and vertical (D_v) eye diameters. Eels were then killed instantly by beheading. The Fulton's condition factor (K), fin (I_F) and ocular (I_O) indexes, and the eye area (A_E) at the time of necropsy were calculated as above, and sex was determined. The 54 eels were males. The liver (M_L) and gut (M_{GU}) of each eel were weighed to the nearest milligram. Hepatosomatic (I_H) and gut (I_{GU}) indexes were calculated as $I_H = 100 \times M_L/M_T$ and $I_{GU} = 100 \times M_{GU}/M_T$. Testes mass was not considered to be reliable enough because of incomplete separation of the gonad lobules from the connective tissue. For the recovery of parasites, swim bladders (wall and lumen) were examined using a binocular microscope. The developmental stages (L_3, L_4, or adult stages) were determined. We checked for the presence of L_2 larvae and eggs in the lumen of the swim bladder. Adult parasites of each swim bladder were weighed to the nearest milligram.

The eyes, gills, gonads, intestine, and kidney were immersed in RNAlater® (Ambion Inc., Austin, Texas) and were stored at −20 C for further molecular analysis.

Choice of silvering parameters

The silvering process is characterized by multiple changes, among which 12 were selected for investigation. Their characteristics are presented in Table I. Choice of parameters was made in order to study the impact of the nematode on the 4 aspects of the metamorphosis, e.g., adaptation to marine environment, growth stop, oceanic migration, and initiation of sexual maturation, but also at the morpho-anatomic and physiological levels at which the modifications take place. Increase in body condition, enlargement of the eye area and pectoral fins, degeneration of the alimentary tract, and increase in the liver mass are well documented morpho-anatomic changes involved in the silvering, for which standardized estimators are available (Table I). Physiological changes have recently been characterized at the molecular level. Quantitative RT-PCR analyses in Japanese eels revealed that the expression of freshwater rod opsin (FWO) in the eyes decreases during the silvering process (Zhang et al., 2000). Experimental studies showed that the Na^+/K^+-ATPase β1 (NKAβ1) and $Na^+/K^+/2Cl^-$ cotransporter (NKCC1a) genes, which code for transmembranous proteins, were significantly up-regulated following seawater transfer (Kalujnaia et al., 2007). Here, the effect of *A. crassus* on adaptation of the vision and osmoregulation was inferred by analyzing the expression of the FWO in the eyes, NKAβ1 in the intestine and in the gills, and NKCC1a in the gills. Hemoglobin and hematocrit were found to be higher in silver eels than in yellow eels (Johansson et al., 1974). Muscle oxygenation capacity was studied by analyzing the expression of hemoglobin α-chain (Hbα) in the kidney (Soldatov, 2005). Finally, we examined the expression of androgen receptors α (ARα) and activin B in the testes because of the close relationship between their gene expression and the 11-KT level in the context of spermatogenesis induction (Miura and Miura, 2003).

Molecular analysis

Total RNA of each organ was extracted using TRIzol (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. RNA concentrations were read spectrophotometrically. Two micrograms of RNA were used to synthesize cDNA in a total volume of 20 μl containing 1× first-strand buffer, 0.5 μg of oligo(dT) as primer, 0.5 mM deoxynucleoside triphosphates, 10 mM dithiothreitol, 40 U of RNaseOUT (Invitrogen), 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen), and diethyl pyrocarbonate water, according to the manufacturer's protocol, and stored at −20 C until use.

Real-time PCR was carried out using a LightCycler (Roche Diagnostics, Basel, Switzerland). Reactions were set up in microcapillaries using the following concentrations in a final volume of 10 μl: 0.5 μM each primer, 3.5 mM of $MgCl_2$, 1× SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany), and 1 μl of cDNA. Primers for the real-time PCR were designed using the LightCycler Probe Design software (Roche

