Differential gene expression analysis in European eels (Anguilla anguilla, L. 1758) naturally infected by macroparasites

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ABSTRACT: We analyzed the relationships between the macro-parasite community of the European eel and the expression of genes involved in the host physiology during its continental life. The genes studied are implicated in: (1) host response to environmental stress, i.e. Heat Shock Protein 70 (HSP70) and metallothionein (MT); (2) osmoregulation, i.e., ß Thyroid Hormone Receptor (ßTHR) and Na^+/K^+ATPase; and (3) silvering, i.e., ßTHR, freshwater rod opsin (FWO) and deep-sea rod opsin (DSO). All were enumerated by quantitative RT-PCR. The epizootiological results for 93 yellow eels caught in the Salses-Leucate Lagoon (France) included 11 species, i.e., 1 nematode, 2 acanthocephalans, 1 monogenean, and 7 digeneans. The molecular results revealed**:** (1) a significant negative relationship between digenean abundance and the expression level of all the tested genes, except FWO; (2) a significant negative relationship between the abundance of the nematode *Anguillicola crassus* and the expression level of Na^+/K^+ATPase gene; and (3) a significant positive relationship between the *A. crassus* abundance and the expression level of MT gene. Eels infected with digeneans had, on average, a lower level of expressed genes. We hypothesize that the parasites may disturb the eel's ability to withstand environmental stress and delay their migration to the Sargasso Sea because of degeneration of the gut. We further propose that the effect of the invasive species, *A. crassus*, on the gene expression was mainly

linked to an increased trophic activity of infected eels. Moreover, it is possible that the parasite may have an effect on the fish's migratory behavior, which is tied to reproductive purposes. Additional work, including an experimental approach, is required to confirm our hypotheses.

Macroparasites are ubiquitous components of natural ecosystems. Their ecological impact on wildlife populations is currently a significant growth area in parasitological research as they may play a role in structuring ecosystems (Thomas et al., 2005). The mechanisms by which macroparasites may regulate natural host populations still needs investigation, especially in order to characterize the pathogenic effects at the organismic level.

Proteomics and genomics are fast-moving disciplines that may give a new dimension to host-parasite interaction studies (Biron et al., 2005). Differential gene expression has been recently used in fish-parasite models to compare parasitized and non parasitized hosts (Collins et al., 2007), and to estimate the immune gene expression related to pathogen and parasite infections (Harms et al., 2003; Lindenstrøm, Secombes, and Buchmann, 2004). In both cases, gene expression appeared to be a powerful tool to analyze the physiological response of a host infected by a given parasite. However, there is still a lack of knowledge regarding the nature of host functions affected and the consequences for gene expression when hosts are infected with parasites. While experimental studies with a single parasite species are probably more efficient to detect effects, hosts in wild condition are confronted with parasite species that may have synergistic or antagonistic effects on host physiology.

The European eel (*Anguilla anguilla*) is considered as a threatened species (Wirth and Bernatchez, 2003). The life cycle of this species is characterized by a growth period in continental waters (yellow-stage), followed by a catadromous migration to reach the spawning area in the Sargasso Sea (silver-stage) (Schmidt, 1922; Tesch, 1977). The quality of habitat in continental water and the subsequent migration from freshwater to sea water will determine

the reproductive success of the species. Among the stresses faced during continental life, the acquisition of a rich parasite fauna (Bruslé, 1994) may have an effect on the survival of the eels. Eels acquire their first macroparasites after arriving in continental waters, i.e., when they begin to feed. Thereafter, they contend with parasites throughout their growth phase, lasting between 3 and 20 yr (Tesch, 1977). Much attention has been paid on the effects of the on eel physiology by recently introduced parasite species, namely the swimbladder nematode *Anguillicola crassus* and the gill monogeneans *Pseudodactylogyrus* spp. (Kennedy, 2007). Besides epizootiological studies and the effects of salinity, nematodes are known to affect the histological structure of the swimbladder (Molnár et al., 1993; Würtz and Taraschewski, 2000; Lefebvre et al., 2002) and blood properties of the eel (Boon et al., 1989; Kelly et al., 2000). Other studies have highlighted a decrease in the host's swimming performances (Sprengel and Lüchtenberg, 1991) and their ability to withstand stress (Molnár, 1993; Kirk, 2003; Gollock et al., 2004). Previous work on the gill monogeneans *Pseudodactylogyrus* spp. has mainly focused on their geographical distribution and on the immune response induced by the parasite (Buchmann et al., 1987; Slotved and Buchmann, 1993; Monni and Cognetti-Varriale, 2002).

