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Molecular ontogeny of larval immunity in European eel at increasing temperatures

Running title: Immunity in European eel larvae

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Abstract:

Temperature is a major factor that modulates the development and reactivity of the immune system. Only limited knowledge exists regarding the immune system of the catadromous European eel, *Anguilla anguilla*, especially during the oceanic early life history stages. Thus, a new molecular toolbox was developed, involving tissue specific characterisation of 3 housekeeping genes, 9 genes from the innate and 3 genes from the adaptive immune system of this species. The spatial pattern of immune genes reflected their function, e.g. complement component *c3* was mainly produced in liver and *il10* in the head kidney. Subsequently, the ontogeny of the immune system was studied in larvae reared from hatch to first-feeding at four temperatures, spanning their thermal tolerance range (16, 18, 20, and 22°C). Expression of some genes (*c3* and *igm*) declined post hatch, whilst expression of most other genes (*mhc2*, *tlr2*, *il1β*, *irf3*, *irf7*) increased with larval age. At the optimal temperature, 18°C, this pattern of immune-gene expression revealed an immunocompromised phase between hatch (0 dph) and teeth-development (8 dph). The expression of two of the studied genes (*mhc2*, *lysc*) was temperature dependent, leading to increased mRNA levels at 22°C. Additionally, at the lower end of the thermal spectrum (16°C) immune competency appeared reduced, whilst close to the upper thermal limit (22°C) larvae showed signs of thermal stress. Thus, protection against pathogens is probably impaired at temperatures close to the critical thermal maximum (CT_{max}), impacting survival and productivity in hatcheries and natural recruitment.

Key words (3-6):

Fish Larvae; Innate Immunity; *Anguilla anguilla*; Early Life History, Gene Expression; Aquaculture; Climate change

1. Introduction:

With more than 34,000 species, teleost fish represent the largest class of vertebrates [1]. This diversity reflects their successful adaption to a great variety of aquatic environments. However, common to most species is an elevated natural mortality during early life [2]. Thus, subtle differences in survivorship can cause large differences in annual offspring production [3]. This is especially important for species that may spawn only once in a lifetime, such as the European eel (*Anguilla anguilla*, Linnaeus 1758), as survival during early-life represents a substantial component of variation in lifetime fitness. An increased understanding of the physical and biological factors that influence mortality rates during these 'critical' developmental stages can enable aquaculture

hatchery production, enhance recruitment predictions for fisheries, and aid in the conservation of this critically endangered species [4].

Teleost fish possess both the innate and adaptive arm of the immune response, characteristic to higher vertebrates [5]. However, evidence has accumulated that newly hatched fish larvae are highly sensitive to pathogens as their immune system is not fully developed [6]. Marine fish larvae are particularly vulnerable to pathogen-induced mortality as it can take up to three months until their immune response is fully functional [7]. During this time, the larvae solely rely on the innate arm of the immune system, which acts in a non-specific manner. During the first stages of larval development (i.e. hatching, mouth opening, first-feeding) exposure to pathogens intensifies [8]. Knowledge of the development of the immune system is hence needed to design preventative methods against pathogens such as the *anguillid herpesvirus 1* (AngHV-1) and the parasite *Anguillicoloides crassus*, which pose an important threat to the European eel [9], in order to prevent losses in forthcoming aquaculture hatcheries. The European eel is a commercially high-value fish species with a long tradition in European fisheries and fish farming. Recruitment and stock size of European eel have decreased substantially in the last decades [10] and a European-wide management strategy is being implemented, while efforts to establish hatchery technology for this species are ongoing [11,12]. However, up-to-date farming as well as restocking of European eel relies on wild-caught juveniles as the life-cycle has not been closed in captivity. It is therefore vital that breeding-technologies and hatchery techniques are being established. Recent advances have enabled the stable production of eggs and larvae, which allow the development and optimisation of hatchery protocols [12,13].

During development organisms are influenced by extrinsic factors (e.g. temperature, pathogens), intrinsic factors (e.g. genetic makeup), and their associated interactions [14,15]. Temperature is one of the main factors influencing marine ecosystems, as it defines the geographical distribution of populations and affects the physiology of individual organisms at all life stages [16]. Furthermore, physiological processes, and therefore development and survival, in ectothermic organisms are generally controlled by the environmental temperature [17]. Here, early life history stages are known to be particularly sensitive to temperature as they have a narrower thermal tolerance window than juveniles or adults and thus are more profoundly affected by even minor temperature changes and short heatwaves [18]. Moreover, temperature is a fundamental modulator of the immune system of fish [19] and has been shown to affect immunity during fish

early life history [20]. The consideration of temperature as an immunomodulatory factor is therefore not only important in the development of hatchery technology of a species such as the European eel in order to optimise offspring rearing protocols, but also in the light of environmental changes in the natural habitat of the larval stages of this species. Here, warming temperatures of the ocean may influence the recruitment of the critically endangered European eel [21,22].