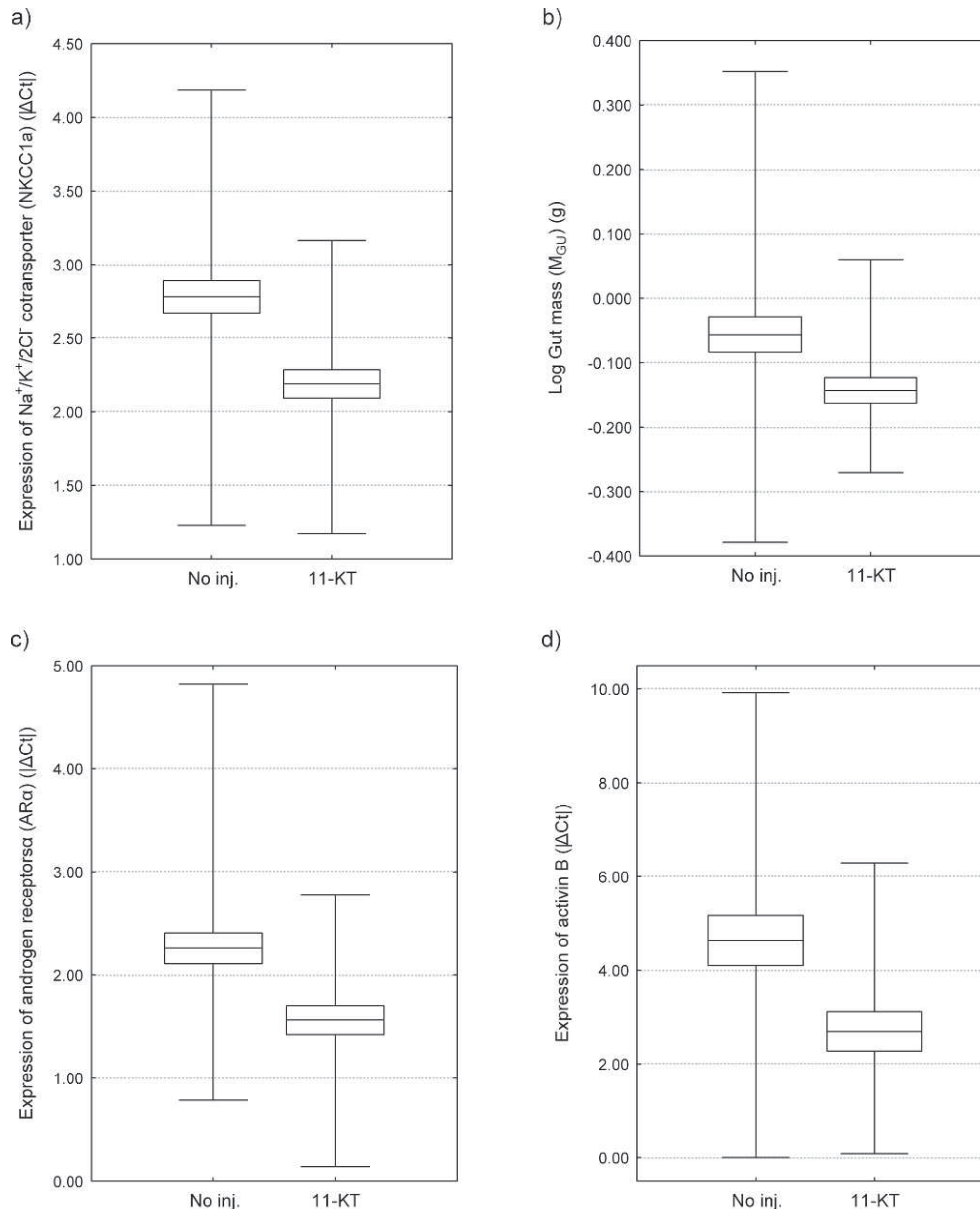

FIGURE 2. Significant effect of 11-ketotestosterone treatment on (**a**) the expression of the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1a) gene, (**b**) the gut mass (M_{GU}), (**c**) the expression of the androgen receptors α (ARα) gene, and (**d**) the expression of the activin B gene in male eels. The solid line represents the mean, the box stands for ± standard error of the mean, and the whiskers for the minimum and maximum values of the sample. 11-KT = 11-ketotestosterone–treated eels; no inj. = eels that received no injections. |ΔCt| = absolute value of relative level of cycle threshold.

Diagnostics, PE Applied Biosystems) on the basis of the European or Japanese eel cDNA sequences. Accession numbers, primer characteristics, and amplicon lengths are shown in Table II. The PCR cycling conditions were as follows: cDNA denaturation for 10 min at 95 C, followed by 40 cycles of 15 sec at 95 C, primer annealing for 5 sec at the specific temperature (see Table II), and a specific time extension at 72 C (see Table II), with fluorescence measured at the end of every annealing and extension step. Reactions for each sample were performed in duplicate. Each PCR was calibrated with a pool of cDNA and a negative control (1 μl of H_2O was used). The specificity of the amplification of each sample was ensured by examination of the melting curves. PCR efficiency (E) was calculated as $E = 10^{(-1/\text{slope})}$ (where slope was calculated performing a PCR on serial dilutions of a pool of cDNA). Our results revealed that all E values were higher than 1.95. Choice of a reference gene in real-time RT-PCR is always difficult to make, because no single gene has a constant expression level. However, in fish studies, β-actin is considered to be one of the most stable reference genes (Olsvik et al., 2005).