The present study aims to estimate the macroparasite load and detect if possible a relationship between parasite numbers and the expression of key genes involved in the physiology of the eels during their continental life. This period is characterized by 3 major physiological challenges: (1) the response to environmental stress such as temperature or pollution; (2) effective osmoregulation, which is of some importance in the highly changing ecosystems represented by coastal lagoons; and (3) the metamorphic process (silvering), a fundamental phase in the eel life cycle, which marks the starting point of the migration to the Sargasso sea for reproduction. The response to environmental stress was tested by estimating the expression of the Heat-Shock Proteins (HSPs) and Metallothioneins (MTs) genes in the

liver. The osmoregulation capacity of eels was estimated by examining the level of the ß Thyroid Hormone Receptor (ßTHR) and the Na^+/K^+ATPase gene expressions in the liver and gills, respectively. Finally, the success of the silvering process was evaluated by analyzing the expression level of the ßTHR and the rodopsin genes in the liver and eyes, respectively. Considered together, we attempted to focus on key genes that, if over- or under-expressed in relation to the presence of the parasite, may alter the host's homeostatic equilibrium.

MATERIALS AND METHODS

Sample collection

Ninety-three eels were collected by professional fishermen in Salses-Leucate Lagoon (France, 42°50'N, 03°00'E). Eels were brought to the laboratory alive and maintained in freshwater (for no more than 36 hr after capture) before analysis. It is possible that these holding conditions may influence gene expression. However, since eels were randomly chosen at the time of dissection, we assumed that uninfected and infected eels were both affected by the same stressful conditions.

Each fish was measured (total length) to the nearest mm. Age was estimated by counting the winter rings on sagital otoliths (Lecomte-Finiger, 1985). The liver, the 4 arches of the same side of the gills, and the eyes, were immersed in liquid nitrogen and stored at –80 C for molecular analysis. The parasites of the swim bladder were counted during the dissection. Later, the 4 arches of the other side of the gills and the digestive tract were examined using a binocular microscope. All the parasites were recovered. For each parasite taxon, some individuals were mounted under a cover slide for further identification. Species identification was done when possible and classical epizootiological parameters were enumerated (Bush et al., 1997), i.e., prevalence is the percentage of infected hosts, mean abundance is the total number of individuals of a parasite species divided by the total number of dissected hosts, individual intensity is the number of individuals of a parasite species in an

infected host, and mean intensity is the total number of individuals of a parasite species divided by the number of infected hosts.

Target genes

HSPs are ubiquitous molecular chaperones expressed in response to a wide range of stressors (Feder and Hofmann, 1999). In teleost fishes, it has been shown that the HSP70 gene expression can be induced by elevated temperatures, heavy metals, pesticides, and hydrocarbons (Sanders, 1993; Iwama et al., 1998). Among the Salmonidae, the HSP70 are involved in the immune response against pathogenic bacteria (Ackerman and Iwama, 2001). MTs are ubiquitous intracellular proteins, which present a strong affinity for metallic ions and thus are involved in detoxification processes. In teleost fishes, the Metallothionein (MT) gene expression is induced by heavy metals (de Smet and Blust, 2001; Langston et al., 2002), free radicals (Kling and Olsson, 2000), and cold thermal shock (Hermesz et al., 2001).

In teleost fishes, THs and ßTHR are involved in growth control, osmoregulation (Parker and Specker, 1990), and metamorphosis (Inui and Miwa, 1985). βTHR is a protein ligand of T_3 and T_4 Thyroid Hormones (THs), which can activate or repress TH gene transcription (Wu and Koenig, 2000). Na^+/K^+ATPase is a transmembranous enzyme that uses the energy liberated from ATP hydrolysis to exchange extracellular K^+ with intracellular Na^+ (Møller et al., 1996). In euryhaline fishes such as the European eel, it plays a major role in the osmoregulatory processes (Cutler et al., 2000).

