Experimental infection with *Anguillicola crassus* alters immune gene expression in both spleen and head kidney of the European eel (*Anguilla anguilla*)

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Experimental infection with *Anguillicola crassus* alters immune gene expression in both spleen and head kidney of the European eel (*Anguilla anguilla*)

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Abstract

Invasive parasites have been implicated in the declines of several freshwater species. The swim bladder nematode *Anguillicola crassus* was introduced into Europe in the 1980s and is considered a threat to the European eel (*Anguilla anguilla*). Infection affects stress resistance and swimming behaviour. European eels produce an immune response against the parasite during the late stages of infection and after repeated infections. We used RNA-seq to examine the molecular response to infection during the poorly understood early stage and identify expression of genes and associated processes that are modified in two immune organs of European eels 3 days post infection with *A. crassus*. In the spleen, 67 genes were differentially expressed, 32 of which were annotated. Most of these were involved in immune processes and their regulation. Other differentially expressed genes in the spleen were important for heme metabolism and heme turn-over. In the head kidney, 257 genes (134 annotated) were differentially expressed. Several of these were associated with immune functions. Other differentially expressed genes in the head kidney were related to renal function, in particular osmoregulation and paracellular flow. We conclude that the early response of European eels to *A. crassus* is complex and involves various processes aside from the immune system. We identified molecular changes occurring early during the infection and identified candidate genes and processes which will facilitate future studies aimed at determining the factors affecting European eel viability in the face of this invasive parasite.

Keywords

Immune response, invasive parasite, RNA-seq, differential gene expression

1. Introduction

Recent decades have seen an increase of newly emerging diseases all over the world. One reason for this is the accidental introduction of non-native parasites by humans (Daszak, et al., 2000, Peeler, et al., 2011). These parasites can be highly virulent in new hosts and there are many examples of translocated parasites posing a considerable threat to their new host species (Dunn and Hatcher, 2015, Smith, et al., 2009). In European fresh waters, invasive parasites have been implicated in the decimation of fish, amphibian, and invertebrate populations (Gozlan, et al., 2005, Kozubikova, et al., 2007, McKenzie and Peterson, 2012, Peeler, et al., 2011). In many cases the reason for the high susceptibility of naïve hosts and the physiological processes underlying the response to invasive parasites are poorly understood.

The introduction of the parasite *Anguillicola crassus* Kuwahara, Niimi & Hagaki, 1974 into Europe is considered to be one of the factors that has led to the decline of the European eel (*Anguilla anguilla* L.) population (Kirk, 2003). *A. crassus* is a trophically transmitted swim bladder nematode native to the Japanese eel (*A. japonica* Temminck & Schlegel, 1846). It was introduced into Europe in the early 1980s from Taiwan (Wielgoss, et al., 2008), first detected in wild eels in Germany in 1982, and reported from other countries soon afterward (Kirk, 2003, Peters and Hartmann, 1986). It has now reached a prevalence of 50-90% across most of the distribution range of the European eel (ICES, 2012, Lefebvre and Crivelli, 2004).

Several effects of *A. crassus* infection have been documented in infected European eels, including histopathological changes of the swim bladder wall and altered gas composition in the swim bladder which likely affects its functioning (Würtz and Taraschewski, 2000, Würtz, et al., 1996). Although some studies have reported no adverse effects of *A. crassus* on performance of European eels during the freshwater stage of their life cycle under normal conditions (Kelly, et al., 2000, Lefebvre, et al., 2013), additional stressors such as hypoxia have strong effects on the viability of infected eels and can increase mortality (Gollock, et al., 2005, Lefebvre, et al., 2007, Molnar, et al., 1991). *A. crassus* infections also appear to accelerate and interfere with the silvering process of

European eels, which prepares them for the long distance spawning migration across the ocean (Fazio, et al., 2012, Pelster, et al., 2016). This occurs in combination with increased energy consumption and behavioural alterations during extensive swimming (Newbold, et al., 2015, Palstra, et al., 2007). *A. crassus* infections might therefore be costly, reducing fitness and quality of spawners and hampering successful completion of migration and reproduction.

A parasite that has a strong effect on the fitness of its host should favour adaptation by the host and therefore the development of a specific immune response. There is some evidence for adaptation by the parasite, as infection intensity and size of European *A. crassus* differed from those of Taiwanese *A. crassus* in experimental infections (Weclawski, et al., 2013, Weclawski, et al., 2014). On the other hand, there is only little evidence that European eels may be adapting to *A. crassus*. Although infection intensities have stabilized, they are still high (Audenaert, et al., 2003, Lefebvre and Crivelli, 2004) and there are only occasional reports of European eels confining the parasite (Audenaert, et al., 2003, Molnár, 1994). Immune responses differ markedly among individuals of *A. anguilla*, with some individuals exhibiting a strong response and others not responding at all (Knopf, et al., 2000, Molnár, 1994). In cases of host responsiveness, inflammatory cells infiltrate the swim bladder following infection. Within the same host, some *A. crassus* larvae were encapsulated and necrotized while others were not (Molnár, 1994), and the infiltrating cells appeared primarily to remove cellular debris caused by the migrating activity of the larvae (Würtz and Taraschewski, 2000). Antibody production varies considerably among individuals and appears to be elicited by adult nematodes rather than the invading larval stage (Knopf, et al., 2000). With respect to immune response, little is known regarding alterations induced at the molecular level by the parasite, and few studies have examined changes in gene expression after *A. crassus* infection. Using a qPCR approach, Fazio et al. (2009, 2012) found indications that the expression of genes involved in osmoregulation, haematopoiesis, and silvering were altered in experimentally infected European eels. Gene expression of a range of processes was differentially regulated in the swim bladder during silvering of naturally infected versus uninfected eels, affecting the modifications necessary for long distance

migration in open waters (Pelster, et al., 2016). Naturally infected eels also regulated expression of genes associated with swim bladder functioning and with the immune response (Schneebauer, et al., 2017). The expression of several inflammatory genes was altered, thus providing additional evidence that a localized immune response can develop in the swim bladder once *A. crassus* has established.

