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Encapsulation of *Anguillicola crassus* reduces the abundance of adult parasite stages in the European eel (*Anguilla anguilla*)

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Abstract

Encapsulation of the parasitic nematode *Anguillicola crassus* Kuwahara, Niimi & Hagaki is commonly observed in its native host, the Japanese eel (*Anguilla japonica* Temminck & Schlegel). Encapsulation has also been described in a novel host, the European eel (*A. anguilla* L.), and there is evidence that encapsulation frequency has increased since the introduction of *A. crassus*. We examined whether encapsulation of *A. crassus* provides an advantage to its novel host in Lake Müggelsee, NE Germany. We provide the first evidence that encapsulation was associated with reduced abundance of adult *A. crassus*. This pattern was consistent in samples taken 3 months apart. There was no influence of infection on the expression of the two metabolic genes studied, but the number of capsules was negatively correlated with the expression of two *mhc II* genes of the adaptive immune response, suggesting a reduced activation. Interestingly, eels that encapsulated *A. crassus* had higher abundances of two native parasites compared with non-encapsulating eels. We propose that the response of *A. anguilla* to infection by *A. crassus* may interfere with its reaction to other co-occurring parasites.

KEYWORDS

Anguilla anguilla, *Anguillicola crassus*, gene expression, invasive parasite, parasite community

1 | INTRODUCTION

Invasion by non-native parasites can affect the viability of novel hosts (Daszak et al., 2000; Peeler et al., 2011), posing strong selection pressure on the host to adapt (Penczykowski et al., 2011). Adaptation to novel parasites has been suggested for a number of species. For example, a Hawaiian honeycreeper species shows signs of increased tolerance to avian malaria after severe population declines following the parasite's introduction (Atkinson et al., 2013). Both increased tolerance and increased resistance were reported in blue mussels after the introduction of a parasitic copepod (Feis et al., 2016). Increased resistance was also observed in a rainbow trout population in response to an invasive myxozoan parasite (Miller & Vincent, 2008).

Anguillicola crassus Kuwahara, Niimi & Hagaki is a parasitic swim bladder nematode that is invasive in the European eel (*Anguilla anguilla*, L.). It was first detected in European freshwaters in 1982, and it has rapidly spread across the entire range of its new host (Kirk, 2003). Infections with the parasite were suggested to hamper the trans-oceanic spawning migration and reproduction of the European eel (Palstra et al., 2007; Pelster, 2015; Sures & Knopf, 2004). Thus, it may contribute to the dramatic population decline observed in recent decades (Bornarel et al., 2017; Diekmann et al., 2019; Drouineau et al., 2018) and the European eel's status as critically endangered (Jacoby et al., 2015). Consequently, European eel individuals with an effective defence against *A. crassus* should have an advantage over non-responders.

In naturally infected Japanese eels (*A. japonica* Temminck & Schlegel), the parasite's original host, capsules of dead *A. crassus* larvae are found in the swim bladder wall (Heitlinger et al., 2009; Münderle et al., 2006). Infection experiments with the Japanese eel and the European eel indicate that the original host is more effective

in defending against *A. crassus*, resulting in killing and encapsulating a large proportion of larvae (Knopf & Lucius, 2008; Knopf & Mahnke, 2004; Weclawski et al., 2013). Encapsulation of helminth parasites has been described as a defence mechanism involving an immune response in several fish species, including eels (Dezfuli et al., 2015, 2016). Naturally infected European eels can encapsulate *A. crassus* larvae, and the immune system was shown to take part in this process (Molnár, 1994). However, the associated costs to the host are not known. Nonetheless, the frequency of encapsulation increased from 0% in 1990 to 20% in 1997 and 2000 in Flanders, Belgium (Audenaert et al., 2003). This suggests that the European eel may be capable of developing strategies to cope with the novel parasite.

European eels infected with *A. crassus* are susceptible to adverse environmental conditions (Molnár et al., 1991). Mortality during hypoxia increases with severity of infection (Lefebvre et al., 2007; Molnár, 1993). Infected eels consume more oxygen during activity than non-infected, thus having a higher energy demand (Palstra et al., 2007). This may be due to increased allocation of resources to the immune system and resource consumption by the parasite itself. Consistent with higher oxygen demand with infection severity, the expression of the haemoglobin α gene was correlated with parasite biomass in experimental infections (Fazio et al., 2009). Down-regulation of several cytochrome genes of the cell respiration pathway indicated that energy provision may be compromised in experimentally infected European eels, which may be due to alteration of resource allocation in infected individuals (Bracamonte, Johnston, Monaghan, et al., 2019).

Populations regularly differ in their resistance to parasites, and differences appear to be related to the degree of exposure and adaptation (MacColl & Chapman, 2010; Weber et al., 2017). Populations that are adapted to a particular parasite have been observed to mount a stronger immune response when challenged with that parasite (Kalbe & Kurtz, 2006; Scharsack & Kalbe, 2014). Increased immune gene expression has been associated with higher resistance in fish (Lenz et al., 2013; Lohman et al., 2017), birds (Bonneaud et al., 2011) and mammals (Guo et al., 2016). At the same time, there is evidence that the expression of non-immune genes is differently affected in populations that differ in parasite resistance (Bonneaud et al., 2011). Additionally, individuals that are better at coping with a particular parasite tend to grow more, have better body conditions and have higher metabolic condition when infected, suggestive of reduced metabolic and energetic costs (Kalbe & Kurtz, 2006; Kurze et al., 2016; MacColl & Chapman, 2010). Based on these observations in other species, we hypothesize that encapsulating *A. crassus* leads to lower infection intensities, an increased immune response and reduced metabolic costs in the European eel.