In the European eel, vision adaptation to oceanic water occurs during the silvering. Freshwater rodopsin (FWO) and the deep-sea rodopsin (DSO) are retinal proteins that permit vision adaptation to the different spectral radiation, respectively, of freshwater and seawater fishes (Pankhurst and Lythgoe, 1983). The differential expression of the FWO and the DSO genes control the progressive switch between these 2 proteins (Wood and Partridge, 1993; Hope et al., 1998; Zhang et al., 2000).

Molecular analysis

Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. RNA concentrations were read spectrophotometrically. Five µg of RNA were used to synthesize cDNA in a total volume of 20 µl containing 1X First-Strand Buffer, 0.5 µg oligo dT as primer, 0.5 mM dNTPs, 10 mM DTT, 40 U RNaseOUT (Invitrogen), 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen), and DEPC water, according to the manufacturer's protocol and stored at –20 C until use.

Real-time PCR was carried out using a LightCycler (Roche, Basel, Switzerland); reactions were set up in microcapillaries using the following concentrations in a final volume of 15 µl, i.e., 0.5 µM of each primer, 4 mM of $MgCl_2$, 1 x SYBR Green master mix, and 5 µl cDNA. Primers for the Real-time PCR were designed using the Light Cycler Probe Design software (Roche, PE Applied Biosystems, Mannheim, Germany). Available sequences of anguillid species were used to design primers for the ß-actin (house-keeping gene), Na^+/K^+ATPase, ßTHR, and FWO. Alignment of other sequences from teleost species was used to design primers for HSP70 and MT. HSP70 sequences from *Oryzias latipes* (Accession number ORZHSC70), *Carassius auratus* (Accession number AB092839), *Silurus meridionalis* (Accession number EF406131), and *Danio rerio* (Accession number NM 131397) were used. MT sequences from *Cyprinus carpio* (Accession number AF001983), *Sparus aurata* (Accession number U58774), *Danio rerio* (Accession number NM131075) and *Paralichthys olivaceus* (Accession number EF406132) were also employed. In both cases, sequences were aligned and primers were designed using the conserved region. For the DSO gene, we employed primers defined by Zhang et al. (2000). Primer characteristics and amplicon lengths are shown in Table I. 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 I), and a specific time extension at 72 C (see Table I), with fluorescence measured at the end of each 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 (5 µl of H_20 was used). Since the amplified portion of the gene is generally conserved at the species level, we did not sequence the target gene products after amplification. The specificity of the amplification of each sample was ensured by examination of the melting curves. Choice of the reference gene in real time RT-PCR is always difficult to undertake, as no single gene has a constant expression level. However, in fish studies β-actin could be considered as one of the most stable (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: $\Delta Ct = \text{mean } Ct_{\text{target gene}} - \text{mean } Ct_{\text{ß-actin}}$; the effect is a negative relationship between the ΔCt value and expression level (EL) of target genes. PCR efficiency (E) was calculated as: $E = 10^{(-1/\text{slope})}$ (slope was calculated performing a PCR on serial dilutions of a pool of cDNA). When $E \geq 1.95$, the relative ratio of the target gene expression (relR) between 2 samples "a" and "b" was calculated as: $relR = 2^{-\Delta\Delta Ct}$ with $\Delta\Delta Ct = \Delta Ct_a - \Delta Ct_b$. When $E < 1.95$, relR was corrected by E as:

$relR = E_{target\ gene}^{Ct_{target\ gene(a)} - Ct_{target\ gene(b)}} \; x \; E_{actin}^{Ct_{target\ gene(b)} - Ct_{target\ gene(a)}}$, according to the manufacturer recommendations. In our work, all E values were found to be higher than 1.95, except for the FWO gene ($E_{FWO} = 1.91$).

Statistical analysis

Parasite species for which prevalence was less than 5% were not incorporated in the analysis. Samples for which difference between duplicates was more than 0.2 cycles were removed from statistical analysis. Non parametric Mann-Whitney *U*-tests were performed to

compare target gene EL between 2 samples (uninfected eels and infected eels). Stepwise multiple regressions were used to test the relationship between target gene EL and continuous variables (Scherrer, 1994). Normality of dependent variables was tested with Shapiro-Wilks test. All distributions of gene ΔCts were normally distributed (P>0.05) except for that of ΔCt DSO. This last gene was, therefore, removed from the multiple regression analysis. Tested variables included: eel standard length, eel age, and intensity of parasite species and classes for which prevalence was more than 5%. The absence of high co-linearity between the independent variables was assessed by the partial correlation coefficients, which were all < 0.80 (Scherrer, 1994).