Eels, i.e. Anguilliformes, are basal bony fish (Teleostei) which belong to the ancient superorder of Elopomorpha, at the phylogenetic basis of Teleostei [23]. The current knowledge of eel immunity has recently been reviewed [24]. Whilst the immune system of fish is well studied in some model species, very little research has been conducted regarding the immune system of Elopomorpha with their unique leptocephalus larvae. Research conducted on the immune system of European and Japanese eels (*A. japonica*) has up to date focused on the cellular innate immune response to infections and have rarely involved molecular studies [24]. For example, in Japanese eel it has been shown that some immune factors (i.e. lectin) are present 8 days post-hatch (dph; rearing temperature unknown), whilst the appearance of most immune organs occurs late during larval development [25]. In this study, we aimed to elucidate certain aspects of the development of the immune system in European eel larvae. Specifically, as part of the innate immune system we investigated two complement components (*c3* and *c1qc*), which aid microbial killing, phagocytosis, inflammatory reactions, immune complex clearance, and antibody production (reviewed by [26]). Moreover, we monitored gene expression of the antimicrobial protein C-type lysozyme (*lysc*), the cytokines interleukin 10 (*il10*) and 1β (*il1β*), as well as tumor necrosis factor alpha (*tnfα*). Cytokines aid both the innate and the acquired immune system by interacting with cells, ligands, and receptors to activate cell-mediated immune responses [27]. IL-10 is an anti-inflammatory cytokine whilst IL-1β expression leads to activation of lymphocytes and synthesis of acute phase proteins and thus activation of the complement system [28]. Furthermore, TNF-α is involved in the control and local restriction of infection. In lieu of measuring antiviral type 1 interferon expression, we analysed interferon regulating factors 3 and 7 (*irf3* and *irf 7*) as they have previously been described for European eel [29]. Additionally, we analysed the expression of a pathogen recognition receptor, the toll like receptor 2 (*tlr2*), which is suggested to recognize bacterial and fungal pathogens in eel [30], while the adaptive immune response was studied using the major histocompatibility complex II (*mhc2*), immunoglobulin M (*igm*) and the cluster of differentiation 3 (*cd3*). Here, MHC II is responsible for the presentation of antigens to adaptive immune cells and thus the initiation of an adaptive immune response. This recognition ultimately leads to the

119 destruction of the pathogen through the immune response [28]. Moreover, IgM is the first
120 immunoglobulin to be produced after activation of B cells and can then interact with the
121 complement component C1 to activate the classical complement pathway [28]. Last, CD3, a protein
122 complex, associated to the T-cell receptor and acting as co-receptor, is involved in the activation of
123 T-cells. Together, these immunological insights may be critical to close the life cycle in captivity
124 for this commercially high-value fish species within aquaculture, and to better understand potential
125 impacts of ocean warming on early life stages in nature.
126
127 This study therefore aimed to i) develop tools to specifically study innate (i.e. complement
128 components, antimicrobial peptides, cytokines) and adaptive (i.e. MHC II, immunoglobulin M)
129 immunity in European eel; ii) shed light on the molecular ontogeny of their immune system during
130 early larval development; and finally iii) investigate the interaction of immune gene expression with
131 temperature during early life history.
132
133 **2. Material and methods:**
134 In order to fulfil the above aims we carried out three independent studies. To develop the
135 molecular toolbox to study immune related genes, we characterised the tissue specific expression of
136 the studied genes (see 2.1) and their regulation in response to an AngHV-1 infection (see 2.2). For
137 the purpose of studying thermally modulated immune gene ontogeny, we carried out a study on eel
138 larvae, which analysed immune-related gene expression every 2 dph at 4 different temperatures (see
139 2.3). In all studies, gene expression was analysed using molecular methods (see 2.4).
140
141 **2.1. Generation of tissue library from farmed immature eels**
142 The tissue specific expression of the studied immune genes was investigated using three
143 immature female European eels at the yellow eel stage raised from the glass eel stage to a size of 58
144 ± 1.6 cm and weight of 470 ± 39.7 g at a Danish commercial fish farm (Stensgård Eel Farm A/S).
145 The eels were euthanized by submersion in an aqueous solution of ethyl p-aminobenzoate
146 (benzocaine) at 20 mg L^{-1} (Sigma-Aldrich, Missouri, USA) and organ tissue samples dissected from
147 hind-gut, gills, head kidney, kidney, liver, skin, spleen, whole brain, heart, and muscle. Samples
148 were stored in RNA-later at -80°C until further use. For further processing see 2.4.
149

2.2 AngHV-1 infection of Juvenile European eel tail explants

In addition to the tissue specific expression, we characterised the response of the targeted immune genes to AngHV-1 infection. The alloherpesvirus AngHV-1 is a highly virulent infection agent and considered the most significant viral threat to the eel population. The virus causes extensive necrosis in the gills as well as necrotic lesions in the skin [31]. Here, we used an *in vitro* model to study the immunological response to an AngHV-1 infection in compliance with the 3R rule. As the *in vivo* infection system is not well established yet for AngHV-1 in European eel, we conducted the infection using explants of the tail tissue. Tail tissues (~10 mm of the body end) were sampled from 10 euthanized (with 0.5g l^{-1} of MS-222) European glass eels during routine health checks at the Veterinary University Hannover, Germany. The fish from which the explants had been collected were confirmed to be AngHV-1 negative by qPCR described earlier [9]. The explants were collected into PBS supplemented with 10 IU ml-^{1} penicillin, 100 mg ml^{-1} streptomycin, 100 mg ml^{-1} gentamycin, and 1 mg ml^{-1} amphotericin B (all Sigma), and thereafter placed on ice. Explants were placed individually into the wells of 24 well tissue culture plates and 1 ml of culture medium (medium 199 supplemented with 20% FCS, 10 IU ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 100 mg ml^{-1} gentamycin and 1 mg ml^{-1} amphotericin B [Sigma]) was added to each well. Explants were incubated at 25°C in a humidified atmosphere containing 2% CO_2. After 1 h, half of the explant cultures (n = 5) were randomly infected by adding 10 µl of AngHV-1 virus suspension [32]. The final concentration of the virus was 5 x 10^{5} $TCID_{50}$ ml^{-1}. Controls received 10 µl of uninfected medium. After 48 h the explants were placed in RNA-later and stored in -80 °C until further processing.