We aimed to identify changes in gene expression and infer the corresponding processes during *A. crassus* infections. We were particularly interested in identifying possible systemic immune responses against the parasite, therefore we focussed on two immune organs of fish, the spleen and head kidney. The spleen is a major secondary lymphoid organ (Ellis, 1980, Whyte, 2007) and it plays an important role in the progression of innate and adaptive immune responses locally and systemically (Bronte and Pittet, 2013). It is involved in clearing the blood and retains antigens for long periods of time. It is also an important site for the destruction of erythrocytes and it functions as metabolic dump (Press and Evensen, 1999). The head kidney is a fish-specific organ equivalent to the bone marrow of higher vertebrates (Alvarez-Pellitero, 2008, Whyte, 2007). It is both a primary and secondary immune organ. As such it is the main site of haematopoiesis. It is important for initiation and progression of an immune response by trapping, processing, and presenting antigens to lymphocytes which trigger an adaptive immune response and it is the main site of antibody production.

We sequenced total mRNA of spleen and head kidney tissue from European eels that were experimentally infected with *A. crassus* and compared expression levels with those from uninfected control eels at three days post-infection (dpi). We hypothesized that this approach would allow us to determine if invading larvae initiate a systemic immune response which is a necessary step for mounting a protective response. While an early time point may not capture the fully mounted immune response, we were interested in the tissue migrating phase of parasites that is considered crucial for their establishment (Mulcahy, et al., 2005). We selected 3 dpi because *A. crassus* larvae were reported in the swim bladder as early as 4 dpi (Knopf, et al., 1998) and we wanted to capture the migrating phase that is considered crucial for the establishment of a helminth. The response

elicited by that stage, or a lack thereof, may hint at why European eels have a low capacity of confining *A. crassus* infections.

2. Materials and Methods

2.1. Infection and sampling

European eels were purchased in 2004 from a commercial eel farm that is free of *A. crassus* (Domäne Voldagsen, Einbeck, Germany). Experimental infections were carried out in November 2014. Eels were kept individually in 40-l compartments within 200-l tanks in a recirculating system with aerated tap water at a water temperature of 22 °C prior to, and during, the experiment. At the beginning of the experiment, five treatment eels were infected with *A. crassus* and five control eels were sham-infected following Knopf et al. (1998). In short, second stage larvae (L_2) of *A. crassus* were isolated from swim bladders of infected wild eels (*A. anguilla*) caught in Lake Müggelsee, Germany, in October 2014 and fed to wild-caught copepods from the same lake. Nineteen to 23 days post infection (dpi), third stage larvae (L_3) were isolated from the copepods using the potter method (Haenen, et al., 1994). Each of the treatment eels was administered 25 randomly selected L_3 suspended in 100 µl of PBS, pH 7.2 with a stomach tube. The same amount of PBS containing no *A. crassus* larvae was administered to control eels. Eels were decapitated 3 dpi, when the parasite is migrating from the intestine to the swim bladder (Knopf, et al., 1998). The spleen and the head kidney (defined according to (Tesch, 2003)) were removed and immediately stored in RNA*later*® (Life Technologies, Darmstadt, Germany) following the manufacturer's recommendations. Tissues were then kept in RNA*later*® at -20 °C until extraction of RNA. The swim bladders of all eels were examined and all *A. crassus* were counted. Encapsulated *A. crassus* were not present. At the end of the experiment, eels weighed 115.9 ± 24.8 g. The study was approved by the Berlin State Office for Health and Social Affairs (LaGeSo) in Berlin, Germany (approval number G 0021/15).

2.2. RNA extraction and sequencing

RNA was extracted from the spleen and the head kidney using TRIzol® Reagent (Life Technologies, Darmstadt, Germany) following the manufacturer's recommendations for fatty tissue with slight modifications as described below. Each tissue type was homogenized in 850 µl of TRIzol twice for 1.5 min at 18/s in a TissueLyser II (Qiagen, Hilden, Germany) and centrifuged at 12,000 x g for 10 min at 4 °C. An additional 150 µl of TRIzol was added to the aqueous phase prior to chloroform (200 µl) addition. RNA was precipitated from the aqueous phase with 500 µl isopropanol. The pellet was washed with 1 ml 70 % ethanol, dried on a heat block for 5 min at 28 °C, and resuspended in 50 µl DEPC water (Life Technologies). RNA was then incubated on a heat block for 2 min at 50 °C. RNA quality and quantity were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The samples were diluted to a concentration of 40 ng/µl in TE buffer pH 7.5 and shipped on dry ice to Beckman Coulter Genomics (Danvers, USA) for mRNA library preparation, paired-end (100 bp) sequencing on an Illumina HiSeq 2000, and quality control. The spleen and the head kidney of six samples (3 infected, 3 control) were sequenced in one run (batch A, n = 3), while another three spleen samples (2 infected, 1 control) and four head kidney samples (2 infected, 2 control) were sequenced in a different run (batch B, n = 2). RNA for the two batches of samples was extracted at different time points but using the same procedure.

2.3. Transcriptome assembly and annotation

A single *de novo* assembly was produced using reads from all samples of both tissues with Phred scores > 30. Reads were assembled using Trinity v2.1.1 (Grabherr, et al., 2011, Haas, et al., 2013) with the default settings. The Trimmomatic option for trimming low quality reads was used with the default settings (SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25). In silico normalization was applied to restrict the maximum read coverage to 50. The transcriptome was annotated by blastx searches against the UniProtKB/Swiss-Prot (www.uniprot.org) and RefSeq (www.ncbi.nlm.nih.gov/refseq/) databases. The E-value cut-off for both was set to 1e-3. Trinotate v3.0.0 (https://trinotate.github.io/) was used to obtain corresponding Gene Ontology (GO) assignments for UniProtKB/Swiss-Prot-derived annotations. The taxonomic composition for best

matches obtained from RefSeq was examined to check for signs of obvious contamination with non-eel RNA.