RESULTS

Biological, morphological, and epizootiological characteristics

The age and the total length of the 93 European eels that were sampled, ranged from 2 to 8 yr (mean ± standard deviation = 4.5 ± 1.2 yr) and from 190 to 480 mm (317 ± 50 mm), respectively. The epizootiological results are presented in Table II. We found 11 species of macroparasites, i.e., 1 Nematoda, 2 Acantocephala, 1 Monogenea, and 7 Digenea. The prevalences of the different species of digeneans ranged from 1.1% for species 1 and 2 (for which only 1 fish was found to be infected), to 51.1% for *Lecithochirium gravidum*. Digeneans were also characterized by having the highest intensities, in particular *Prosorhynchus aculeatus* (688 parasites in 1 intestine). Of the 7 digenean species, 5 were niche specific (stomach for digenean species 1 and 2, and intestine for *Prosorhynchus aculeatus*, *Bucephalus* sp., and *Deropristis inflata*), and 2 (*L. gravidum* and *Helicometra* sp.) were not.

Expression level of target genes and presence of parasites

Table III shows the ΔCt values of uninfected and infected fish with each of the parasite class for the HSP70, MT, ßTHR, Na^+/K^+ATPase, FWO, and DSO genes. Expression of the

HSP70 gene was lower in the liver of fish when digeneans were present in the stomach as compared to fish without digeneans in their stomach ($n_{uninfected}$ = 40, $n_{infected}$ = 38, U = 554, P = 0.04) and when at least 1 digenean was present relative to fish without any digenean ($n_{uninfected}$ = 23, $n_{infected}$ = 55, U = 417, P=0.02). Fish that were uninfected by digeneans had 1.5 and 1.7 times (relR) higher levels of liver HSP70 gene expression than infected fish. Liver MT gene expression was significantly higher when *A. crassus* was present ($n_{uninfected}$ = 69, $n_{infected}$ = 10, U = 147, P=0.004). Fish that were infected with *A. crassus* had a MT gene expression level that was 3.8 times higher than that of uninfected fish. Moreover, MT gene expression was significantly lower when digeneans were present in the stomach as compared to fish without digeneans in their stomach ($n_{uninfected}$ = 40, $n_{infected}$ = 39, U = 535, P=0.02) and when fish were infected by at least 1 digenean compared to fish without digeneans ($n_{uninfected}$ = 24, $n_{infected}$ = 55, U=433, P=0.02). Fish that were uninfected by digeneans had 2.0 and 2.2 times higher levels of MT gene expression than infected fish.

Liver βTHR gene expression was significantly lower when digeneans were present in the stomach when compared to fish without digeneans in their stomach ($n_{uninfected}$ = 41, $n_{infected}$ = 39, U=455, P<0.001) and when at least 1 digenean was present relative to fish without any digenean ($n_{uninfected}$ = 24, $n_{infected}$ = 56, U=343, P<0.001). Uninfected fish had 2.2 and 2.3 times higher levels of ßTHR gene expression than fish infected with digeneans.

The levels of gill Na^+/K^+ATPase gene expression were significantly lower when *A. crassus* was present relative to fish without *A. crassus* ($n_{uninfected}$ = 67, $n_{infected}$ = 8, U=93, P=0.003). The uninfected fish had 2.4 times the level of Na^+/K^+ATPase gene expression of the infected fish.

The levels of DSO gene expression in the eyes were significantly greater when *A. crassus* was present ($n_{uninfected}$ = 39, $n_{infected}$ = 5, U=42, P=0.04). Fish infected with *A. crassus* had 16.7 times the level of DSO gene expression when compared to uninfected fish.

Moreover, DSO gene expression was significantly lower when digeneans were present in the stomach relative to fish without digeneans in their stomach ($n_{uninfected}$ = 20, $n_{infected}$ = 24, *U*=150, *P*=0.03) and when at least 1 digenean was present relative to fish without any digenean ($n_{uninfected}$ = 10, $n_{infected}$ = 34, *U*=99, *P*=0.04). Fish that were uninfected by digeneans had 5.7 and 13.1 times the level of DSO gene expression when compared to infected fish. There were no other statistically significant differences in gene expression for the remaining comparisons between infected and uninfected fish.