RNA was extracted from the tip of the tail as described above and diluted to a common concentration of 30 ng $µl^{-1}$ with HPLC water. RNA (250 ng) was transcribed to cDNA (-RT controls were included) and expression in these samples were analysed using the qPCR Biomark™ HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips) as described below in section 2.4. Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method, in relation to the non-infected control [33]. Gene expression for these samples was normalised against the geometric mean of *rps18* and *ef1* (= Δct) as these genes showed the highest stability (see 2.4).

2.3 Experimental broodstock management and offspring production

Female silver eels were obtained from a freshwater lake, Vandet Sø, Denmark. Male eels were obtained from Stensgård Eel Farm A/S. Females used for experiments (n = 4) had a mean (± SEM) standard length and body weight of 65 ± 4 cm and 486 ± 90 g, respectively. Male eels (n = 11) had a mean (± SEM) standard length and body weight of 40 ± 3 cm and 135 ± 25 g, respectively. Experiments were conducted at a DTU Aqua research facility located at Lyksvad Fish Farm, Denmark. For detailed information on fish handling, maturation and strip spawning, as well as gamete collection and fertilisation see [11,12,34]. The experimental protocol for the study was approved by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries (permit number: 2012-15-2934-00458). All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (EU Dir 2010/63).

2.3.1 Experimental conditions

Eggs from each female were fertilised by a milt pool from 4 males [11] to experimentally create four parental crosses. In total, 11 males were used. Within 30 min post fertilization, ~500 floating zygotes per 100 mL, with a mean size (± SD) of 1.5 ± 0.1 mm (n = 4 females), were distributed in replicated 600 mL flasks [182.5 cm^2 sterile tissue culture flasks with plug seal caps (VWR®)] [34]. Larvae were reared in thermal controlling incubators (MIR-154 Incubator, Panasonic Europe B.V.) at five temperatures (16, 18, 20, 22, and 24 ± 0.1°C), with a salinity of 36 ppt. Seawater was 0.2 µm filtered, UV sterilized and supplemented with rifampicin and ampicillin (each 50 mg L^{-1}, Sigma-Aldrich, Missouri, USA) [35]. Rearing of embryos and larvae took place in darkness, while handling and sampling was performed under low intensity light conditions (< 2.2 µmol m^{-2} s^{-1}) [36]. Light and salinity levels as well as the temperature range were chosen to resemble the environmental conditions prevailing between 0 and 600 m's depth in the Sargasso Sea, i.e. the assumed spawning area of European eel, and to account for projected climate-driven ocean warming.

2.3.2 Data collection

For molecular analysis, ~30 larvae (× 2 replicates) from each temperature and parental combination were randomly sampled at hatch and every second day post-hatch until the corresponding first-feeding stage. As feeding trials were beyond the scope of this study, rearing was not conducted beyond these time points. Larvae were euthanized, using an aqueous solution of tricaine methane sulphonate (MS-222, Sigma-Aldrich, Germany) at 500 ppm, rinsed with deionized

water, preserved in RNA-later (Qiagen, Germany), and kept at -20°C. No larvae hatched at 24°C and therefore this treatment was excluded from the statistical analysis.

2.4. Molecular analyses

The present study is part of a wider project in which various aspects of larval development were investigated. In this regard, various molecular pathways were investigated in the same larvae. Whilst this study focusses on the immune system, other studies have focussed on the expression of genes relating to stress and growth [34] as well as the thyroid hormone signalling pathway [37].

The present study took advantage of the assembly of the European eel genome [38] and obtained sequences (blastn) were checked for high similarity with other fish species (see supplementary material ST3). Primers (Table 1) were designed for real-time PCR with Primer3plus (http://primer3plus.com/). Molecular analysis was performed at GEOMAR, Helmholtz Centre for Ocean Research in Kiel, Germany. Total RNA from all samples was extracted using a combination of Tri-Reagent (Sigma-Aldrich, Germany) and the InviTrap® Spin tissue RNA MiniKit (Stratec) following the manufacturer's instructions. RNA concentration was determined by Nanodrop ND-1000 (Peqlab, Germany) and normalized to a common concentration of 100 ng μl^{-1} with HPLC water. Consequently 680 ng RNA were transcribed with the Quanta qScript cDNA Synthesis Kit (QuantaBio, Germany) as described by the manufacturer including a genomic DNA wipe-out step [Quanta PerfeCta DNase I Kit (QuantaBio, Germany)]. Controls for gDNA efficiency were also included and cDNA was stored at -20°C until further use.

Tissue specific expression was measured for 14 genes using the StepOnePlus qPCR system (Applied Biosystems, Germany). For this purpose, a mix of 2 µl cDNA, 5x EvaGreen qPCR Mix Plus Rox (Solis Biodyne, Estonia), 2.5 pmol of each primer, and HPLC water was used in a total volume of 10 µl. The cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by 95°C for 15 s, 60°C for 1 min and 95°C for 15 s.