2.4. Differential gene expression and functional analysis

Different tissues are known to respond differently to parasite infections (Huang, et al., 2016, Robledo, et al., 2014, Skugor, et al., 2008), therefore we contrasted gene expression in the spleen and the head kidney between infected and control eels separately for each tissue. Reads were re-aligned separately for each of the 9 spleen and 10 head kidney samples using the assembled transcriptome as a reference for abundance estimates using RSEM v1.2.26 and the script provided by Trinity. These estimates were used for calculating differential gene expression between infected and control eels separately for the spleen and the head kidney with DESeq2 v1.10.1 (Love, et al., 2014). Aside from the treatment, the sequencing run was included into the model to control for batch (A, B, see above) effects. Genes with a mean coverage below 10 (low coverage) were excluded from the analysis, as recommended by Todd et al. (2016), and a local fit was used to estimate the dispersions. Genes were considered significantly differentially expressed if they had an adjusted p-value < 0.05 and a $\log_2$ fold change $\geq \pm 1$. Adjustment of p-values followed the Benjamini-Hochberg procedure for multiple testing (Benjamini and Hochberg, 1995) implemented in DESeq2. A principal component analysis (PCA) was done to determine the overall effect of treatment on sample relationship within tissues and the differences between the spleen and the head kidney. For the PCA, samples of both tissues were combined into one dataset which only included genes that passed the mean coverage filter (see above) in both tissues. The prcomp function in R (R Core Team, 2016) was used on the variance-stabilized counts of the RSEM abundance estimates to perform the PCA.

Enriched GO terms were identified by conditional hypergeometric tests using the GOstats package v2.36.0 (Falcon and Gentleman, 2007) for R. Custom GO annotations obtained from the Trinotate annotation of the transcriptome were used as reference for the GO terms of the differentially expressed genes (DEG) to be compared. The analysis was restricted to GO terms from the domain “biological processes”. The significance level for enriched GO terms was set to 0.01.

3. Results

All eels that were administered *A. crassus* had living L_3 in their swim bladders at the end of the experiment. Mean infection intensity was 2.4 parasites per eel (range: 1-3). More advanced parasitic stages were not present and the majority of larvae were likely still migrating from the intestine to the swim bladder. None of the control eels were infected.

3.1. Transcriptome assembly and annotation

Sequencing of spleen and head kidney mRNA resulted in >860 M reads (438,649,866 reads in batch A and 423,602,528 reads in batch B; see section 2.2). Of these, 95.3% and 96.0% passed quality control (QC). The mean number of high quality reads per sample was 34.8 M in batch A and 50.8 M in batch B. There were no significant differences in number or quality of reads between spleen and head kidney samples. All reads that passed QC were assembled into 578,084 contigs and 823,359 isoforms with an average length of 620 bases and an N50 of 948 bases. The GC content for these sequences was 46.03%. UniProt annotations were assigned to 74,796 contigs (13% of the total genes) and RefSeq annotations were assigned to 50,311 contigs (8.7%). Twelve of the top 15 matches from RefSeq were from fish genera. Most matches came from *Lepisosteus*, *Danio*, and *Astyanax* and these together provided best matches for about 44% of all annotated contigs.

3.2. Differential gene expression

Excluding contigs with a low coverage (see section 2.4) reduced the number that was used for the analysis to 59,666 in the spleen and 63,672 in the head kidney. Of these, 49.5% and 50.9%, respectively, had UniProt annotations. We refer to those contigs with sufficient coverage as genes throughout the rest of the manuscript. Treatment and control samples clustered together in a tissue-specific manner in the PCA (Fig. 1), indicating that the spleen and the head kidney differed considerably in their expression profiles regardless of the infection status. Within tissues, samples did not separate into control and treatment groups when considering the expression profiles of all genes. One head kidney sample of the control group and one head kidney sample of the treatment

clustered separately from all other head kidney samples (Fig. 1). GO enrichment of DEG between those two samples and the rest did not allow us to identify the reason for the differences. Therefore, they were included in all further analyses.

The number of DEG between infected and control individuals was different in the two tissues. In the spleen, 67 genes were differentially expressed and in the head kidney, 257 genes were differentially expressed (Table S1). Of these, 32 in the spleen and 134 in the head kidney had either UniProt or RefSeq annotations. In the spleen, the number of up- and down-regulated genes was very similar (32 vs 35) (Fig. 2a). In the head kidney more genes were up-regulated (196) than down-regulated (61) (Fig. 2b). The $\log_2$ fold changes ranged from -4.9 to 2.9 in the spleen and from -5.3 to 4.8 in the head kidney. Only 13 genes were differentially expressed in both tissues. Of these, 7 were up-regulated and 6 were down-regulated in both spleen and head kidney (Table S1). Four of each were annotated.

3.3. Functional analysis

The enrichment analysis revealed 32 GO terms to be enriched in the up-regulated genes and 20 GO terms in the down-regulated genes in the spleen (Table S2). In the head kidney, 99 GO terms were enriched in the up-regulated genes and 20 GO terms in the down-regulated genes (Table S3). In the spleen, 18 out of 32 enriched GO terms for the up-regulated genes belonged to the categories "immune system process" or "response to stimulus", the majority of which were related to inflammatory processes (Table 1). Another 4 enriched GO terms were related to regulation of cytokine biosynthesis and production. Furthermore, "leukotriene biosynthetic process" was enriched (Table S2). Among the enriched GO terms for down-regulated genes 3 belonged to the categories "immune system process" or "response to stimulus" and 3 enriched GO terms were related to the regulation of chemokine production and secretion. Furthermore, processes involved glycopeptide and carbohydrate metabolism were enriched (Tables 1 & Table S2).