Relationships between expression level (EL) of target genes and intensity of parasites or biological parameters of the eel

Most of the tested variables were not significantly correlated with levels of target gene expression. When the regression coefficient is positive, the gene expression is negatively correlated with the corresponding variable. It is the opposite when the regression coefficient is negative (Table IV).

We found significant positive correlations between: (1) ΔCt_{HSP70} and the increasing age of the eels; (2) ΔCt_{MT} and *Bucephalus* sp. intensity; and (3) $\Delta Ct_{\beta THR}$ and both the age of the eels and *L. gravidum* intensity.

DISCUSSION

The parasite richness reported in our study is typical of that found in European eels in Spain (Maillo et al., 2000), Germany (Sures et al., 1999), Italy (Kennedy et al., 1997; 1998), Belgium (Schabuss et al., 1997), England (Kennedy, 1997), Denmark (Køie, 1988), and Ireland (Conneely and McCarthy, 1986).

Molecular analyses revealed significant correlations between the macroparasite community and the expression of all but 1 (FWO gene) of the target genes. The study of the response to environmental stresses revealed 2 different patterns of gene expression between eels infected by digeneans and the ones by the nematode *A. crassus*. In the presence of

digeneans, both HSP70 and MT gene expressions were significantly reduced. Furthermore, there was a significant correlation between the intensity of infection with the digenean *Bucephalus* sp. and reduced MT gene expression. Lower levels of expression of these genes in the presence of digeneans may influence the ability of eels to survive when confronted with changes in their environment, i.e., temperature variation, pollution. Our data suggest that the presence of digeneans may have an effect on stress-related physiology. In the present study, fish infected with *A. crassus* had higher levels of liver MT gene expression when compared to uninfected fish. One explanation for this result could be related to the parasite's life cycle involving small fish as paratenic hosts (Belpaire et al., 1989). Thus, trophic activity of the eel should increase the likelihood of being parasitized when consuming infected intermediate hosts, thereby facilitating bioaccumulation of pollutants. Thereafter, MT gene expression would probably be higher in eels infected with *A. crassus*.

The study of the osmoregulation revealed that lower levels ßTHR and Na^+/K^+ATPase gene expression were correlated with the presence and the intensity of digeneans for the ßTHR and with the presence of *A. crassus* for the Na^+/K^+ATPase. Reduced ßTHR gene expression in the presence of digeneans supports our previous interpretation regarding the negative impact of digeneans on the ability of eels to respond to environmental stresses. Among all other possible stresses encountered by eels during their life, parasites seem also to play a role in their ability to osmoregulate properly. The lower levels of expression of the Na^+/K^+ATPase gene may appear in contradiction with the absence of any relationship between the plasma ion concentration and the presence of the nematode *A. crassus* found by Kelly et al. (2000). However, our study focused on mRNA quantification, i.e., a pre-transcriptional level, while the Kelly et al. (2000) investigation was undertaken at the post-transcriptional level by quantifying the action of the transmembranous protein. Regulation processes during the protein synthesis may have induced the difference between the results.

In the study on silvering, none of the parasites present was found to significantly influence FWO gene expression. However, expression of the DSO gene was significantly lower when digeneans were present. Modification of the host behavior by parasites with complex life cycles has been largely reported when the host is an intermediate host (Moore, 2002; Sasal and Thomas, 2005). Unfortunately, examples when considering fish as the final host are rare. When eels begin their sexual silvering, in addition to the switch between FWO and DSO gene expressions generally observed, there is also a degeneration of the digestive tract (Pankhurst and Sorensen, 1983). We hypothesize that, in terms of number of eggs laid, it should be more advantageous for the digeneans in the digestive tract to delay the more possible the eel migration. Although we did not observe intestine degeneration on the dissected eels, we also believe that the presence of digeneans in eels, coupled with the higher gene expression, could be more a consequence than the cause of this difference. In any case, it is interesting to note that the gene was, on average, 13.1 times more highly expressed in eels uninfected with digeneans; more work is needed to unravel this relationship. Finally, we found that fish infected by *A. crassus* had higher DSO gene expression than uninfected ones. The high virulence of this invasive nematode (see synthesis in Kirk, 2003) should act as a strong selection pressure on eels. We thus hypothesize that infected eels could anticipate their pre-migratory metamorphosis while their swim bladder is still functional. An alternative explanation would be that infected eels spend more time in brackish systems or saltwater as the parasite usually prefers freshwater (Kirk et al., 2000). In both cases, there would be an increase of DSO gene expression. An experimental study with changing salinities is required to provide more information on this effect.