An average value of cycle threshold (Ct) of the duplicates was calculated and corrected with the mean value of the calibrator duplicates for each sample. Relative level of cDNA (ΔCt) of each gene was calculated as $\Delta Ct = \text{mean } Ct_{\text{target gene}} - \text{mean } Ct_{\beta\text{-actin}}$. The relative ratio of the target gene expression (relR) between 2 samples "a" and "b" was calculated as $relR = 2^{-\Delta\Delta Ct}$ with $\Delta\Delta Ct = \Delta Ct_a - \Delta Ct_b$, according to the manufacturer recommendations for $E \geq 1.95$.

Statistical analysis

Samples for which the difference between duplicates was more than 0.2 cycles were excluded from statistical analysis. Because there is a negative relationship between the ΔCt value and the expression level of target genes, ΔCt data were transformed. First, the maximum ΔCt value found for a target gene was subtracted from all the other values for the same gene, resulting in all ΔCt values ≤ 0. Second, the absolute value of each ΔCt value (|ΔCt|) was calculated, resulting in a positive relationship

TABLE V. Parameters and estimators related to the different aspects of the silvering process. Data are presented as mean ± SD (min–max) for each experimental group.

Silvering's aspect	Silvering's parameters and estimators†	Uninfected eels* 11-KT (N = 7)	Uninfected eels* No inj. (N = 10)
Adaptation to the marine environment	A_E (mm^2)	175.2 ± 28.9 (116.9–201.1)	171.4 ± 41.7 (100.2–240.5)
	I_O	11.3 ± 1.6 (8.1–13.2)	11.2 ± 2.2 (7.1–14.4)
	Expression of FWO (|ΔCt|)	2.63 ± 0.63 (1.72–3.52)	2.80 ± 0.87 (1.30–4.03)
	Expression of NKAβ1 in the gills (|ΔCt|)	1.61 ± 0.79 (0.53–2.74)	1.23 ± 0.79 (0.14–2.79)
	Expression of NKAβ1 in the intestine (|ΔCt|)	6.67 ± 0.22 (6.37–6.92)	6.49 ± 0.92 (4.58–8.12)
	Expression of NKCC1a (|ΔCt|)	1.94 ± 0.74 (1.18–2.88)	2.89 ± 0.74 (1.23–4.19)
Growth cessation	M_{GU} (g)	0.759 ± 0.182 (0.553–1.120)	1.106 ± 0.444 (0.587–2.247)
	I_{GU}	0.964 ± 0.285 (0.712–1.571)	1.540 ± 0.777 (0.915–3.196)
Preparation to the spawning migration	M_T (g)	80.1 ± 11.7 (65.0–101.1)	77.8 ± 24.1 (48.2–114.7)
	K (g/cm^3)	0.139 ± 0.009 (0.121–0.152)	0.139 ± 0.016 (0.116–0.161)
	L_F (mm)	20.5 ± 1.3 (18.0–21.8)	19.2 ± 2.1 (16.3–22.5)
	I_F	5.3 ± 0.2 (5.0–5.7)	5.1 ± 0.3 (4.6–5.4)
	M_L (g)	0.734 ± 0.081 (0.641–0.866)	0.738 ± 0.262 (0.393–1.140)
	I_H	0.922 ± 0.066 (0.839–1.009)	0.961 ± 0.243 (0.550–1.486)
	Expression of Hbα (|ΔCt|)	2.51 ± 1.13 (0.85–3.71)	2.18 ± 1.36 (0.10–4.01)
Sexual maturation	Expression of ARα (|ΔCt|)	1.27 ± 0.98 (0.14–2.78)	2.07 ± 0.40 (1.23–2.46)
	Expression of activin B (|ΔCt|)	2.58 ± 1.86 (0.77–5.47)	4.82 ± 2.72 (0.00–8.65)

* 11-KT = 11-ketotestosterone–treated eels; no inj. = eels that received no injections.
† A_E = eye area; I_O = ocular index; FWO = freshwater rod opsin; |ΔCt| = absolute value of relative level of cycle threshold; NKAβ1 = Na^+/K^+-ATPase β1; NKCC1a = $Na^+/K^+/2Cl^-$ cotransporter; M_{GU} = gut mass; I_{GU} = gut index; M_T = total mass; K = Fulton's condition factor; L_F = pectoral fin length; I_F = fin index; M_L = liver mass; I_H = Hepatosomatic index; Hbα = hemoglobin α-chain; ARα = androgen receptors α.

between the |ΔCt| value and the expression level of target genes. A Kolmogorov–Smirnov test was used to assess the normality of variable distributions. The homogeneity of variances was tested using the Levene *F*-test (Zar, 1999). To meet these assumptions, gut mass (M_{GU}) was log-transformed prior to analysis (Zar, 1999), and 1 extreme value was removed from the following variables: expression of NKAβ1 in gills and in the intestine, and expression of NKCC1a. Nonparametric Mann–Whitney *U*-tests and Kruskal–Wallis tests were performed to compare silvering's parameters of eels that were under identical experimental conditions in terms of parasite exposition and steroid treatment but distributed in 2 or 3 tanks (Zar, 1999). No differences were found between eels reared in different tanks under identical experimental conditions ($P > 0.05$). Progress in the silvering process was evaluated using paired *t*-tests for M_T, L_T, K, I_F, and I_O between times of infection and dissection.