From a natural population of European eels in Lake Müggelsee, Berlin, Germany, we identified *A. crassus* infection intensity and macroparasite community composition and compared them between eels encapsulating *A. crassus* and those not encapsulating it. We further tested for temporal variation of these parameters between August and October. We chose these two dates to determine whether the observed pattern was temporally stable, because *A. crassus* infections varied temporally in some locations (Lefebvre et al., 2002; Schabuss et al., 2005) but not in others (Kennedy & Fitch, 1990; Würtz et al., 1998) and we had no information about the situation in Lake Müggelsee. We used quantitative PCR to test whether eels encapsulating and those not encapsulating the parasite differed in immune (*mhc II*), energy-related (*cox1*) and haematopoietic (*epor*) gene expression, suggestive of an increased immune response and reduced metabolic costs (see Section 2 for information on target genes). As for infection intensity and parasite community, we tested whether gene expression responses showed temporal variation. Genes were selected based on differential expression in transcriptome-wide expression studies on European eels and Japanese eels experimentally infected with *A. crassus*. *Mhc IIA* and *mhc IIB* both had altered expression profiles in experimentally infected European eels (Bracamonte, Johnston, Knopf, et al., 2019; Bracamonte, Johnston, Monaghan, et al., 2019). We hypothesized that encapsulation in a natural population may trigger an immune response and lead to increased expression of *mhc II* genes. *Cox1* expression was reduced in European eels experimentally infected with *A. crassus* (Bracamonte, Johnston, Monaghan, et al., 2019). We hypothesized that the disruption of the energy balance would be mitigated by encapsulation, resulting in higher expression of *cox1*. The expression of *epor* was increased in Japanese eels following *A. crassus* infections (Bracamonte, Johnston, Monaghan, et al., 2019). We expected increased expression in more heavily infected European eels, especially in the presence of blood-feeding adults. Furthermore, we expected reduced expression in individuals encapsulating *A. crassus* if encapsulation led to reduced infection intensity.

2 | MATERIALS AND METHODS

2.1 | Sampling

European eels were caught by electrofishing near Surferwiese (52.448°N 13.656°E) in Lake Müggelsee, Germany, on 8 August 2017 ($n = 13$) and on 10 and 17 October 2017 ($n = 25$). Electrofishing for sampling the fish for this study was approved by the responsible fisheries authority (Fischereiamt Berlin). Eels were immediately decapitated, immobilized by destruction of the spinal cord and kept on ice for transportation back to the laboratory. Dissections were carried out approximately 1 hr after electrofishing. The spleen and the head kidney were removed and stored at −20°C in RNA*later* (Life Technologies) following the manufacturer's instructions. For all individuals, weight was determined to the nearest g and total length (TL) to the nearest 0.5 cm. Relative condition factor (K_{rel}) was calculated according to Le Cren (1951) using the values available from FishBase (Froese & Pauly, 2019). *Anguillicola crassus* in the swim bladder and other parasites on the gills, in the gut, the anal fin and the eyes were counted using a stereomicroscope (7×–70× magnification). The intestinal cestodes *Bothriocephalus claviceps* and *Proteocephalus macrocephalus* were combined, because they were assumed to affect

their host in a similar way and because they could not always be distinguished during dissection. Similarly, the gill monogeneans *Pseudodactylogyrus bini* and *Pseudodactylogyrus anguillae* were not distinguished. Cysts formed by the myxozoans *Myxobolus portucalensis* on the anal fin and *Myxidium giardi* on the gills and the gill monogenean *Pseudodactylogyrus* spp. were categorized into abundance classes of 0, 1–5, 6–20 and >20 (on the anal fin or per gill arch; Table S1). The number of encapsulated *A. crassus* larvae (Figure S1) in the swim bladder wall was recorded. We performed PCRs on a subset of capsules following Heitlinger et al. (2009). We checked the size of PCR products on a gel to confirm that the capsules contained *A. crassus* tissue.

2.2 | Analysis of parasite communities

All analyses were done in R v3.5.3 (R Core Team, 2019). Prevalence, mean infection intensity and mean abundance of larval *A. crassus* in the third (L_3) and fourth (L_4) larval stages, adults and all stages combined (simply referred to as *A. crassus*) were calculated for all eels and separately for each month. We determined the prevalence of encapsulated larvae. We also calculated infection intensity and abundance of larval and adult stages only including eels that contained living *A. crassus* in the swim bladder and for which the encapsulation status (presence/absence of capsules) could be determined unambiguously ($n = 32$). For these eels, we used Wilcoxon rank-sum tests to estimate whether weight and length differed between encapsulation status or sampling month. Six eels either did not contain living *A. crassus* in their swim bladders or were of uncertain encapsulation status and were excluded from further analyses (i.e. parasite community analysis and gene expression analysis; Table S1).

We assessed differences in total abundance, larval abundance and adult abundance of *A. crassus* with generalized linear models (GLMs) that included encapsulation status, sampling month and their interaction as factors, each with a negative binomial distribution with a log-link function using the glm.nb function of the MASS package. Model assumptions were tested with the DHARMa package v0.3.2.0 (Hartig, 2020). We further used GLMs with the same parameters to assess whether abundances were a function of the number of capsules rather than the encapsulation status. We ran these latter models including all individuals and including only individuals encapsulating *A. crassus*. We correlated the total number of *A. crassus*, the number of larval *A. crassus* and the number of adult *A. crassus* with weight and TL using Spearman's rank correlation tests with the cor.test function in base R. We performed these tests once for all eels and then separately for eels sampled in August and October and for eels of the non-encapsulating group (i.e. without capsules, NC) and the encapsulating group (i.e. with at least one capsule, C).