ACKNOWLEDGMENTS

This work was financially supported by the French Ministère de l'écologie et du développement durable, the CNRS, and the University of Perpignan. G.F. was financially

supported by a grant from the French Ministère de l'enseignement supérieur et de la recherche. Thanks are due to M. Cyprien, professionnal fisherman in Salses-Leucate Lagoon, for providing eels.

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Table 1. Sequences, amplicon sizes, annealing temperature and extension time of the primers used for target gene amplification. F: forward, R: reverse. *: HSP70 and MT primers were designed into conserved regions of at least four teleost cDNA sequences. HSP70 and MT cDNA sequences of *Danio rerio* are shown as references. Ampl. size: Amplicon size, Ann.: Annealing, Ext.: Extension

Target gene	Tissue	Accession No	Primers	Sequences	Ampl. size (in bp)	Ann. (in °C)	Ext. (in s)
Actine	liver, gills, eyes	AB074846	Actin F	5'-CGGAATCCACGAGACC-3'	205	48	12
			Actin R	5'-TCCAGACGGAGTATTTGC-3'			
Heat Shock Protein 70	liver	NM131397*	HSP70 F	5'-ACCCAGACCTTCACCACCTACTC-3'	377	67	20
			HSP70 R	5'-CTTCATGTTGAAGGCGTAGGACTC-3'			
Metallothionein	liver	NM131075*	MT F	5'-CCAAGACTGGAGCTTGCAACTG-3'	120	60	10
			MT R	5'-CAGCCAGAGGCGCACTTGCTG-3'			
β Thyroid Hormone Receptor	liver	AF302241	βTHR F	5'-CACTAACGCTCAGGGC-3'	229	60	15
			βTHR R	5'-CGCATGGTAACTCACAG-3'			
Na^+/K^+ATPase	gills	AJ239317	NaK F	5'-CACAATGAACACTGAGCTGC-3'	157	60	8
			NaK R	5'-GAAATGGGGGAGAGAGAAAG-3'			
Deep Sea Opsin	eyes	AJ249203	DSO F	5'-TTTGTGGTGGGTCGGTGCCTTATGC-3'	446	60	20

Fresh Water Opsin	eyes	AJ249202	DSO R	5'-ACTTCTCCATCCCCCTCACCATT-3'	319	60	17
			FWO F	5'-CATCTCATTCCTGGTC-3'			
			FWO R	5'-TCGTGGTAATATGCCGTG-3'			

Table 2. Epidemiological parameters, calculated according to Bush et al. (1997), of the parasite species found on 93 European eels *Anguilla anguilla* at Salses-Leucate lagoon (Pyrénées-Orientales, France). SB: swimbladder, S: stomach; I: intestine, G: gills, M. Ab. ± S.D.: mean abundance ± standard deviation, P.%: prevalence in percent, M. Int .± S.D. (min-max): mean intensity ± standard deviation (minimal and maximal values of individual intensity)

PARASITE		Niche	M. Ab. ± S.D.	P. %	M. Int. ±S.D. (min-max)
Class	Species				
Nematoda	*Anguillicola crassus*	SB	0.32 ± 1.48	10.6	3.00 ± 3.68 (1-13)
Acantocephala	*Acantocephalus anguillae*	I	0.10 ± 0.64	4.3	2.25 ± 2.50 (1-6)
	Acanthocephala species 1	I	0.19 ± 1.86	1.1	18
Monogenea	*Pseudodactylogyrus anguillae*	G	1.00 ± 5.97	6.4	15.67 ± 19.67 (1-53)
Digenea	*Lecithochirium gravidum* (Hemiuridae)	S	4.19 ± 14.79	44.7	9.38 ± 21.12 (1-134)
		I	0.09 ± 0.35	6.4	1.33 ± 0.52 (1-2)
		S+I	4.28 ± 10.63	51.1	8.38 ± 19.91 (1-134)
	Helicometra sp. (Opecoelidae)	S	0.18 ± 0.83	6.4	2.83 ± 1.94 (1-6)
		I	0.87 ± 2.67	23.4	3.73 ± 4.53 (1-17)
		S+I	1.05 ± 2.00	27.7	3.50 ± 4.10 (1-17)
	Prosorhynchus aculeatus (Bucephalidae)	I	20.18 ± 91.45	22.3	90.05 ± 179.54 (1-688)