In the head kidney, none of the 99 enriched GO terms for the up-regulated genes belonged to the category "immune system process" and only 11 were subcategories of "response to stimulus", 4

of them relating to DNA damage response and cellular senescence (Table 2). Additionally, GO terms related to the regulation of arachidonic acid and icosanoid secretion were enriched. Also, cell adhesion processes and anatomical structure development, mainly related to kidney development, were enriched (Table S3). For the down-regulated genes 15 enriched GO terms were subcategories of "immune system process". Of the remaining 5 GO terms, 4 belonged to the category "response to stimulus" (Table 2). Enriched GO terms indicate that immunoglobulin- and lymphocyte-mediated immune responses as well as Fc receptor signalling were down-regulated.

Closer examination of the functions of DEG revealed that 11 in the spleen and 26 in the head kidney were classified as genes of immune system process or response to stimulus according to UniProt annotations (Tables 3 & 4). Among them, up- and down-regulated genes in the spleen were involved in inflammation, cell migration, and differentiation and activation of macrophages, mast cells, and lymphocytes. In the head kidney, the up-regulated genes were mostly associated with the cytoskeleton and extracellular matrix. Also genes involved in B cell maturation and inflammation were up-regulated. Down-regulated genes in the head kidney were involved in immunoglobulin formation and T cell activation.

The vast majority of annotated DEG in the spleen and down-regulated genes in the head kidney was related to immune response or the regulation thereof, although they are not classified as genes of immune system process or response to stimulus by UniProt annotations (Tables 3, 4 & S1). They were associated with inflammation, cell proliferation, activation, and migration, and wound healing. In the head kidney several up-regulated genes that were not classified as genes of immune system process or response to stimulus were implicated in wound healing, B cell development, and cell migration as well (Table S1).

Ferrochelatase, an enzyme of the heme biosynthesis pathway, and the scavenger receptor cysteine-rich type 1 protein M130, which is involved in haemoglobin scavenging, were both up-regulated in the spleen. In the head kidney, several genes involved in osmoregulation and

paracellular flow were upregulated, among them aquaporin-5, claudin-8, and multiple PDZ domain protein 1.

4. Discussion

Anguillicola crassus is a parasitic nematode that was introduced into European fresh waters in the 1980s and has acquired the European eel (*Anguilla anguilla*) as a new host (Kirk, 2003). Previous studies of the *A. anguilla* immune response to *A. crassus* have found localized inflammation in the swim bladder (Schneebauer, et al., 2017, Würtz and Taraschewski, 2000) and an antibody response against adults (Knopf, et al., 2000). We wanted to know whether the European eel is capable of mounting an immune response during the early stages of infections and used transcriptome-wide gene expression in two immune organs of European eels, the spleen and the head kidney, to detect immunological changes occurring 3 days post-infection (dpi) during the larval migrating phase of *A. crassus*. This early stage has been proposed to be critical for establishment of helminthic infections (Mulcahy, et al., 2005). We found expression of genes involved in an immune response to be modified in both organs. Infection also led to modified expression of genes related to heme metabolism, the O_2-binding unit of haemoproteins such as haemoglobin and cytochrome, in the spleen, and of genes involved in osmoregulation and renal function in the head kidney. Therefore, the European eel activates the immune system, but the overall response appears to be more complex. Below we first discuss genes and processes associated with an immune response to the parasite. We then discuss non-immune-related genes and processes that may influence the performance of infected European eels.

4.1. Immune-related processes

There were several indicators in both organs that *A. crassus* elicited an immune response in *A. anguilla*. Specifically, genes of the major histocompatibility complex class II (MHC II) were up-regulated in both organs. MHC II displays parasitic antigens that are recognized by the T cell receptor (TCR) (Morris, et al., 1994). Successful binding to MHC II-antigen complexes activates helper T cells

298 and induces a specific immune response (Murphy, 2012). European eels express up to four different
299 MHC IIB variants (Bracamonte, et al., 2015). Whether up-regulation of MHC IIB gene expression
300 implies presentation of *A. crassus*-derived antigens and increased TCR signalling cannot be
301 determined with our data, but suggests some immune response. Genes that modulate inflammatory
302 processes were modified in both head kidney and spleen. The phospholipase A2 receptor gene
303 (*pla2r*) was up-regulated in the head kidney. Ligand binding to PLA2R induces the production of pro-
304 inflammatory mediators (Granata, et al., 2005, Park, et al., 2003) and controlled cell death
305 (apoptosis) upon DNA damage through the production of arachidonic acid (Augert, et al., 2009, Pan,
306 et al., 2014). Cathelicidins, up-regulated in the spleen in this study, are anti-microbial peptides with
307 immunomodulatory properties (Brown and Hancock, 2006). They may promote either a pro-
308 inflammatory or an anti-inflammatory response. Elevated expression of the scavenger receptor
309 cysteine-rich type 1 protein M130 gene (*cd163*) also suggests suppression of inflammation. CD163 is
310 characteristic for alternatively activated macrophages (Kowal, et al., 2011, Van Gorp, et al., 2010).
311 These macrophages are generally associated with a T helper type 2 (Th2) immune response,
312 suppression of an inflammatory response, and wound healing (Gause, et al., 2013). Whether CD163
313 plays a role in the protective function against helminths has not yet been resolved.

314 There were also several indicators that *A. anguilla* did not produce an immune response at 3 dpi.
315 The early growth response protein 1 gene (*egr-1*) was down-regulated in the spleen. Egr-1 is a
316 transcription factor that is involved in the activation of lymphocytes, primarily Th2 cells, and the
317 stimulation of interleukin-4 expression (Lohoff, et al., 2010), an important regulator of the humoral
318 immune response. It further promotes the differentiation of macrophages and activation of mast
319 cells (Li, et al., 2006, McMahon and Monroe, 1996). All of these processes play an important role in
320 an effective immune response in mice and humans against helminths (Gause, et al., 2013). Down-
321 regulation of *egr-1* also indicates decreased proliferation of mature B cells (Gururajan, et al., 2008).
322 Impairment of B cells, the antibody (Ab)-producing cells, was further supported by the up-regulation
323 of the protein kinase C δ gene (*pkcd*) that we observed in the head kidney of infected individuals.