For eels with unambiguous encapsulation status, prevalences and, if applicable, mean abundances and mean intensities for all other parasites were estimated overall and separately for August and October and for each encapsulation status (NC and C). GLMs were used to test whether prevalences of each parasite differed between encapsulation status and sampling month applying a binomial distribution with a logistic link function. Similarly, GLMs with a Poisson distribution with a log-link function were used to test for differences in abundances. Differences in parasite community composition, excluding *A. crassus* and species with <10% overall prevalence, were determined with an analysis of similarity (Clarke, 1993) on Bray–Curtis distances using the function anosim of the vegan package v2.4–4 (Oksanen et al., 2019) with 100,000 permutations. Parasite communities were compared between the two months and the two encapsulation status. Species that contributed most to parasite community dissimilarities were identified with a similarity percentage analysis (simper) implemented in the vegan package. For visualization, non-metric multidimensional scaling plots were produced with the function metaMDS of the vegan package.

2.3 | RNA extraction and cDNA synthesis

RNA was extracted from spleen and head kidney tissue as described in Bracamonte, Johnston, Monaghan, et al. (2019) and quantified on a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Remnant DNA was removed from RNA extracts with DNase I, Amplification grade (Thermo Fisher Scientific) following the manufacturer's instructions. Purified RNA was reverse-transcribed in duplicates with MMLV High Performance Reverse Transcriptase (Biozym) following the manufacturer's instructions. For quantitative real-time PCR (qPCR), duplicate reverse transcriptions were pooled.

2.4 | Target genes

The selected genes responded to experimental infection with *A. crassus* in previous studies, based on transcriptome-wide gene expression (Bracamonte, Johnston, Knopf, et al., 2019; Bracamonte, Johnston, Monaghan, et al., 2019). Genes had either increased expression (*mhc IIB* and *epor*) or decreased expression (*mhc IIA* and *cox1*) in the European eel or the Japanese eel (divergence time approx. 20 Mya; Minegishi et al., 2005). Furthermore, the genes are involved in physiological processes that were previously shown to respond to infection in the European eel (Fazio et al., 2009; Knopf & Lucius, 2008; Palstra et al., 2007). The major histocompatibility complex class II (MHC II) is essential for initiating an adaptive immune response against extracellular parasites, which ultimately results in highly specific antibody production (Morris et al., 1994). An MHC II molecule is composed of two chains encoded by genes *mhc IIA* and *mhc IIB*. *Mhc IIB* is usually more polymorphic, providing higher antigen specificity (Brown et al., 1993; Reche & Reinherz, 2003). However, in European eels *mhc IIA* may be equally variable (Bracamonte et al., 2015). Cytochrome c oxidase subunit 1 (COX1) is a core protein of the respiratory chain, which is responsible for energy generation (Hosler et al., 2006). The erythropoietin receptor (EPOR) is expressed on the progenitors of erythrocytes during

their maturation and promotes their proliferation and differentiation (Elliott et al., 2014).

2.5 | Primer design and qPCR

We carried out qPCR using a combination of newly designed and published primers (Table 1). We newly designed primers for *cox1*, *epor* and the housekeeping gene β-actin (*actb*) using sequences from two European eel transcriptome assemblies (Bracamonte, Johnston, Knopf, et al., 2019; Bracamonte, Johnston, Monaghan, et al., 2019) and other data as follows. For *cox1*, we included sequences of European eels and American eels (*A. rostrata*) available on NCBI (acc. nos: NC_006531.1 and NC_006547.2) and sequences from EeelBase (Coppe et al., 2010). For *epor*, no anguillid sequences were available in public databases; therefore, we included sequences of a Japanese eel transcriptome assembly for primer design (Bracamonte, Johnston, Monaghan, et al., 2019). For *actb*, we used a published reverse primer (Fazio, Mone, et al., 2008) and a new forward primer designed using European eel and Japanese eel sequences available on NCBI (acc. nos: DQ286836.1, KJ021893.1 and GU001950.1) and EeelBase (Coppe et al., 2010). Primers for both *mhc II* genes were modified from Bracamonte et al. (2015).

Primers were validated in regular PCRs using the Biozym Probe qPCR Kit (Biozym). PCRs were carried out in a volume of 20 µl containing 10 µl of 2x qPCR Probe Mix, 400 nM of forward and reverse primer, and 2 µl of cDNA for the genes *actb*, *cox1*, *mhc IIA* and *mhc IIB*. PCR for *epor* contained 500 nM of each primer. For genes *actb*, *cox1*, *mhc IIA* and *mhc IIB*, cycling conditions were as follows: initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 5 s and 65°C for 30 s, and a final elongation at 65°C for 10 min. For *epor*, the number of cycles was increased to 40. A Mastercycler nexus GSX1 (Eppendorf) was used for all PCRs. PCR products were purified and sequenced at Macrogen Europe. Sequences were aligned back to those used for primer design, and they were blasted against the nr protein database of NCBI for identity confirmation.

For the qPCRs, the reaction mix was identical to that used for regular PCRs (above) except that 0.0006 µl 10,000× SYBR Green I Nucleic Acid Gel Stain (Invitrogen) was added. Reactions were run on a Stratagene Mx3005P qPCR System (Agilent Technologies) with the cycling conditions described above, but omitting the final elongation. The number of cycles was set to 40 for all genes. qPCRs were run in duplicates, and a fivefold dilution series was added on each plate as standard curve. One plate was prepared for each gene and tissue containing all samples and duplicates. Ct values were determined with MxPro QPCR Software (Agilent Technologies) using default parameters. Samples for which Ct values between duplicates differed by more than 0.5 were repeated (mixed plates for genes and tissues).