Multivariate general linear models were used with Type III sum of squares (SS) to analyze the variation in the 12 parameters of the silvering (Table I). The predictors were (1) the infection state (uninfected/infected eels); (2) the biomass of adult parasites; (3) the 11-KT treatment (11-KT–treated eels and eels that received no injections); (4) the interaction between the infection state and the 11-KT treatment (infection state × 11-KT treatment); and (5) either the fish total mass (M_T) or total length (L_T) as a covariate. The way we selected the appropriate covariate depended on the nature of the parameters, i.e., organ mass/length or gene expression. For the morpho-anatomic variables, we 'logically' analyzed gut and liver masses while controlling for M_T, and eye area and pectoral fin length while controlling for L_T. With regard to the physiological variables, choice of the covariate was made by testing the goodness of fit of the statistical models. Total mass and total length, but also the other explanatory variables (infection state, parasite biomass, 11-KT treatment, and infection state × 11-KT treatment), were included in the analysis. We calculated Akaike's information criterion (AIC) to rank the reduced models, i.e., to determine the best subsets of predictors. Only candidate models with an AIC value within 2 points of the best-fitting model, i.e., the candidate model with the lowest AIC, were considered to have substantial empirical support (Burnham and Anderson, 2002). We then selected the covariate that was included in the candidate model with the closest AIC of the best-fitting model. Expression of FWO, expression of NKAβ1 in gills, expression of NKCC1a, gut mass (M_{GU}), liver mass (M_L), and expression of activin B were examined in a single multivariate analysis of covariance (MANCOVA) with total mass (M_T) as a covariate. Eye area (A_E), expression of NKAβ1 in the intestine, pectoral fin length (L_F), expression of Hbα, and expression of ARα were examined in a single MANCOVA with total length (L_T) as a covariate. The homogeneity of variance–covariance matrix was tested using the *F*-test from Box's *M* statistics, and Pillai's trace criterion was chosen to assess the significance of the test because of its robustness when experimental group sizes are unequal (Johnson and Field, 1993). When a MANCOVA was significant, univariate analyses of covariance (ANCOVAs), which included only the significant predictors, were subsequently performed for each parameter alone. Variation in eel body condition was investigated using an ANCOVA, which allows, after log-transformation of the data, one to estimate directly the effects of both parasite and steroid treatment on fish mass while controlling for a concomitant variable of influence, i.e., fish length (García-Berthou, 2001; Freckleton, 2002). The importance of individual predictors was assessed by the squared semipartial correlation coefficient ($r^2_{semipartial}$), calculated as $r^2_{semipartial} = SS_{effect}/SS_{total}$, which represents the proportion of total variation accounted for by a factor over and above what is explained by the other factors (Cardinal and Aitken, 2006). The contribution of continuous predictors was assessed by the sign of the unstandardized regression coefficients (b). Analyses were carried out using Statistica 6.0 software.

RESULTS

Ocular and fin indexes and Fulton's condition factor were significantly different between times of infection and dissection (paired *t*-test, $P < 0.05$; Table III). All experimentally infected eels were found to be parasitized at necropsy, and no *A. crassus* was found in unexposed eels (Table IV). All recovered parasites were at the adult stage, except 1. These adults reproduced in 29 of 37 eels. Their biomasses ranged from 1 to 1,725 mg, which is consistent with that typically found in naturally infected eels.

Considering together expression of FWO, expression of NKAβ1 in gills, expression of NKCC1a, gut mass (M_{GU}), liver mass (M_L), and expression of activin B, we found a significant multivariate relationship with the total mass (M_T) (Pillai's trace = 0.627, $F_{6,39} = 10.94$, $P < 0.001$), 11-KT treatment (Pillai's trace = 0.533, $F_{6,39} = 7.42$, $P < 0.001$), infection state (Pillai's trace = 0.269, $F_{6,39} = 2.40$, $P = 0.046$), and interaction between the

TABLE V. Extended.