From all larval samples (4 crosses × 4 temperatures × 2 replicates) and the AngHV-1 infection samples, the expression of 14 genes (*rps18*, *tubb*, *ef1*, *c3*, *c1qc*, *cd3*, *igm*, *irf3*, *irf7*, *il1β*, *lysc*, *mhc2*, *tnfα*, *tlr2*; Table 1) was analysed with three technical replicates using the qPCR BiomarkTM HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips), as previously described in [39]. In brief, a pre-amplification step was performed with a 500 nM pool of all primers in TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.3 µl cDNA per sample at 10 min at 95°C; 14 cycles: 15 s at 95°C and 4 min at 60°C. Obtained PCR products were diluted 1:10

with low EDTA-TE buffer. The pre-amplified product was loaded onto the chip with SsoFast-EvaGreen Supermix Low Rox (Bio Rad) and DNA-Binding Dye Sample Loading Reagent (Fluidigm). Primers were loaded onto the chip at a concentration of 50 µM in Assay Loading Reagent (Fluidigm) and low EDTA-TE Buffer. The chip was run according to the Fluidigm 96.96 PCR protocol with a Tm of 60°C. qBase+ software verified stability of housekeeping gene expression throughout analysed samples (M < 0.4; according to [40]). Gene expression was normalised (ΔCt) to the geometric mean of the two most stable housekeeping genes (*rps18*, *ef1*). Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method, in relation to the 16°C sample of Day 0 from female 1 [33]. This calculation allowed us to test for effects induced by temperature and larval age at specific developmental stages and in real time.

Table 1: Oligos used for molecular analysis of immune-related gene expression in *Anguilla anguilla.*

Function	Gene name	Abbre-viation	Primer sequence (FW: Forward, RV: Reverse)		Accession
Housekeeping	18S ribosomal RNA	RSP18	FW	AGAGCAGGGGAACTGACTGA	AZBK01681648
			RV	ACCTGGCTGTATTTGCCATC	
	Tubulin β	Tubb	FW	TGATGAGCACGGTATTGACC	AZBK01756733.1
			RV	TGGCACATACTTTCCACCAG	
	Elongation Factor 1	EF1	FW	CTGAAGCCTGGTATGGTGGT	EU407824.1
			RV	CATGGTGCATTTCCACAGAC	
Complement system	Complement component C3	C3	FW	AATATGTGCTCCCAGCCTTC	GBXM01025382.1
			RV	GATAACTTGCCGTGATGTCG	
	Complement Component 1, Q Subcomponent, C Chain	C1qc	FW	ACAATGTCGACACAGGCAAG	GBXM01013997.1
			RV	ACTTGGTTGAGGTTGGAGGTC	
Pro-inflammatory Cytokines	Tumor necrosis factor α	TNFa	FW	TCTGCGATGCTATTCCACTG	JQ793636.1
			RV	TTCAAGTTCTGCTGGTGCTC	
	Interleukin 1β	IL1β	FW	ATTGGCTGGACTTGTGTTCC	AZBK01652159.1
			RV	CATGTGCATTAAAGCTGACCTG	
Anti-inflammatory cytokine	Interleukin 10	IL10	FW	CCTGCAAGAAACCCTTTGAG	AZBK01749637.1
			RV	TGAACCAGGTGTCAATGCTC	
Induce type I Interferon (ant-viral)	Interferon Regulatory Factor 7	IRF7	FW	TTCCTTGGAAGCACAACTCC	KF577784.1
			RV	TGTCGTTCGGATTCTCTCTG	
	Interferon Regulatory Factor 3	IRF3	FW	GAAGAGGTGGCAGCAAAATC	KF577783.1
			RV	GGAAAAAGAGGGGGATTCAC	
Antibacterial	Lysozyme Type	LysC	FW	ACGGCATCTTCCAGATCAAC	AZBK01554584

response	C		RV	TGGAGCACGGGATATTACAG	
Pathogen recognition	Toll like receptor 2	TLR2	FW RV	TGGTTCTGGCTGTAATGGTG CGAAATGAAGGCATGGTAGG	AZBK01853964.1
Antigen presentation to immune cells	Major histocompatibility complex, Class II	MHC 2	FW RV	TCAAATTGACCTGGCTGAGAG TTTCCATTAGCCAGCTCCTC	AF134926.1
Antibody	Immunoglobulin M	IgM	FW RV	CCAAGGACCATTCTTTCGTC ACTGGCTTTCAGGAAGATGC	EU551246.1
T-cell co-receptor	Cluster of differentiation 3	CD3	FW RV	AACCGATGATGCTGGAGAAG ATGTGTATTCGCCCGAACTG	AZBK01640579.1

2.5. Statistical analysis

Tissue specific expression was tested using one-way ANOVAs with parental cross stated as random term. The difference between control samples and samples infected with AngHV-1 was statistically analysed using Mann-Whitney tests for each gene. Statistical models were used to investigate temperature effects on larval morphology and gene expression throughout early larval development (0 to 18 dph) and at specific developmental stages (Stages 1-3). Across the temperature treatments, Stage 1 represents the day of hatch, Stage 2 represents the timing of teeth formation, and Stage 3 represents the first-feeding stages [12]. Together, this allowed us to decipher changes in temperature in real-time and at standardized developmental intervals.