2.6 | Gene expression analysis

Relative expression of the target genes was calculated for every sample following the ΔΔCt method of Pfaffl (2001) using *actb* as reference gene. The 1:5 dilution of the standard curve was used as a calibrator to standardize among plates. Since tissues are known to differ in gene expression, the spleen and the head kidney were analysed separately. Analyses were performed separately for every gene in R. GLMs were used to analyse relative gene expression changes as a function of encapsulation status, sampling month and their interaction. Model assumptions were tested as above. Tukey's HSD post hoc tests were performed for models with significant factors using the multcomp v1.4-8 (Hothorn et al., 2008) package. Relative gene expression changes were further analysed with infection intensity of *A. crassus* as continuous predictor, sampling month as a factor and their interaction. The same analyses were performed with the number of adult *A. crassus* as continuous predictor, sampling month as

TABLE 1 Oligonucleotide sequences used for PCRs and qPCRs and amplicon size

Gene	Primer name	Sequence 5'→3'	Amplicon size	Source
Actb	ACTBF2	GAGACCACCTTCAACTCC	196 bp	Present study
	Actin R	TCCAGACGGAGTATTTGC		Fazio, Mone, et al. (2008)
Cox1	COX1F2	CTACTCCTCTCCCTGCCAGT	150 bp	Present study
	COX1R2	GTATACTTCTGGGTGGCCGA		Present study
Epor	EPORF1	ACAATGACACGGACAGGGAA	142 bp	Present study
	EPORR1	CCTTCACCAATTCCCGCTTG		Present study
Mhc IIA	MHCIIAE3F	GATCCTCCTCAGAGCACAATCT	250 bp	Modified from Bracamonte et al. (2015)
	MHCIIAE3R	TGTGCTCCACGCTGCAGGAA		Modified from Bracamonte et al. (2015)
Mhc IIB	MHCIIBE3F3	TTCTACCCCAGAGGAATCAAAATGAC	167 bp	Bracamonte et al. (2015)
	MHCIIBE3R	TGCTCCACCTTGCAGGAGATTT		Modified from Bracamonte et al. (2015)

Abbreviation: bp, base pairs.

TABLE 2 Prevalence (P, %), mean intensity ± *SE* (I) and mean abundance ± *SE* (A) of *Anguillicola crassus* stages. The prevalence of encapsulated *A. crassus* is indicated. Parameters are given for all sampled eels (overall) and separately for each sampling month and each encapsulation status

		Sum			Larvae ($L_3 + L_4$)			Adults			Capsules
	n	P	I	A	P	I	A	P	I	A	P
Overall	38	89	8.7 ± 1.6	7.8 ± 1.5	79	6.8 ± 1.3	5.4 ± 1.2	55	4.3 ± 1.0	2.4 ± 0.8	39
August	13	92	7.8 ± 2.0	7.2 ± 1.9	85	6.1 ± 2.1	5.2 ± 2.0	46	4.3 ± 1.4	2.0 ± 1.0	46
October	25	88	9.2 ± 2.2	8.1 ± 2.1	76	7.3 ± 1.7	5.5 ± 1.5	60	4.3 ± 1.3	2.6 ± 1.0	36
NC[a]	19	100	10.2 ± 2.6	10.2 ± 2.6	84	7.2 ± 2.0	6.1 ± 1.8	79	5.1 ± 1.6	4.1 ± 1.4	0
C[a]	13	100	5.6 ± 1.5	5.6 ± 1.5	92	5.2 ± 1.6	4.8 ± 1.5	38	2.0 ± 0.5	0.8 ± 0.3	100

Abbreviations: C, encapsulating eels;*n*, number of eels examined; NC, non-encapsulating eels.

[a]Includes only infected individuals for which the encapsulation status could be determined unambiguously.

TABLE 3 GLM statistics for (a) infection intensity with *Anguillicola crassus* and (b) gene expression. Only parameters of interest are shown. Significant deviance values ($p < .05$) are indicated with bold text

	Group		Month		Group × month	
Response variable	Deviance	*p*-value	Deviance	*p*-value	Deviance	*p*-value
a						
Sum	3.65	0.056	0.19	0.66	1.18	0.28
Larvae	0.39	0.53	0.65	0.42	2.20	0.14
Adults	**9.24**	**0.002**	0.03	0.87	0.01	0.93
b						
Cox1 spleen	0.29	0.17	0.40	0.11	0.01	0.74
Cox1 head kidney	2.29	0.14	**6.56**	**0.012**	1.29	0.27
Epor spleen	0.49	0.56	**7.17**	**0.027**	<0.01	0.99
Epor head kidney	0.38	0.37	0.04	0.76	<0.01	0.97
Mhc IIA spleen	0.95	0.11	**5.49**	**<0.001**	1.19	0.07
Mhc IIA head kidney	0.41	0.44	0.20	0.59	**5.84**	**0.003**
Mhc IIB spleen	0.29	0.44	**2.94**	**0.014**	0.06	0.72
Mhc IIB head kidney	1.09	0.12	0.09	0.65	**2.18**	**0.028**

Note: Group = non-encapsulating versus encapsulating and Month = August versus October.

a factor and their interaction separately for the NC group and the C group. For the C group, an additional model included the number of capsules as continuous predictor, sampling month as a factor and their interaction. qPCR plate was included as a fixed factor in all models. The models for *mhc IIA* additionally included all two-way interactions with qPCR plate. For the *cox1* gene, residuals were normally distributed, and thus a Gaussian distribution was used. For the genes *mhc IIA*, *mhc IIB* and *epor*, a Gamma distribution was used with an inverse link function with few exceptions detailed below. For *epor* in the spleen, starting values of 0.5 for the intercept and 0 for the predictors were supplied to the model with capsules as continuous predictor. For *mhc IIA* in the spleen, a Gaussian distribution was used for all models including adult *A. crassus* as a predictor. For *mhc IIA* in the head kidney, a Gaussian distribution was used for the models of the C group including capsules or adult *A. crassus* as predictor. Spearman's rank correlation tests were used to assess correlation between the relative expression levels of *mhc IIA* and *mhc IIB*.