Bucephalus sp. (Bucephalidae)	I	0.65 ± 2.07	13.8	4.69 ± 3.54 (1-13)
Deropristis inflata (Acanthocolpidae)	I	2.32 ± 12.39	16.0	14.53 ± 28.81 (1-109)
Digenea species 1	S	0.05 ± 0.52	1.1	5
Digenea species 2	S	0.01 ± 0.10	1.1	1

Table 3. Mean ΔCt values of uninfected and infected fishes with each of the parasite class, for each of the six target genes. * indicates that ΔCt values are significantly different between uninfected and infected eels (Mann-Whitney test, $p<0.05$). n.s: not significant, n.t: not tested because of a too small sample size, SB: swimbladder, G: gills, S: stomach; I: intestine, S.D.: standard deviation

Parasite class (niche)	Status	Mean ΔCt target gene ± S.D.											
		HSP70		MT		βTHR		Na^+/K^+ ATPase		FWO		DSO	
Nematoda	Uninf.	1.36±1.51	n.s	1.80±2.07	*	0.07±1.48	n.s	0.03±1.28	*	-0.95±0.95	n.s	1.94±3.64	*
(SB)	Inf.	1.52±1.09		-0.11±1.20		0.00±1.69		1.23±0.59		-0.98±0.75		-2.12±4.68	
Monogenea	Uninf.	1.37±1.48	n.s	1.64±2.11	n.s	0.09±1.54	n.s	0.08±1.26	n.s	-0.95±0.94	n.t	1.88±3.19	n.t
(G)	Inf.	1.47±1.40		0.55±1.33		-0.31±0.83		1.09±1.17		-0.92±0.85		-7.45±0.61	
Digenea	Uninf.	1.07±1.54	*	1.03±2.01	*	-0.48±1.30	*	0.29±1.60	n.s	-1.15±0.86	n.s	-0.12±4.64	*
(S)	Inf.	1.69±1.32		2.09±2.03		0.64±1.49		0.02±0.80		-0.77±0.95		2.38±2.76	
Digenea	Uninf.	1.11±1.48	n.s	1.42±2.05	n.s	-0.13±1.56	n.s	0.02±1.38	n.s	-1.09±0.89	n.s	0.84±4.38	n.s
(I)	Inf.	1.66±1.40		1.70±2.12		0.26±1.42		0.32±1.16		-0.83±0.95		1.66±3.40	

Digenea	Uninf.	0.83±1.43	*	0.77±2.03	*	-0.77±1.17	*	0.00±1.68	n.s	-1.32±0.91	n.s	-1.62±5.42	*
(S+I)	Inf.	1.60±1.43		1.90±2.02		0.42±1.49		0.25±1.08		-0.83±0.91		2.09±2.92	

Table 4. Significant parameters of the stepwise multiple regressions. RC: regression coefficient, R^2: multiple correlation coefficients. Differences in sample size (N) are due to individuals from which molecular results were not available (high difference between duplicates, non specific amplification curves …). I: intestine, S: stomach

ΔCt target gene	Variables	N	RC	R^2	p
ΔCt HSP70	age	74	0.56	0.22	< 0.001
ΔCt MT	*Bucephalus* sp.	76	0.25	0.07	< 0.05
ΔCt βTHR	age	76	0.40	0.11	< 0.01
	L. gravidum (I+S)		0.08	0.20	< 0.001
ΔCt Na^+/K^+ATPase	(none significant)	70			
ΔCt FWO	(none significant)	53			
ΔCt DSO	total length	46	-0.40	0.26	< 0.001
	A. crassus		-0.51	0.37	< 0.001
	Helicometra sp. (I)		0.45	0.43	< 0.001