To examine the effect of temperature on gene expression throughout early development, we used two statistical approaches. In the first approach, we analysed the data using a series of repeated measures mixed-model ANOVAs (PROC MIXED; SAS Institute 2003). Models contained the temperature (16, 18, 20 and 22°C) and age (0 to 18 DPH) or stage (1, 2 and 3) main effects as well as the temperature × age (or stage) interaction term. Akaike's (AIC) and Bayesian (BIC) information criteria were used to assess which covariance structure (compound symmetry, autoregressive order, or unstructured) was most appropriate [41]. Temperature and age (or stage) were considered fixed, whereas parental cross was considered random. Tukey's post-hoc analyses were used to compare means between treatments. If a significant temperature × age (or stage) interaction was detected, the model was decomposed into a series of reduced one-way ANOVA models to determine the effect of temperature for each age (or stage) and of age (or stage) for each temperature. This was the case for *il1β*, *lysc*, *irf7*, and *mhc2*. Reduced one-way ANOVA models involved only pre-planned comparisons and did not include repeated use of the same data, so alpha level corrections for *a posteriori* comparison were not necessary.

In the second approach, we examined variation in gene expression, throughout development at each temperature, by fitting linear, quadratic, or cubic equations (PROC REG; SAS Institute 2003). This allowed us to create predictive models to explore patterns of variation throughout early development at each temperature. Linear, quadratic, or cubic equations were chosen a-priori to fit the data [42]. Final equation selection (linear, quadratic, or cubic) was based on an F-statistic: $d.f._j \times (R^2j - R^2i)/(1 - R^2j)$, where: R^2i = the R^2 for the i-th order, R^2j = the r^2 for the next higher order, $d.f._j$ = the degrees of freedom for the higher-order equation with j degrees of freedom in the numerator and $d.f._j = n - j - 1$ degrees of freedom in the denominator [42]. Graphs and regressions were prepared in SigmaPlot® (Version 13.0).

3. Results:

3.1 Characterisation of tissue specific gene expression:

Target genes were characterised for their expression in various tissues of on-grown farmed European eels (Fig. 1). Only baseline levels of *irf3* expression could be detected in all studied organs (Fig. 1B). On the contrary, interferon regulator factor 7 (*irf7*) demonstrated low expression in brain, heart, muscle as well as head kidney but significantly higher mRNA levels ($P \leq 0.05$; Fig. 1C) were detected in the hind-gut (145 ± 26-fold compared to head-kidney). Tissue specific expression also varied amongst cytokines: mRNA levels of interleukin (*il1β*) were highest in gills (2844 ± 1517-fold) and skin (1450 ± 953-fold) (both $P \leq 0.05$ compared to brain; Fig. 1D), followed by the immune organs. Variation between individuals was high and no significant differences between organs were detected for the expression of tumor necrosis factor α (*tnfα*) (Fig. 1E). However, *il10* was mainly expressed in head kidney (606 ± 326-fold compared to heart with $P \leq 0.05$; Fig. 1F). In regard to the adaptive immune system, immunoglobulin M (*igm*) seemed to be expressed in the hind-gut, gills, and head kidney, with the highest expression in kidney (91 ± 26-fold compared to the liver with $P \leq 0.01$; Fig. 1G). High intra-individual variability was observed for *cd3* and *mhc2*, which led to no significant differences between tissues to be detected (Fig. H, I). Differential tissue expression was detected for the two complement components analysed: whilst *c3* was mainly expressed in liver ($P \leq 0.05$ compared to all other organs; Fig. 1J), *c1qc* was least expressed in this organ but highest in the head kidney and kidney ($P \leq 0.05$; Fig. K). The expression of the toll like receptor 2 (*tlr2*) was 160 ± 52-fold higher in the hind-gut than in the other investigated organs ($P \leq 0.05$; Fig. 1L).