3 | RESULTS

Mean weight ± *SE*, mean TL ± *SE* and mean K_{rel} ± *SE* were 122 ± 15 g, 40.6 ± 1.5 cm and 0.948 ± 0.014, and they did not differ significantly between C and NC eels (Wilcoxon's rank-sum test, weight: $W = 97$, $p = .32$; length: $W = 94.5$, $p = .27$; K_{rel}: $W = 135$, $p = .67$). Weight and TL were greater in August than in October ($W = 171$, $p = .029$ for both weight and TL, August: 166 ± 26 g and 45.5 ± 2.6 cm, October: 100 ± 16 g and 38.0 ± 1.6 cm), but there were no differences in K_{rel} ($W = 111$, $p = .87$, August: 0.941 ± 0.022, October: 0.951 ± 0.018).

3.1 | Parasite community

The abundance of *A. crassus* in the swim bladder ranged from 0 to 46, with 0–35 larvae and 0–25 adult worms. Encapsulated *A. crassus*

were found in 39% of the eels. Infection parameters are summarized in Table 2. Mean infection intensity was 1.8 times higher in NC eels than in C eels, although the difference was not significant (Tables 2 and 3). Mean abundance of adult *A. crassus* was 5.3 times lower in C eels than in NC eels (Figure 1a; Table 3). Mean abundance of larval *A. crassus* did not differ with encapsulation status (Figure 1b; Table 3). Neither infection intensity nor abundance differed with sampling month. Infection intensity and adult abundance decreased with an increasing number of capsules when including both NC and C eels (intensity: Dev = 5.42, p = .020; abundance: Dev = 9.52, p = .002). However, this relationship did not hold in C eels only (intensity: Dev = 2.59, p = .11; abundance: Dev = 1.37, p = .24). There was no relationship between larval abundance and the number of capsules (NC + C: Dev = 1.15, p = .28; C: Dev = 1.40, p = .24). Heavier and larger individuals did not harbour more *A. crassus* or more larval stages for any encapsulation status or sampling month. The number of adult parasites was positively correlated with eel weight and TL for the NC group (Spearman's rank correlation test, rho = 0.50, p = .028, for both weight and TL) but not the C group (weight: rho = −0.14, p = .64; TL: rho = −0.20, p = .50). There was no correlation between the number of adult parasites and weight or TL in August or October.

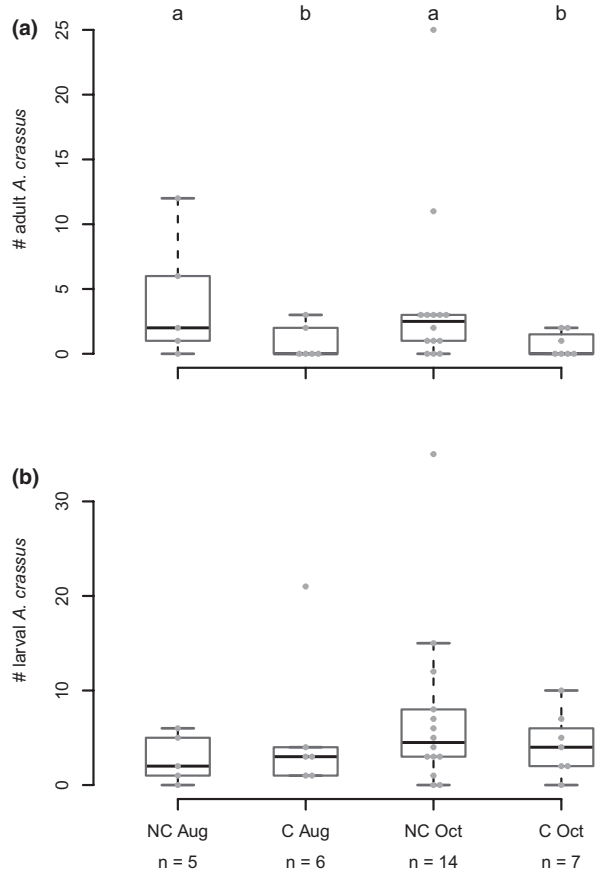

FIGURE 1 Infection intensities of (a) larval *Anguillicola crassus* and (b) adult *A. crassus* in the swim bladder of eels with unambiguous encapsulation status. Aug and Oct indicate the sampling months, and NC and C indicate the absence and presence of capsules. Statistically significant differences (Tukey's HSD) are indicated with different lower case letters. Boxes indicate the interquartile range, and whiskers extend to the largest and smallest values within the 1.5× interquartile range

Overall prevalence of the native parasites across both sampling months and encapsulation status ranged from 6% for *Camallanus lacustris* to 78% for *Myxidium giardi* (Table 4). The invasive *Pseudodactylogyrus* species (*P. bini* and *P. anguillae*) had a prevalence of 100%. Overall mean infection intensities ± *SE* ranged from 1 for *Diplostomum* sp. to 1.5 ± 0.3 for cestodes (*B. claviceps* and *P. macrocephalus*) and *Ergasilus gibbus* and were thus low compared with *A. crassus*. Infections with *Pseudodactylogyrus* spp., *M. giardi* and *Myxobolus portucalensis* were categorized into unequally sized intervals; therefore, abundances and intensities could not be calculated. *Diplostomum* sp. and *C. lacustris* had low prevalences (<10%) and were excluded from analyses of parasite community composition. The prevalence of cestodes and *E. gibbus* was 2.6 times higher in C group eels compared with NC group eels and that of *M. portucalensis* was 2.2 times higher, though none of the differences was significant (Dev = 3.68, p = .055, for cestodes and *E. gibbus* and Dev = 2.25, p = .13, for *M. portucalensis*; Table 4). The prevalence of *M. giardi* did not differ between groups. *M. portucalensis* was 7.6 times more prevalent in August than in October (Dev = 12.37, p = .0004; Table 4). The abundance of cestodes and *E. gibbus* was significantly higher in C group eels than NC group eels (Dev = 4.00, p = .046, for both). Although the categorization of *Pseudodactylogyrus* spp., *M. portucalensis* and *M. giardi* did not allow for the testing of differences in