Uninfected eels*	Infected eels		
Total (N = 17)	11-KT (N = 17)	No inj. (N = 20)	Total (N = 37)
173.0 ± 36.0 (100.2–240.5)	192.8 ± 31.4 (141.0–248.8)	190.2 ± 21.8 (141.0–246.0)	191.4 ± 26.3 (141.0–248.8)
11.3 ± 1.9 (7.1–14.4)	12.1 ± 1.5 (9.0–14.0)	12.2 ± 1.1 (9.9–14.5)	12.1 ± 1.3 (9.0–14.5)
2.73 ± 0.76 (1.30–4.03)	1.92 ± 1.02 (0.00–4.05)	1.97 ± 0.89 (0.95–3.74)	1.95 ± 0.94 (0.00–4.05)
1.38 ± 0.79 (0.14–2.79)	1.39 ± 0.38 (0.69–2.04)	1.40 ± 0.50 (0.64–2.35)	1.40 ± 0.45 (0.64–2.35)
6.56 ± 0.71 (4.58–8.12)	6.45 ± 0.42 (5.90–7.45)	6.66 ± 0.61 (5.26–8.23)	6.56 ± 0.53 (5.26–8.23)
2.57 ± 0.85 (1.18–4.19)	2.27 ± 0.32 (1.83–3.17)	2.72 ± 0.54 (1.60–3.92)	2.51 ± 0.50 (1.60–3.92)
0.963 ± 0.392 (0.553–2.247)	0.729 ± 0.180 (0.536–1.149)	0.849 ± 0.293 (0.418–1.740)	0.794 ± 0.252 (0.418–1.740)
1.303 ± 0.675 (0.712–3.196)	0.838 ± 0.267 (0.529–1.616)	0.982 ± 0.295 (0.567–1.491)	0.916 ± 0.288 (0.529–1.616)
78.7 ± 19.5 (48.2–114.7)	90.5 ± 20.1 (58.2–131.7)	87.5 ± 17.8 (58.7–126.8)	88.8 ± 18.7 (58.2–131.7)
0.139 ± 0.013 (0.116–0.161)	0.143 ± 0.016 (0.119–0.171)	0.145 ± 0.010 (0.128–0.163)	0.144 ± 0.013 (0.119–0.171)
19.8 ± 1.9 (16.3–22.5)	20.7 ± 1.9 (17.5–23.8)	20.3 ± 0.9 (18.0–22.3)	20.5 ± 1.4 (17.5–23.8)
5.2 ± 0.3 (4.6–5.7)	5.2 ± 0.3 (4.5–6.1)	5.2 ± 0.2 (4.8–5.7)	5.2 ± 0.3 (4.5–6.1)
0.736 ± 0.203 (0.393–1.140)	0.895 ± 0.248 (0.501–1.347)	0.961 ± 0.168 (0.646–1.183)	0.931 ± 0.208 (0.501–1.347)
0.945 ± 0.187 (0.550–1.486)	0.987 ± 0.157 (0.827–1.315)	1.123 ± 0.230 (0.861–1.775)	1.060 ± 0.209 (0.827–1.775)
2.32 ± 1.24 (0.10–4.01)	3.90 ± 1.97 (1.16–7.36)	3.35 ± 1.61 (0.96–6.24)	3.60 ± 1.78 (0.96–7.36)
1.74 ± 0.79 (0.14–2.78)	1.68 ± 0.52 (0.68–2.71)	2.35 ± 0.97 (0.79–4.82)	2.05 ± 0.85 (0.68–4.82)
3.89 ± 2.60 (0.00–8.65)	2.74 ± 2.18 (0.09–6.29)	4.54 ± 3.10 (0.00–9.92)	3.71 ± 2.83 (0.00–9.92)

infection state and the 11-KT treatment (Pillai's trace = 0.290, $F_{6,39}$ = 2.66, P = 0.029). There was no multivariate effect of the biomass of adult parasites (Pillai's trace = 0.182, $F_{6,39}$ = 1.45, P = 0.22). Considering together the eye area (A_E), expression of NKAβ1 in the intestine, pectoral fin length (L_F), expression of Hbα, and expression of ARα, we found a significant multivariate effect for the total length (L_T) (Pillai's trace = 0.470, $F_{5,43}$ = 7.64, $P < 0.001$), biomass of adult parasites (Pillai's trace = 0.678, $F_{5,43}$ = 18.14, $P < 0.001$), and 11-KT treatment (Pillai's trace = 0.429, $F_{5,43}$ = 6.46, $P < 0.001$). There was no multivariate effect with respect to the infection state (Pillai's trace = 0.061, $F_{5,43}$ = 0.55, P = 0.73) and interaction between the infection state and the 11-KT treatment (Pillai's trace = 0.077, $F_{5,43}$ = 0.71, P = 0.62).