PKCD negatively affects B cell development and proliferation (Limnander, et al., 2011, Mecklenbräuker, et al., 2002, Miyamoto, et al., 2002). Accordingly, genes encoding immunoglobulin chains, which compose the B cell receptor and Ab, were down-regulated in the head kidney. We conclude that Ab production might not be initiated at 3 dpi, in agreement with previous findings showing that European eels produce no Ab against *A. crassus* larvae (Knopf, et al., 2000).

An anti-inflammatory Th2 response is thought to have evolved in response to macroparasitic infections (e.g. heminths) to protect the host from excessive damage by the immune response itself. A pro-inflammatory Th1 response is usually produced to fight microparasitic infections (e.g. bacteria) (Graham, et al., 2005). However, there is evidence that migrating parasitic larvae may be better controlled by a Th1 response prior to chronic infections and that this may prevent their establishment (Moreau and Chauvin, 2010, Mulcahy, et al., 2005). Our data did not clearly indicate a Th1 response or a Th2 response. An unresolved mix of Th1 response and Th2 response may be the initial state upon encountering a novel parasite. Selection can then drive the response into a Th1 response, which may prevent establishment of infection, or a Th2 response which may reduce damage while permitting the parasite to establish. Infection intensities of *A. crassus* have stabilized since its introduction in the 1980s (Audenaert, et al., 2003, Lefebvre and Crivelli, 2004) and there are signs of adaptation (Weclawski, et al., 2013). Monitoring the early stages of the immune response in recent eel generations at a higher resolution may indicate if the T helper response is targeted by selection and which trajectory the European eel will follow to cope with this invasive parasite.

4.2. Non-immune-related processes

In addition to immune responses, *A. crassus* infections affected genes related to metabolism of *A. anguilla*. CD163, although associated with inflammatory processes, is best known for its function as scavenger of haemoglobin-haptoglobin complexes (Fabriek, et al., 2005). Haemoglobin is released from old and defective erythrocytes. Degradation of heme, the O_2-binding unit of haemoglobin, induces an anti-inflammatory response by alternatively activated macrophages (Fabriek, et al., 2005). The increased expression of *cd163* that we observed in infected individuals may therefore indicate

higher erythrocyte turnover following *A. crassus* infections. This hypothesis is further supported by our observed up-regulation of the gene encoding ferrochelatase (*fech*), the last enzyme in heme biosynthesis (Layer, et al., 2010). Infected eels might have a reduced ability to bind and distribute O_2 and up-regulation of heme biosynthesis may be an attempt to meet the O_2 requirement. Höglund et al. (1992) reported slightly decreased values for haemoglobin in naturally infected eels containing adult blood-sucking *A. crassus* and no differences in numbers of erythrocytes. In contrast, Fazio et al. (2009) found increased expression of the gene encoding haemoglobin α in experimentally infected eels, which is in line with our finding of increased expression of *fech*. Heme is also necessary for cellular respiration (Paoli, et al., 2002), which provides energy for metabolic processes. Increased *fech* expression may indicate a higher demand of O_2 by infected individuals due to increased energy consumption. The metabolism of carbohydrates and polypeptides was enriched among down-regulated genes in the spleen. We conclude that the energy balance might be disturbed in eels infected with *A. crassus*.

GO enrichment revealed that renal integrity and osmoregulatory function were affected in the head kidney of infected eels. Genes encoding the tight junction proteins claudin and multiple PDZ domain protein 1 were up-regulated. Tight junctions determine epithelial permeability and regulate paracellular transport of solutes, mainly through claudins (Koval, 2006, Yu, 2015). Aquaporins, also up-regulated in the head kidney of infected eels in our study, form water channels and are important for osmoregulation (Cerda and Finn, 2010, Madsen, et al., 2015). Fazio et al. (2009) reported an effect on osmoregulation in the intestine, but not until 8 weeks post infection. Furthermore, they did not observe an effect on the expression of the aquaporin-encoding gene in the gills, although we observed it to be up-regulated in the head kidney.

4.3. Organ differences

The head kidney may be more strongly affected by the infection than the spleen. The number of genes and processes affected was more diverse in the head kidney than in the spleen. The differentially expressed genes in the spleen were almost exclusively associated with the immune

system while in the head kidney, they were associated with both immune system processes and physiological processes. The head kidney of eels is considerably different in shape and location from those of other fish (Tesch, 2003) and our data suggests that it might contribute extensively to renal processes in addition to its function as an immune organ. Altered reabsorption of solutes and osmoregulation could therefore indicate that the induction of an immune response by *A. crassus* also interferes with renal excretion and osmotic homeostasis.

Two head kidney samples had expression profiles that differed from those of all other head kidney samples. Although we cannot exclude that the samples were contaminated with trunk kidney tissue or higher amounts of blood during removal of the head kidney tissue, GO enrichment gave no such indication, The genes differing between the two outlier samples and all other samples were enriched for >2000 GO terms that were associated with very diverse processes. Eels cannot be bred efficiently and the individuals used for the experiment were originally caught from the wild for aquaculture. Also, sex-determination of immature eels is difficult and unreliable. Thus, eels were of unknown sex and genetic background, both of which can have an influence on gene expression. Furthermore, the eels had been held in captivity for a length of time similar to the time span of their continental phase (ca. 10 yr) and were likely close to silvering, i.e. preparing for migration in saltwater and initiating sexual maturation. The silvering process involves major physiological modifications (Tesch, 2003) and if eels were at different developmental stages then this may affect gene expression.

4.4. Conclusion

Gene expression results indicated that European eels modify immune processes early during *A. crassus* infections. The mix of Th1 and Th2 processes may be one of the reasons why *A. crassus* is not confined immediately after infection; however, the observed response was complex and affected processes other than those involved in immunity but which may interfere with an immune response. Interestingly, this was more pronounced in the head kidney than in the spleen, supporting a potentially important role of the head kidney in immune response. The molecular modifications that

we identified here provide a basis for determining processes that may influence European eel performance following infection.