TABLE 4 Prevalence (P, %) of parasites other than *Anguillicola crassus* in eels that were included in the gene expression analyses. Parameters are given for all eels and separately for each encapsulation status and each sampling month

	Location	Overall	NC	C	August	October
Cestodes[a]	Intestine	34	21	54	36	33
Camallanus lacustris	Intestine	6	5	8	9	5
Pseudodactylogyrus spp.	Gills	100	100	100	100	100
Myxidium giardi	Gills	78	79	77	64	86
Ergasilus gibbus	Gills	34	21	54	45	29
Myxobolus portucalensis	Fins	31	21	46	73	10
Diplostomum sp.	Eyes	9	11	8	18	5
n		32	19	13	11	21

Abbreviations: C, encapsulating eels; *n*, number of eels examined; NC, non-encapsulating eels.

[a]*Bothriocephalus claviceps* and *Proteocephalus microcephalus*.

abundance, categories suggest higher abundance in August than October for *Pseudodactylogyrus* spp. and *M. portucalensis*, but not *M. giardi*, and no difference between C and NC group eels for any of the three parasites. An analysis of similarity revealed moderate differences in parasite community composition between the two months ($R = 0.30$, $p = .001$, Figure 2a), but no significant difference between C and NC group eels ($R = 0.09$, $p = .066$, Figure 2b). *Myxobolus portucalensis* and *Pseudodactylogyrus* spp. contributed significantly to the parasite community differences between August and October ($p = .001$ for both taxa).

3.2 | Gene expression

Encapsulation status had no significant effect on relative expression of any studied gene in spleen or head kidney (Figure 3; Table 3). *Cox1* was about 1.8 times more highly expressed in the head kidney of eels sampled in August than in those sampled in October (Figure 3b; Table 3). Post hoc tests indicated higher expression in the NC group in August than in October (Tukey's HSD, $z = 2.64$, $p = .041$). Relative expression of *epor* and both *mhc II* genes in the spleen differed between sampling months (Figure 3c,e,g; Table 3). The expression of *epor* was elevated 2.5-fold, *mhc IIA* 2.2-fold and *mhc IIB* 1.8-fold in August compared with October. For both *mhc II* genes, temporal expression patterns in the head kidney differed with encapsulation status (Figure 3f,h).

The expression of both *mhc II* genes in the head kidney correlated negatively with the number of capsules (*mhc IIA*: Dev = 101.8, $p = .016$, *mhc IIB*: Dev = 3.22, $p = .008$). There was no correlation between gene expression and either infection intensity or the number of adult *A. crassus* in either organ. For *mhc IIA* and *mhc IIB*, there was a strong overall correlation between relative expression levels (Spearman's rank correlation, $\rho = 0.749$, $p < .001$). The correlation remained highly significant when analysing the spleen and the head kidney separately (spleen: rho = 0. 529, $p < .001$, head kidney: rho = 0.542, $p < .001$).

4 | DISCUSSION

European eels are infected by a wide variety of macroparasites. The number of parasite taxa that we observed in eels of Lake Müggelsee was comparable to those reported from other European locations (e.g. Gérard et al., 2013; Jakob et al., 2009; Sures et al., 1999; Sures & Streit, 2001). Similarly to those reports, the invasive parasites *Anguillicola crassus* and *Pseudodactylogyrus* spp. were the most prevalent. Prevalence and mean infection intensity of *A. crassus* were in the upper range of those reported across Europe (e.g. Audenaert et al., 2003; Becerra-Jurado et al., 2014; Gérard et al., 2013; Knopf, 2006). We found no difference in abundance between August and October. Seasonal dynamics have now been reported in some locations (e.g. Lefebvre et al., 2002; Schabuss et al., 2005) but not others (e.g. Kennedy & Fitch, 1990; Würtz et al., 1998). We observed no relationship between eel size and infection intensity or abundance of *A. crassus*. This was similar to the findings of Norton et al. (2005), although both positive (Becerra-Jurado et al., 2014; Neto et al., 2010; Schabuss et al., 2005) and negative (Barry et al., 2017; Fazio, Sasal, et al., 2008) relationships have been reported.

Invasive parasites can impose strong selective pressures on their novel hosts, which are expected to lead to adaptation of the host (Penczykowski et al., 2011). We found that European eels encapsulating *A. crassus* had fewer adult-stage parasites in their swim bladders compared with eels that did not encapsulate, but all eels