Variation in eye area (A_E) was best explained by total length ($r^2_{semipartial}$ = 0.47, $P < 0.0001$) (model: SS = 26653.30, df = 3, F = 19.42, $P < 0.0001$, R^2 = 0.54). Variation in expression of FWO was best explained by infection state ($r^2_{semipartial}$ = 0.09, P = 0.024) (model: SS = 9.89, df = 4, F = 3.18, P = 0.022, R^2 = 0.21). The uninfected eels had 1.5 times the level of FWO gene expression of the infected eels (Fig. 1a). The candidate model, i.e., total mass, infection state, 11-KT treatment and interaction between the infection state, and the 11-KT treatment, for explaining variation in expression of NKAβ1 in gills was not significant (model: SS = 2.35, df = 4, F = 2.00, P = 0.11, R^2 = 0.14). The candidate model, i.e., total length, biomass of adult parasites, and 11-KT treatment, for explaining variation in expression of NKAβ1 in the intestine was not significant (model: SS = 1.58, df = 3, F = 1.59, P = 0.20, R^2 = 0.09). Variation in expression of NKCC1a was best explained by 11-KT treatment ($r^2_{semipartial}$ = 0.24, P = 0.0003) (model: SS = 5.11, df = 4, F = 4.28, P = 0.005, R^2 = 0.27). Eels that received no injections had a NKCC1a gene expression level that was 1.6 times higher than that of 11-KT–treated eels (Fig. 2a).

Variation in gut mass (M_{GU}, log-transformed data) was best explained by 11-KT treatment ($r^2_{semipartial}$ = 0.13, P = 0.006) and infection state ($r^2_{semipartial}$ = 0.07, P = 0.035) (model: SS = 0.24, df = 4, F = 3.87, P = 0.008, R^2 = 0.24). Since the difference between logs is the log of the ratio, the ratio of the mean M_{GU} of 11-KT–treated eels (mean $M_{GU[11\text{-}KT]}$) to that of eels that received no injections (mean $M_{GU[no\ inj.]}$) was calculated, after reciprocal transformation of the data, as mean $M_{GU[11\text{-}KT]}$/mean $M_{GU[no\ inj.]}$ = $10^{\log(\text{mean } M_{GU[11\text{-}KT]}) - \log(\text{mean } M_{GU[no\ inj.]})}$ = $10^{-0.138+0.032}$ = 0.78. This corresponds to a decrease in size of 22% on average between eels that received no injections and 11-KT–treated eels (Fig. 2b). The ratio of the mean M_{GU} of infected eels to that of uninfected ones was 0.83. This corresponds to a decrease in size of 17% on average between uninfected and infected eels (Fig. 1b).

Variation in pectoral fin length (L_F) was best explained by total length ($r^2_{semipartial}$ = 0.49, $P < 0.0001$) (model: SS = 78.73, df = 3, F = 23.02, $P < 0.0001$, R^2 = 0.58). Variation in total mass (M_T) was best explained by total length ($r^2_{semipartial}$ = 0.77, $P < 0.0001$) (model: SS = 0.45, df = 4, F = 55.62, $P < 0.0001$, R^2 = 0.85). Variation in liver mass (M_L) was best explained by total mass ($r^2_{semipartial}$ = 0.40, $P < 0.0001$) and infection state ($r^2_{semipartial}$ = 0.05, P = 0.015) (model: SS = 1.55, df = 4, F = 16.97, $P < 0.0001$, R^2 = 0.58). The ratio of the mean M_L of infected eels to that of uninfected ones was 1.15 for a fixed total mass of 85.7 g (see Table III), which corresponds to an increase in size of 15% on average between uninfected and infected eels (Fig. 1c). Variation in expression of Hbα was best explained by biomass of adult parasites ($r^2_{semipartial}$ = 0.29, $P < 0.0001$) and total length ($r^2_{semipartial}$ = 0.12, $P < 0.0001$) (model: SS = 84.08, df = 3, F = 19.00, $P < 0.0001$, R^2 = 0.53). The biomass of adult parasites had a positive influence on the expression level of the Hbα (b = +2.50, Fig. 3a).

Variation in expression of ARα was best explained by 11-KT treatment ($r^2_{semipartial}$ = 0.24, $P < 0.0001$), biomass of adult parasites ($r^2_{semipartial}$ = 0.12, P = 0.002), and total length ($r^2_{semipartial}$ = 0.11, P = 0.003) (model: SS = 17.04, df = 3, F = 14.10, $P < 0.0001$, R^2 = 0.46). For a fixed total length of 390 mm (see Table III) and a fixed biomass of parasites of 306 mg (mean value for all eels, including uninfected ones), eels that received no injections had a ARα gene expression level that was 1.8 times

higher than that of 11-KT–treated eels (Fig. 2c). The biomass of adult parasites had a positive influence on the expression level of the ARα (b = +0.78, Fig. 3b). Variation in expression of activin B was best explained by 11-KT treatment ($r^2_{semipartial}$ = 0.11, P = 0.015) (model: SS = 76.38, df = 4, F = 2.92, P = 0.030, R^2 = 0.19). Eels that received no injections had an activin B gene expression level that was 3.8 times higher than that of 11-KT–treated eels (Fig. 2d).