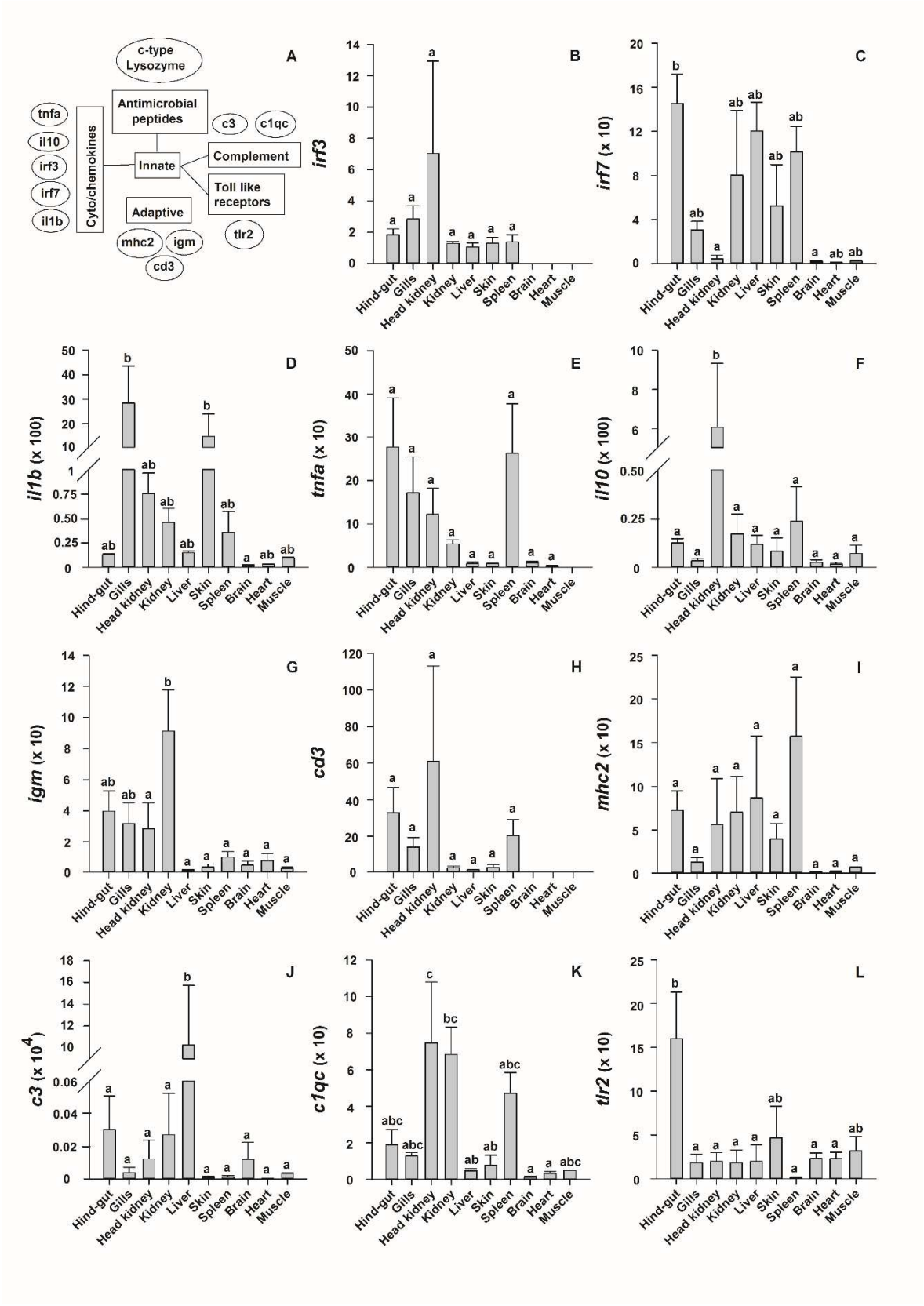


Fig. 1: Tissue specific expression of immune-related genes in on-grown farmed female *A. anguilla*. A) Overview of genes targeted in the present study. B-L) Expression of immune-related

genes in certain tissues of European eel. Data are presented as mean ± SEM (n = 3). Gene expression is displayed as x-fold expression compared to the organ with lowest mRNA levels. Values with the same letters are not significantly different (P > 0.05).

3.2 AngHV-1 infection

At the point of sampling no clinical signs of AngHV-1 were visible. Infection of tail tissue with AngHV-1 increased the expression of two of the characterised genes (Fig. 2). An increase in expression was observed for the innate immune factor *il1β*, which was up-regulated approximately 3-fold (dct *il1β* control 6.70 ± 0.17, infection 5.29 ± 0.45). Interestingly, the strongest response was observed for *mhc2*, which displayed a 6.13 ± 1.65-fold increase in expression due to AngHV-1 (dct 3.25 ± 0.34) compared to the uninfected control treatment (dct 5.68 ± 0.86).

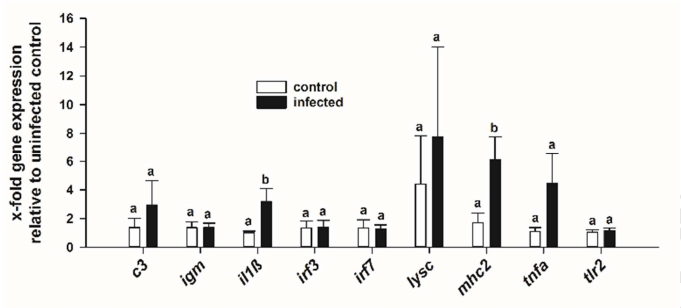


Fig. 2: Expression of immune-related genes in response to AngHV-1 infection.

Tail tissue explants of European eel were infected with 5 x 10^5 $TCID_{50}$ of AngHV-1 *in vitro* for 36 hours. Values represent means (±SEM) of five biological replicates. Treatments with the same letters are not significantly different (P > 0.05).

3.3 Development of the larval immune system:

Generally, increasing temperature accelerated development, resulting in larvae reaching the first-feeding stage in 8 days at 22°C, 10 days at 20°C, 12 days at 18°C, and 16 days at 16°C. Housekeeping (HK) gene expression was stable (M < 0.4) throughout the experiment (see Fig. S1, supplement) and variance in HK expression was clearly coupled to variation in cDNA amount in samples. No mRNA of complement component *c1qc* and T-cell marker *cd3* was detected in the studied larvae. Gene expression of the cytokine *tnfα* was low (ct > 26) and not affected by temperature nor larval age and no temperature × age interaction was detected. For details on the observed regression please refer to Table S1 in the supplementary material. If not otherwise

indicated, x-fold values and Δct-values are given as mean ± SEM of all temperatures combined (indicated as Δct($\overline{T}$)).

Complement component c3 is the central molecule in the activation pathway of complement and thus its protein levels are linked to complement activity. During development, it was observed that larval gene expression was related to age or stage ($P < 0.0001$; Fig. 3A, D), such that gene expression of *c3* significantly decreased with increasing age from 0.77 ± 0.06-fold (Δct($\overline{T}$): 4.18 ± 0.20) on 0 dph to a minimum of 0.13 ± 0.06-fold (Δct($\overline{T}$) 6.61 ± 0.20) on day 12 ph (Fig. 3D). Immunoglobulin M is the first antibody to respond to an infection and has been dubbed a natural antibody in both mammals and teleosts as it can respond to pathogens without prior immunisation [43]. Thus, providing initial protection before the adaptive immune response is formed. Its mRNA levels decreased significantly with increasing stage ($P < 0.0001$; Fig. 3I) and age ($P < 0.0001$; Fig. 3L) displaying a maximum expression (2.16 ± 0.30-fold, Δct($\overline{T}$): 9.42 ± 0.21) on day 0 and a constant expression level of 0.48 ± 0.03-fold (Δct($\overline{T}$): 11.51 ± 0.10) after that.