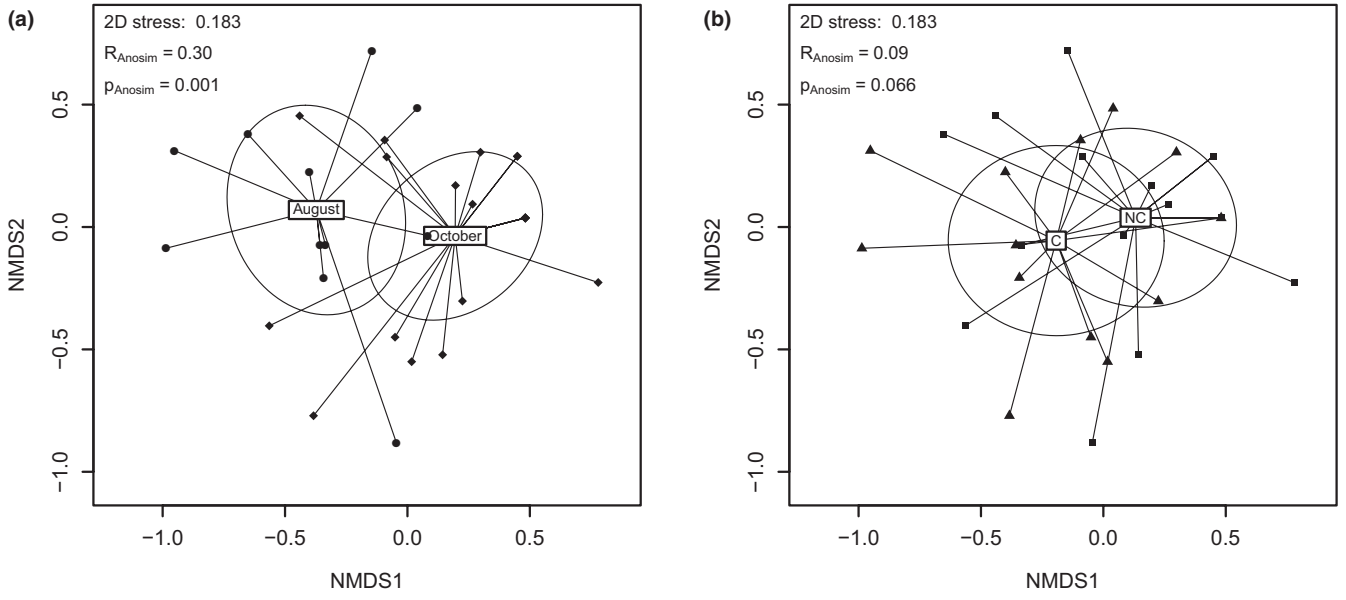

FIGURE 2 Non-metric multidimensional scaling plot of parasite communities in eels with unambiguous encapsulation status (a) in August (●) and October (◆) and (b) for non-encapsulating (NC, ■) and encapsulating (C, ▲) eels

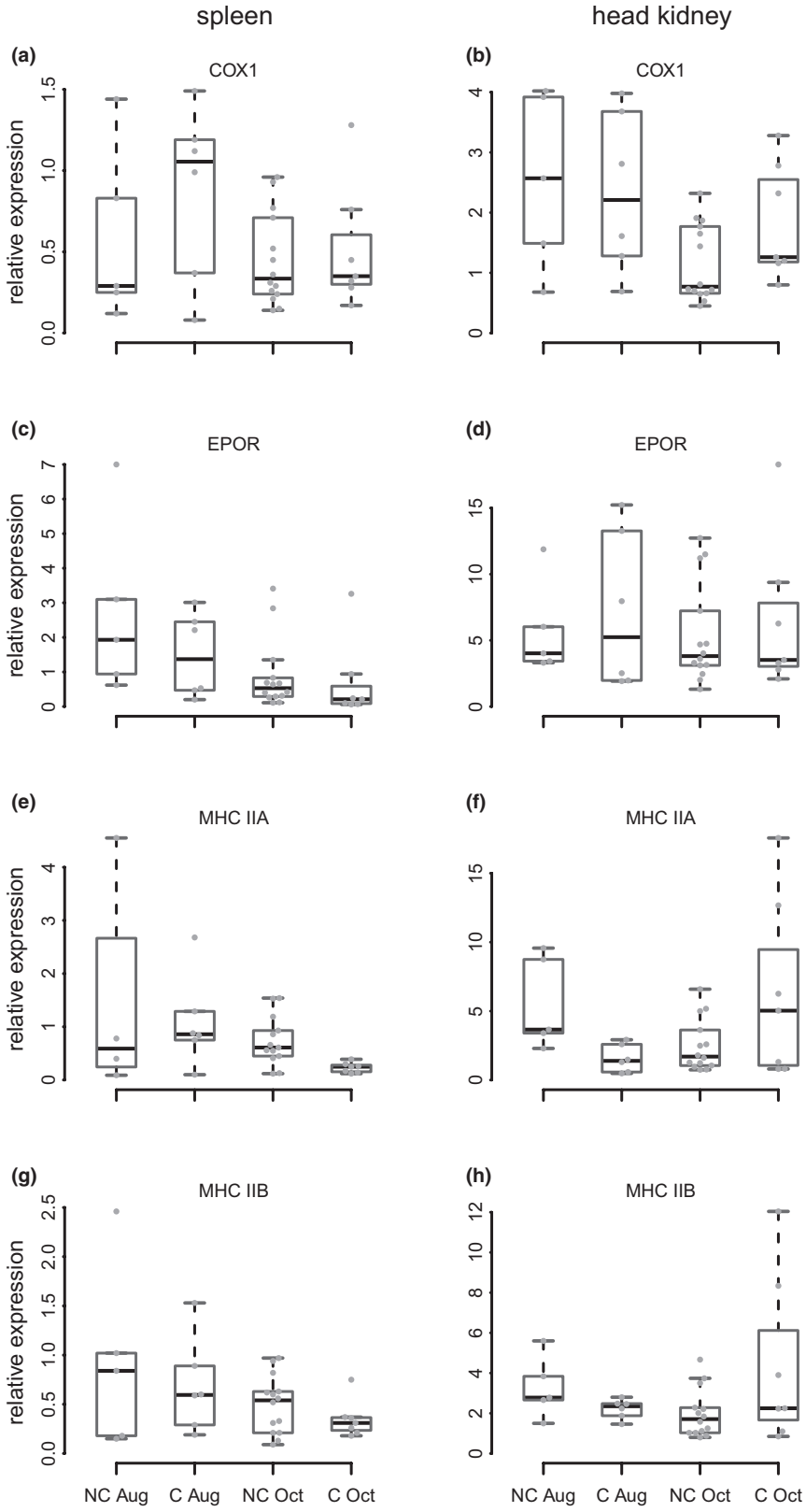

FIGURE 3 Expression of target genes relative to the expression of the reference gene β-actin in the spleen (a, c, e, g) and the head kidney (b, d, f, h). Aug and Oct indicate the sampling months, and NC and C indicate absence and presence of capsules

had similar numbers of larval-stage parasites. Encapsulation may therefore prevent the parasite's development to adulthood rather than prevent its establishment. Adult stages are proposed to have the strongest impact on the European eel (Würtz et al., 1996), and reducing their abundance may diminish adverse effects of severe *A. crassus* infections. This may indicate a first step towards adapting to the novel parasite. However, there was no relationship between the abundance of adult *A. crassus* and the number of capsules for eels encapsulating the parasite and it is possible that both depend on additional factors.