DISCUSSION

Our results showed that the biological characteristics of the eels were different between times of infection and necropsy. Significant differences in total mass and condition factor were suspected to be a consequence of starvation. Significant difference in total length was likely due to caudal fin damage in some eels. The other results confirmed the progress of the silvering process of the experimental fishes.

Surprisingly, the steroid treatment had almost no effect on the silvering process. Only gut mass was negatively influenced by 11-KT injections, thus revealing an effect of the androgen on the degeneration of the alimentary tract. We even found that the steroid treatment had adverse effects on the expression of the NKCC1a, ARα, and activin B genes. This appears to contradict the results obtained by Rohr et al. (2001) in short-finned female eels. These authors showed that 11-KT–treated eels had a significant eye enlargement, a thicker dermis, an epidermis with fewer, or no, mucous cells (adaptation that improves hydro-dynamism), and a larger heart, liver, and gonads. Olivereau and Olivereau (1985) obtained similar results in male European eels treated with the synthetic androgen 17α-methyltestosterone. Our results indicate that 11-KT is inefficient for inducing silvering-related changes in male European eels. Methodological differences, such as the quality of the hormone used or the administration method, seem unlikely to explain this finding, since we found that the treatment had a significant effect on the sex ratio of the parasite (Fazio, Moné, Mouahid, and Sasal, 2008). Stress caused by repeated handling, anesthesia, and injection may have counterbalanced the effect of the steroid treatment.

We showed that *A. crassus* infection had an effect on 5 silvering-related parameters of the 12 tested. Moreover, the 4 aspects of the silvering process were affected in infected eels. First, adaptation to the marine environment was affected by changes in FWO gene expression. Second, growth cessation coincides with changes in gut mass. Third, preparation for the spawning migration begins with changes in liver mass and Hbα gene expression. Fourth, sexual maturation is initiated by changes in ARα gene expression. An important point is the way in which these parameters were influenced, i.e., the liver mass, and the expression of the Hbα and ARα genes were higher in infected eels, whereas the expression of the FWO gene and the gut mass were lower in infected eels. The former variables are believed to increase during silvering, while the latter ones decrease. Accordingly, our results suggest that infected eels were at a more advanced stage in the silvering process than the uninfected individuals. This finding is quite surprising at first glance. Indeed, we expected an increase in expression of the Hbα gene as a result of the blood feeding behavior of the nematode and a delay, even an inhibition, of the entire silvering process because of the energetic cost of parasitism. Nevertheless, this is in agreement

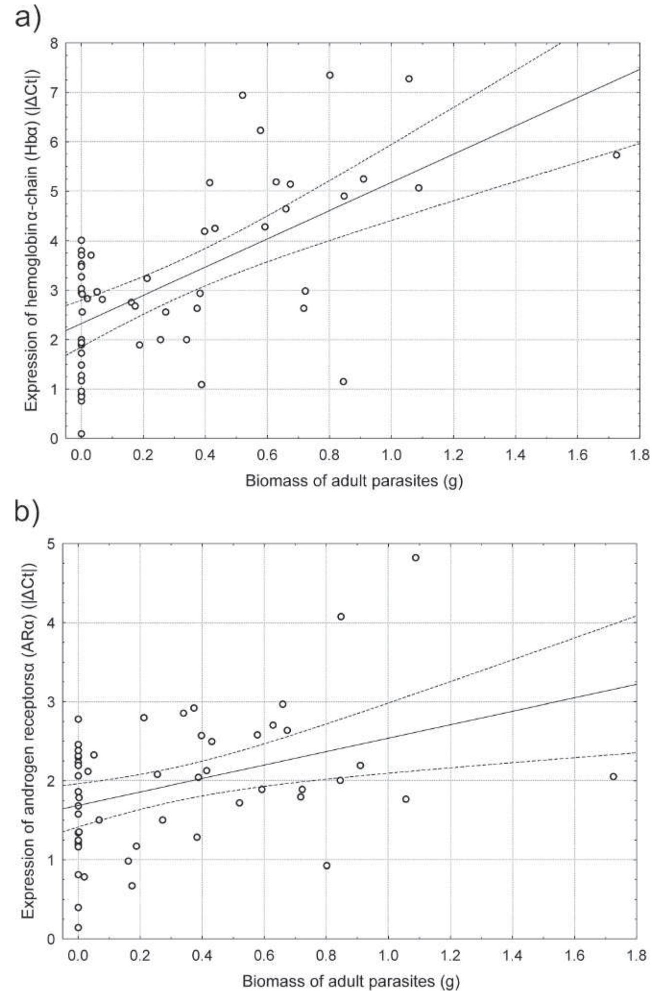

FIGURE 3. Significant effect of the biomass of *Anguillicoloides crassus* adults on the expression of (**a**) the hemoglobin α-chain (Hbα) and (**b**) the androgen receptors α (ARα) genes. The solid line represents the fitted regression line ($y = 2.32 + 2.86x$, and $y = 1.69 + 0.85x$, for (a) and (b) respectively), and the dashed lines stand for the 95% confidence interval. |ΔCt| = absolute value of relative level of cycle threshold.