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Data availability

The Transcriptome Shotgun Assembly project has been deposited at DDBJ/ENA/GenBank under the accession GHAH00000000. The version described in this paper is the first version, GHAH01000000.

Declarations of interest

None.

Figure legends

Figure 1 Principal component analysis (PCA) separating head kidney (●) from spleen (▲) samples but not infected (turquoise) from control (purple) samples within tissues. Head kidney and spleen samples from the same individual are labelled identically.

Figure 2 MA-plots showing differential gene expression between infected and controls for (a) the spleen and (b) the head kidney. Differentially expressed genes are shown as red dots and the number of up- and down-regulated genes is indicated.

Supplementary files

Table S1 List of all differentially expressed genes in the spleen and the head kidney. Annotations, where available, were obtained by blastx searches against UniProt and RefSeq databases. Genes that were differentially expressed in both tissues are shaded in gray. Log2FC is the $\log_2$ fold change between expression in infected and control individuals, Wald stat is the wald statistic calculated by DESeq2, and Direction indicates up-regulation (↑) or down-regulation (↑) in infected individuals. (XLSX 46 kb)

Table S2 List of all enriched GO terms in the spleen. Terms that were also enriched in the head kidney are shaded in gray. (XLSX 14 kb)

Table S3 List of all enriched GO terms in the head kidney. Terms that were also enriched in the spleen are shaded in gray. (XLSX 17 kb)

644 **Tables**

645 **Table 1** Enriched GO terms in the spleen related to “immune system process” or “response to stimulus”.

Category	Go term (biological process)	Expected	Count	Size	Direction	P-value
GO:0006953	acute-phase response	0	2	119	↑	<0.001
GO:0001788	antibody-dependent cellular cytotoxicity	0	1	3	↑	<0.001
GO:0001805	positive regulation of type III hypersensitivity	0	1	6	↑	0.001
GO:0019884	antigen processing and presentation of exogenous antigen	0	2	360	↑	0.001
GO:0006954	inflammatory response	0	3	1754	↑	0.001
GO:0071493	cellular response to UV-B	0	1	13	↑	0.002
GO:0071492	cellular response to UV-A	0	1	14	↑	0.002
GO:0001798	positive regulation of type IIa hypersensitivity	0	1	14	↑	0.002
GO:0002892	regulation of type II hypersensitivity	0	1	14	↑	0.002
GO:0048002	antigen processing and presentation of peptide antigen	0	2	543	↑	0.002
GO:0042742	defense response to bacterium	0	2	550	↑	0.002
GO:0001812	positive regulation of type I hypersensitivity	0	1	21	↑	0.003
GO:0002866	positive regulation of acute inflammatory response to antigenic stimulus	0	1	33	↑	0.004
GO:0002524	hypersensitivity	0	1	39	↑	0.005
GO:0043306	positive regulation of mast cell degranulation	0	1	44	↑	0.006
GO:0002696	positive regulation of leukocyte activation	0	2	962	↑	0.006
GO:0002861	regulation of inflammatory response to antigenic stimulus	0	1	49	↑	0.006
GO:0042590	antigen processing and presentation of exogenous peptide antigen via MHC class I	0	1	76	↑	0.010
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I	0	2	342	↓	0.002
GO:1990523	bone regeneration	0	1	22	↓	0.004
GO:0019882	antigen processing and presentation	0	2	651	↓	0.007

Expected and Count give the number of expected and observed differentially expressed genes assigned to the respective category. Size is the total number of genes in the reference transcriptome assigned to that category. Direction indicates enrichment in up-regulated (↑) or down-regulated (↓) genes in infected individuals.

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Table 2 Enriched GO terms in the head kidney related to “immune system process” or “response to stimulus”.

Category	Go term (biological process)	Expected	Count	Size	Direction	P-value
GO:0007185	transmembrane receptor protein tyrosine phosphatase signaling pathway	0	3	46	↑	<0.001
GO:0046426	negative regulation of JAK-STAT cascade	0	4	163	↑	<0.001
GO:0090403	oxidative stress-induced premature senescence	0	2	16	↑	<0.001
GO:0043517	positive regulation of DNA damage response, signal transduction by p53 class mediator	0	2	26	↑	0.001
GO:0009968	negative regulation of signal transduction	5	13	3839	↑	0.001
GO:0090398	cellular senescence	0	3	158	↑	0.001
GO:0030520	intracellular estrogen receptor signaling pathway	0	3	180	↑	0.002
GO:0042770	signal transduction in response to DNA damage	0	3	275	↑	0.005
GO:0071307	cellular response to vitamin K	0	1	6	↑	0.008
GO:0051387	negative regulation of neurotrophin TRK receptor signaling pathway	0	1	7	↑	0.009
GO:0052697	xenobiotic glucuronidation	0	1	7	↑	0.009
GO:0045087	innate immune response	1	6	2927	↓	<0.001
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I	0	3	342	↓	<0.001
GO:0019882	antigen processing and presentation	0	3	651	↓	<0.001
GO:0006958	complement activation, classical pathway	0	2	342	↓	0.002
GO:0002757	immune response-activating signal transduction	0	3	1532	↓	0.002
GO:0038096	Fc-gamma receptor signaling pathway involved in phagocytosis	0	2	394	↓	0.002
GO:0072376	protein activation cascade	0	2	430	↓	0.002
GO:0002440	production of molecular mediator of immune response	0	2	458	↓	0.003
GO:0016064	immunoglobulin mediated immune response	0	2	513	↓	0.004
GO:0042742	defense response to bacterium	0	2	550	↓	0.004
GO:0034165	positive regulation of toll-like receptor 9 signaling pathway	0	1	25	↓	0.004
GO:0050778	positive regulation of immune response	0	3	2038	↓	0.005
GO:0006959	humoral immune response	0	2	620	↓	0.005
GO:0002682	regulation of immune system process	1	4	4405	↓	0.005
GO:0002449	lymphocyte mediated immunity	0	2	795	↓	0.008
GO:0051707	response to other organism	0	3	2536	↓	0.009
GO:0002460	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	0	2	830	↓	0.009
GO:0038095	Fc-epsilon receptor signaling pathway	0	2	844	↓	0.009
GO:0009607	response to biotic stimulus	0	3	2602	↓	0.009

Columns as described for table 1.