The co-infecting parasite community can determine the outcome of infections and its impact on the host (Abbate et al., 2018; Benesh & Kalbe, 2016; Johnson & Hoverman, 2012). Co-infecting parasite species can interact with each other either directly via competition, for example for resources, or indirectly, for example via host immune response, which can suppress or facilitate co-infections (Pedersen & Fenton, 2007; Poulin, 1999). We found abundances of two native parasites to be higher in eels encapsulating *A. crassus*, suggesting that the ability to encapsulate and the establishment of other parasites may interfere with each other.

The number of capsules was negatively correlated with *mhc II* expression among eels encapsulating *A. crassus*. Western blot analysis of the antibody response of experimentally infected European eels suggested that antigens of adult parasites trigger an adaptive immune response (Knopf et al., 2000) and that this response is stronger with increasing infection intensity (Knopf & Lucius, 2008). Increased *mhc II* expression may thus indicate higher susceptibility and encapsulation may lead to a reduction in the adaptive immune response by reducing the number of adult worms. Here, *mhc II* expression did not correlate with adult *A. crassus* load; however, it may be correlated more strongly with encapsulation and the number of adults than was detected. Our primers do not discriminate the alleles of European eel *mhc II* (Bracamonte et al., 2015). European eels contain at least four expressed *mhc IIA* and *mhc IIB* alleles, and if *A. crassus*-specific alleles exist, their change in expression may be masked by expression changes in other alleles induced by the other parasites. The need to respond to co-infecting parasites could also explain why we did not find a difference in *mhc II* expression between encapsulating and non-encapsulating eels.

Adult *A. crassus* are sanguivorous (De Charleroy et al., 1990), and a high load of adults may stimulate erythrocyte production in eel hosts. In experimental infections, Fazio et al. (2009) found increased expression of a gene associated with red blood cells (haemoglobin α), suggesting an increase in red blood cells with increasing parasite biomass. Our results based on expression of *epor*, a gene involved in erythropoiesis, did not indicate such an increase. Furthermore, we did not find a correlation of expression with the number of adult *A. crassus* or the number of capsules. Additional biotic and abiotic factors may influence the effect of *A. crassus* on red blood cells in the wild. Similarly, we did not find an association between the expression of the energy-associated gene *cox1* and the presence of capsules, their number or the number of adult *A. crassus*. Hence, there was no evidence for energetic benefits of encapsulating *A. crassus*. One reason could be that such benefits only become evident during the spawning migration (Palstra et al., 2007; Palstra & van den Thillart, 2010) and none of the eels we studied were migrating. Another could be that the higher abundance of native parasites (see above) may countervail any energetic benefits of harbouring fewer adult *A. crassus*.

Whole transcriptome gene expression analyses of infected and uninfected individuals indicated reduced cell respiration and the induction of both innate and adaptive immune responses in the presence of *A. crassus* larvae (Bracamonte, Johnston, Knopf, et al., 2019; Bracamonte, Johnston, Monaghan, et al., 2019). Increased immune gene expression in naturally infected eels compared to those without an active infection was also observed after acclimation to a common, stress-free environment (Schneebauer et al., 2017). Because all of our individuals were infected with *A. crassus*, we cannot exclude the possibility that the mere presence of *A. crassus* determines the physiological status and the initiation of an immune response, regardless of infection intensity. Extending the analysis to non-infected individuals may offer further insight into the importance of *A. crassus* on the physiological status of wild continental European eels.

Independently of *A. crassus* or its encapsulation, the expression of all genes varied between August and October. This suggests that environmental factors may affect expression of the selected genes. All genes showed higher expression in August than in October in one of the two tissues. Fish are ectothermic; hence, colder water in October could be one factor driving the difference (Brown et al., 2016; Logan & Somero, 2010). Parasite community composition also differed between sampling dates, and this could lead to variation in gene expression. Translocated sticklebacks adjust the expression profiles of immune genes to those of the local population, which is likely a response to encountering different parasite communities (Stutz et al., 2015). Temporally changing parasite communities can be expected to cause similar adjustments of the response. However, eels caught in August were significantly larger than eels caught in October. Hence, we cannot exclude that body size has an effect on relative gene expression.

In conclusion, the invasive parasite *A. crassus* was one of the most prevalent parasites of eels in Lake Müggelsee. Eels that encapsulated *A. crassus* had fewer blood-feeding adults. We argue that this may be a first step towards adaptation by the novel host, although we have no information on the genetic basis of encapsulation. The lower number of adults may then contribute to the negative relationship between *mhc II* gene expression and the number of capsules because the adaptive immune system was shown to respond to adult *A. crassus*. However, we found that the abundance of two native parasites was higher in eels that encapsulated, suggesting that there may be interference among responses to different parasites or among the parasites themselves. This interference, together with a potentially weak effect of *A. crassus* on the continental stage of eels, may be one reason for the absence of a clear pattern in gene expression, particularly of the energy-related and the haematopoietic genes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

No shared data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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