with the up-regulation of the deep-sea rod opsin gene that we previously observed in wild infected eels (Fazio, Moné, Lecomte-Finiger, and Sasal, 2008). Similar effects have been observed with pollutants in female silver eels. For example, experimental cadmium exposure was found to strongly stimulate the pituitary–gonad–liver axis of eels that had matured sexually under artificial conditions, leading to early and enhanced vitellogenesis (Pierron et al., 2008). Another study, using eels artificially stimulated to sexual maturation, revealed that the smallest and youngest silver-stage eels obtained following hormonal treatment were those migrating from the location that displayed the lowest water quality (in terms of organic matter and mineral pollution) and highest *A. crassus* prevalence (93%) (Durif et al., 2006).

We suggest 2 hypotheses to explain advanced silvering state in infected eels, a physiological one (parasite side-effect) and an evolutionary one (host response). First, *A. crassus* infection could alter the physiological mechanisms involved in the silvering, as a by-product of the infection. One mechanism eventually implicated would be the production of cortisol in infected eels. This hormone was shown to have stimulatory effects on GTH2 synthesis, which

is 1 of the 2 pituitary gonadotropins involved in sexual maturation in fish (Dufour et al., 2003). Moreover, cortisol is the key hormone produced during fasting, which induces the mobilization of lipid and protein stores and stimulates hepatic gluconeogenesis (Dufour et al., 2003). Because larval stages of the nematode increase the cortisol level in early infection (Sures et al., 2001), we hypothesize that the presence of the parasite could have indirect stimulatory effects on silvering mechanisms as a result of stress. Another possibility would be an effect of the parasites due to their blood feeding behavior. Erythropoiesis, which normally increases during silvering (Johansson et al., 1974), would be re-enforced in infected eels by the demand imposed by the parasites and may have stimulatory co-effects on eel physiology via the action of circulating erythropoietin (Lai et al., 2006).

As an alternative hypothesis, we suggest that there is an adjustment in life history traits of infected eels. The potential plasticity of the silvering duration would allow infected eels to accelerate their metamorphosis in order to undertake the reproductive migration before the energetic cost imposed by the parasite becomes too high (swim bladder degeneration and blood sucking activity of adults). Hochberg et al. (1992) described how parasites could affect the timing of first reproduction by their hosts. They showed that hosts affected by virulent parasites and unable to resist by other means, i.e., by the avoidance of infection or an efficient immune response (Combes, 1995), would be favored by selection if they were able to reproduce earlier. To our knowledge, this idea has never been investigated in fish. However, empirical studies in snails and insects have shown that infected individuals had a significantly decreased developmental time and, therefore, matured sexually earlier than uninfected ones (Michalakis and Hochberg, 1994). Another example is given in the snail *Helisoma anceps*, where castrated-populations of this snail due to larval trematode infections benefited most by reproducing at a smaller size (Negovetich and Esch, 2008). Such a response in European eels clearly remains to be demonstrated, as much as its putative origin, i.e., phenotypic plasticity or genetic determinism. However, the absence of an efficient immune response against the parasite (Nielsen, 1999; Nielsen and Esteve-Gassent, 2006) would be consistent with such a modification in life history traits.

Questions remain regarding the potential effect of accelerated silvering on the European eel migration and reproductive success. If such a response to *A. crassus* infection takes its origin in the plasticity of the silvering process, one can reasonably assume that fitness of individuals would be maximized by increasing the probability of reaching the Sargasso Sea. However, whether or not accelerated silvering is a physiological effect resulting from *A. crassus* infection (larval stage—induced production of cortisol or blood feeding behavior of adults), it may be considered as an additional cost of parasitism. Indeed, accelerated silvering may trigger a kind of "physiological discordance" in infected eels if functional adaptations are set in organisms that are not able to physiologically "integrate" them. This may result in the departure, from continental waters, of silver stages of lesser quality and further reduce the ability to migrate and contribute to the future recruitment of the species.

ACKNOWLEDGMENTS

We thank François Lefebvre for valuable comments on an earlier version of the manuscript. We also thank William Hughes for comments and English revision on the last version of the manuscript. We are very grateful to Mr. Molle for supplying eels. This work was financially supported by the French Ministère de l'écologie et du développement durable, the CNRS and the University of Perpignan Via Domitia. G.F. was financially supported by a grant from the French Ministère de l'enseignement supérieur et de la recherche.

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