650 **Table 3** Differentially expressed genes in the spleen associated with the immune system.

Gene ID	Log2FC	Wald stat	P-value	Adj. p-value	Direction	Uniprot and refseq annotations
TRINITY_DN168651_c5_g8	2.14	5.97	2.35E-09	2.26E-05	↑	High affinity immunoglobulin gamma Fc receptor I
TRINITY_DN164847_c1_g5	2.37	5.56	2.71E-08	1.96E-04	↑	HLA class II histocompatibility antigen, DR beta 4 chain
TRINITY_DN85147_c0_g2	1.99	4.67	3.06E-06	0.006	↑	carboxypeptidase N subunit 2-like
TRINITY_DN172142_c2_g9	1.62	4.68	2.84E-06	0.006	↑	Scavenger receptor cysteine-rich type 1 protein M130 (CD163)
TRINITY_DN172142_c2_g4	1.53	4.36	1.31E-05	0.017	↑	Scavenger receptor cysteine-rich type 1 protein M130 (CD163)
TRINITY_DN160149_c1_g6	1.66	4.27	1.94E-05	0.023	↑	Ig heavy chain V-I region HG3
TRINITY_DN150174_c4_g2	1.78	4.17	3.01E-05	0.031	↑	Neprilysin
TRINITY_DN131224_c0_g2	1.33	4.05	5.06E-05	0.044	↑	Cathelicidin
TRINITY_DN137702_c1_g8	-4.88	-12.80	1.56E-37	9.00E-33	↓	Major histocompatibility complex class I-related gene protein
TRINITY_DN162543_c1_g4	-3.11	-7.48	7.62E-14	2.20E-09	↓	CD48 antigen
TRINITY_DN172627_c9_g2	-1.89	-5.38	7.44E-08	4.31E-04	↓	Interferon-induced very large GTPase 1
TRINITY_DN158064_c3_g2	-1.70	-5.23	1.72E-07	7.68E-04	↓	Early growth response protein 1
TRINITY_DN170110_c2_g11	-2.10	-4.93	8.02E-07	0.002	↓	Periostin
TRINITY_DN165611_c2_g3	-1.86	-4.42	9.75E-06	0.014	↓	Major histocompatibility complex class I-related gene protein
TRINITY_DN139249_c2_g10	-1.82	-4.26	2.01E-05	0.024	↓	GTPase IMAP family member
TRINITY_DN162962_c0_g2	-1.69	-4.13	3.61E-05	0.035	↓	Actin-related protein 2/3 complex subunit 1A
TRINITY_DN169065_c0_g2	-1.46	-4.09	4.32E-05	0.038	↓	Early growth response protein 1

Log2FC is the log2 fold change between expression in infected and control individuals, Wald stat is the wald statistic calculated by DESeq2, Adj. p-value is the Benjamini-Hochberg adjusted p-value, and Direction indicates up-regulation (↑) or down-regulation (↓) in infected individuals. Gene IDs in bold indicate differential expression also in the head kidney.

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653 **Table 4** Differentially expressed genes in the head kidney associated with the immune system.

Gene ID	Log2FC	Wald stat	P-value	Adj. p-value	Direction	Uniprot and refseq annotations
TRINITY_DN146291_c0_g1	3.14	5.10	3.41E-07	7.09E-04	↑	Estrogen receptor beta
TRINITY_DN164847_c1_g5	3.83	5.08	3.70E-07	7.43E-04	↑	HLA class II histocompatibility antigen, DR beta 4 chain
TRINITY_DN168593_c4_g3	3.01	4.83	1.39E-06	0.002	↑	Estrogen receptor beta
TRINITY_DN136158_c0_g1	1.77	4.55	5.25E-06	0.006	↑	Receptor tyrosine-protein kinase erbB-3
TRINITY_DN158138_c1_g1	2.05	4.55	5.28E-06	0.006	↑	Receptor-type tyrosine-protein phosphatase delta
TRINITY_DN139426_c1_g2	3.03	4.51	6.54E-06	0.007	↑	Biglycan
TRINITY_DN151863_c1_g1	2.34	4.43	9.29E-06	0.008	↑	Collagen alpha-5(IV) chain
TRINITY_DN149909_c1_g3	1.88	4.45	8.75E-06	0.008	↑	Receptor-type tyrosine-protein phosphatase delta
TRINITY_DN163104_c3_g2	2.43	4.37	1.23E-05	0.010	↑	Atrial natriuretic peptide receptor 1
TRINITY_DN157378_c3_g1	2.13	4.38	1.21E-05	0.010	↑	Receptor-type tyrosine-protein phosphatase delta
TRINITY_DN144825_c0_g1	1.63	4.35	1.37E-05	0.011	↑	SH2 domain-containing protein 3A
TRINITY_DN159222_c0_g1	1.76	4.32	1.57E-05	0.011	↑	Platelet-derived growth factor C
TRINITY_DN171723_c2_g4	1.97	4.24	2.25E-05	0.015	↑	Receptor-type tyrosine-protein phosphatase delta
TRINITY_DN162605_c1_g2	1.68	4.21	2.55E-05	0.016	↑	Wilms tumor protein
TRINITY_DN148691_c0_g1	2.52	4.20	2.68E-05	0.016	↑	Receptor-interacting serine/threonine-protein kinase 4
TRINITY_DN169331_c3_g2	1.40	4.17	3.00E-05	0.018	↑	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta
TRINITY_DN164726_c4_g1	1.50	4.15	3.26E-05	0.018	↑	Secretory phospholipase A2 receptor
TRINITY_DN157378_c3_g2	2.04	4.09	4.35E-05	0.021	↑	Receptor-type tyrosine-protein phosphatase delta
TRINITY_DN160149_c1_g6	1.65	4.05	5.02E-05	0.023	↑	Ig heavy chain V-I region HG3
TRINITY_DN128669_c0_g2	1.90	4.05	5.19E-05	0.023	↑	Serine/threonine-protein kinase pim-3
TRINITY_DN156836_c3_g2	1.42	4.05	5.15E-05	0.023	↑	Receptor-type tyrosine-protein phosphatase delta
TRINITY_DN171512_c0_g1	2.44	4.04	5.45E-05	0.024	↑	Desmoplakin
TRINITY_DN152103_c1_g1	1.65	4.01	6.05E-05	0.026	↑	finTRIM family, member 82
TRINITY_DN153657_c2_g1	2.15	3.97	7.13E-05	0.028	↑	Collagen alpha-5(IV) chain-like
TRINITY_DN161130_c4_g1	2.54	3.95	7.77E-05	0.029	↑	Estrogen receptor beta
TRINITY_DN133663_c0_g1	2.67	3.94	7.99E-05	0.029	↑	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5
TRINITY_DN127233_c1_g1	2.91	3.94	7.98E-05	0.029	↑	Sex hormone-binding globulin
TRINITY_DN127456_c0_g1	2.65	3.90	9.46E-05	0.032	↑	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5
TRINITY_DN119036_c0_g1	2.83	3.87	1.08E-04	0.035	↑	Ras association domain-containing protein 5
TRINITY_DN142758_c0_g1	2.36	3.85	1.16E-04	0.036	↑	basement membrane-specific heparan sulfate proteoglycan core protein-like
TRINITY_DN156129_c0_g2	2.17	3.83	1.26E-04	0.037	↑	UDP-glucuronosyltransferase 1
TRINITY_DN149035_c4_g1	1.71	3.77	1.65E-04	0.041	↑	Beta-1,4 N-acetylgalactosaminyltransferase 2
TRINITY_DN165320_c4_g1	2.36	3.74	1.81E-04	0.044	↑	Protein kinase C delta type