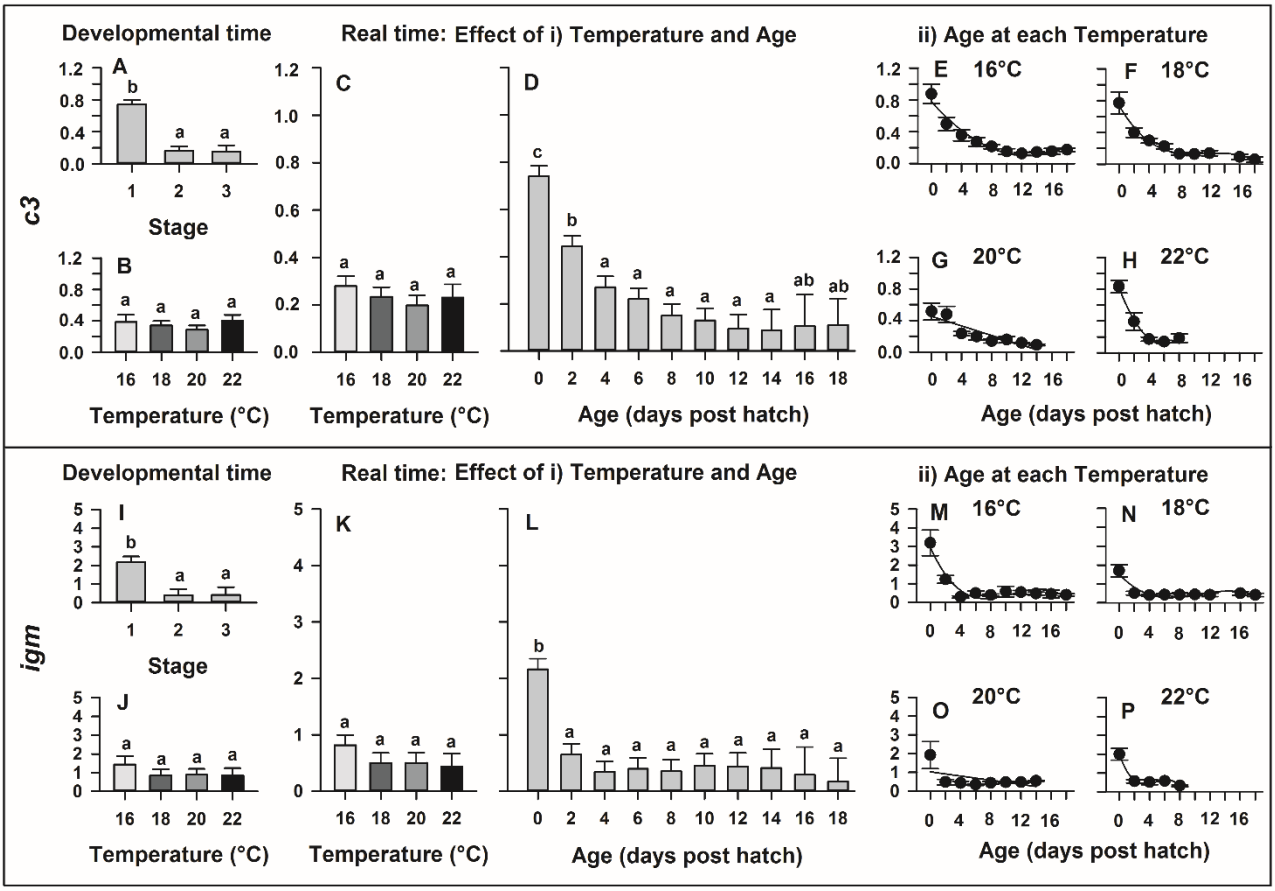


Fig. 3: Gene expression of *c3* and *igm* in European eel (*Anguilla anguilla*) larvae reared under four different temperatures. All y-axes display x-fold expression in relation to the 16°C sample of day 0 from female 1. The expression at specific developmental stages (1: hatching, 2: teeth

formation, 3: first-feeding) is displayed for *c3* (A-B) and *igm* (I-J) whilst expression measured in real time is displayed in C-D for *c3* and K-L for *igm.* E-H and M-P show the effect of age on *c3* and *igm*, respectively. Relationships between age and *c3* expression can be explained by a cubic regression at 18°C, a linear regression at 20°C and quadratic regressions at 16 or 22°C ($P < 0.01$; $R^2 > 0.78$). Relationships between age and *igm* expression can be explained by a linear regression at 20°C and cubic regressions at 16, 18 or 22°C ($P < 0.001$; $R^2 > 0.16$). Values represent means (±SEM) among four crosses at each temperature and treatments with the same letters are not significantly different ($P > 0.05$).