TRINITY_DN145200_c1_g2	2.71	3.73	1.95E-04	0.046	↑	Desmoglein-1
TRINITY_DN142233_c0_g1	1.58	3.72	2.01E-04	0.046	↑	Secretory phospholipase A2 receptor
TRINITY_DN166241_c2_g1	1.89	3.69	2.21E-04	0.048	↑	Homeodomain-interacting protein kinase 2
TRINITY_DN137702_c1_g10	-5.33	-7.64	2.14E-14	6.24E-10	↓	Major histocompatibility complex class I-related gene protein
TRINITY_DN137702_c1_g8	-5.28	-7.68	1.63E-14	6.24E-10	↓	Major histocompatibility complex class I-related gene protein
TRINITY_DN155813_c1_g1	-2.58	-5.89	3.80E-09	1.84E-05	↓	BTB/POZ domain-containing protein 17
TRINITY_DN168857_c2_g5	-2.86	-4.91	9.28E-07	0.002	↓	GTPase IMAP family member 4
TRINITY_DN162543_c1_g4	-3.61	-4.81	1.51E-06	0.002	↓	CD48 antigen
TRINITY_DN152464_c2_g16	-3.15	-4.34	1.43E-05	0.011	↓	Granulins
TRINITY_DN120453_c1_g2	-1.90	-4.16	3.14E-05	0.018	↓	Ig heavy chain V-III region 23
TRINITY_DN144819_c0_g4	-1.60	-4.13	3.64E-05	0.019	↓	P-selectin
TRINITY_DN144089_c4_g6	-1.53	-4.09	4.39E-05	0.021	↓	Ig lambda chain V-V region DEL
TRINITY_DN128201_c0_g3	-3.05	-4.00	6.45E-05	0.026	↓	CMRF35-like molecule
TRINITY_DN152706_c1_g13	-2.58	-3.89	1.02E-04	0.034	↓	GTPase IMAP family member 4
TRINITY_DN156202_c2_g3	-2.91	-3.85	1.18E-04	0.036	↓	IgGFc-binding protein
TRINITY_DN147923_c2_g2	-2.66	-3.81	1.38E-04	0.038	↓	Myosin-11
TRINITY_DN165611_c2_g3	-2.35	-3.80	1.42E-04	0.038	↓	Major histocompatibility complex class I-related gene protein
TRINITY_DN168771_c3_g3	-2.90	-3.78	1.57E-04	0.040	↓	Ig kappa chain V-V region HP 91A3
TRINITY_DN164893_c1_g8	-2.17	-3.78	1.60E-04	0.040	↓	HERV-H LTR-associating protein 2-like
TRINITY_DN151002_c1_g1	-1.55	-3.74	1.85E-04	0.044	↓	High affinity immunoglobulin epsilon receptor subunit beta-like

Columns as described for table 3. Gene ID in bold indicates that this gene was also differentially expressed in the spleen.

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655 **Vitae**

656 Seraina E. Bracamonte is a doctoral candidate studying the immune response of eel hosts to
657 parasites.

658 Dr. Paul R. Johnston is a postdoc in the evolutionary biology group at the Free University of Berlin
659 and the Berlin Center for Genomics in Biodiversity Research. He is interested in host-microbe
660 interactions, especially the evolution of bacterial resistance to host defences, the genomics of gut
661 symbionts, and the consequences of dynamic developmental processes such as metamorphosis for
662 symbiosis.

663 PD Dr. Klaus Knopf is a senior scientist and the leader of the fish parasitology and immunology group
664 at IGB. His research focusses on determining stress and immune parameters as indicators for fish
665 welfare in aquaculture and new ways to control disease in these conditions. He also focusses on how
666 fish parasites affect the role of their hosts in food webs.

667 Michael T. Monaghan is a senior scientist at Leibniz-IGB and a professor of biology at Freie
668 Universität Berlin. His interests include freshwater ecology, evolution, and biodiversity.