Moreover, mRNA levels of major histocompatibility complex II (*mhc2*) were also significantly affected by stage as transcription increased beyond stage 2 (teeth formation; $P = 0.001$; Fig. 4A). MHC II is located on the cell surface of antigen-presenting cells (e.g. macrophages, B-cells) and it fulfils the function of presenting extracellular antigens to immune cells. The expression of *mhc2* increased more than 2-fold at 16 and at 22°C ($P = 0.003$) compared to the other temperatures, though no significant temperature × stage interaction was detected (Fig. 4B). On the contrary, when analysed in real time, a significant temperature × age interaction ($P = 0.043$) was observed, which allowed us to determine the effects of temperature for each age (Fig. 4C) and of age for each temperature (Fig. 4D-G). Here, 22°C showed the largest effect as it led to an increase of *mhc2* levels of 13.71 ± 3.76-fold at 4 dph, 13.27 ± 0.93 at 6 dph, and 39.60 ± 6.54 at 8 dph (all $P \leq 0.01$). Furthermore, larval age significantly influenced expression of *mhc2* (Fig. 4D-F) when larvae were reared at temperatures ranging from 16 to 20°C ($P \leq 0.01$), while no age effect was observed at 22°C (Fig. 4G). This leads to a steady increase in mRNA levels throughout development of the larvae. Initial detection of antigens is amongst others carried out by toll like receptors. In fish, toll like receptor 2 (TLR2) is involved in the recognition of bacterial and parasitic ligands [44]. As shown in Fig. 4H, expression of *tlr2* approximately doubled at stage 2 (teeth formation) and tripled at stage 3 (first-feeding; $P < 0.0001$). It was also significantly affected in real time ($P < 0.0001$) and increased with increasing age from a minimum expression of 0.62 ± 0.09-fold (Δct: 10.52 ± 0.15) on 2 dph to a maximum expression of 4.34 ± 0.23-fold (Δct: 7.48 ± 0.08) on 16 dph (Fig. 4K).

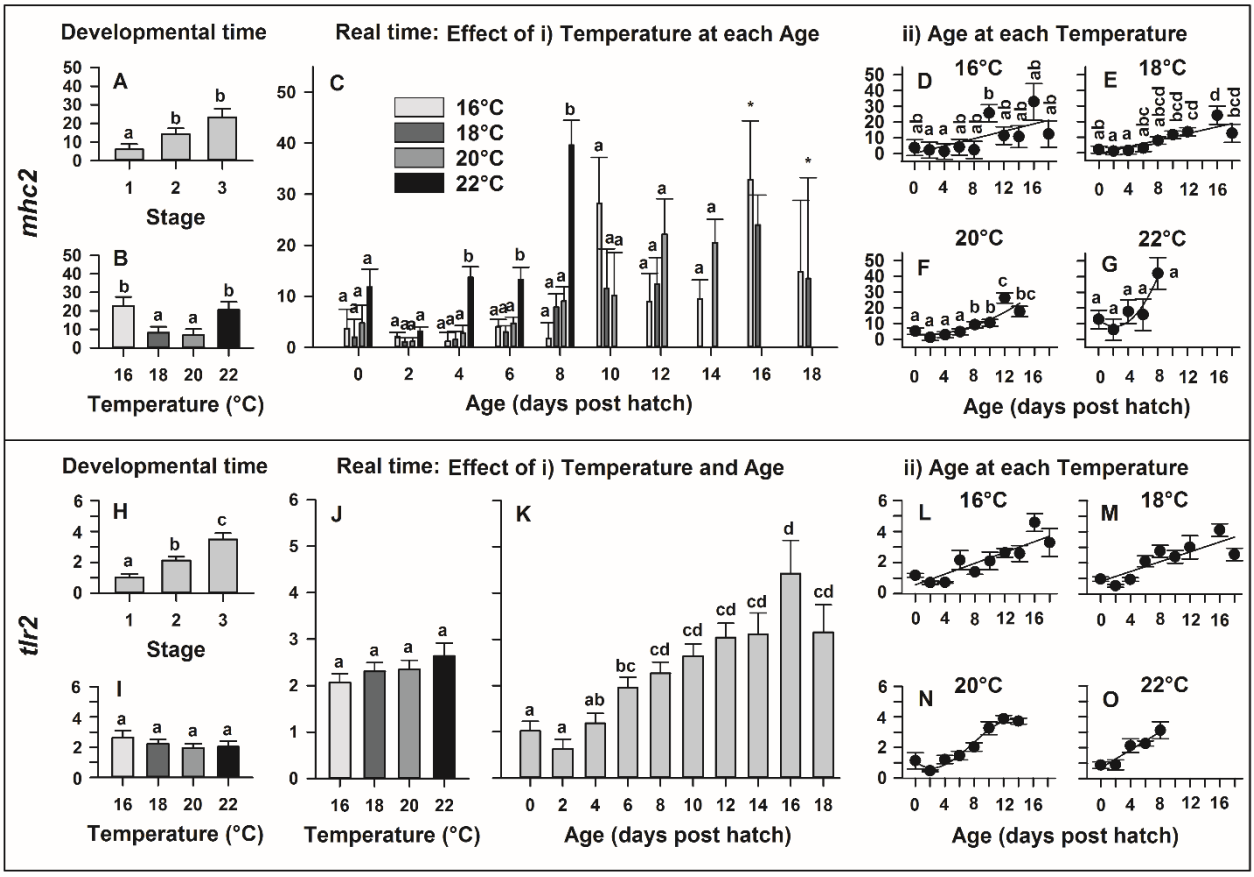


Fig. 4: Gene expression of *mhc2* and *tlr2* in European eel (*Anguilla anguilla*) larvae reared under four different temperatures. All y-axes display x-fold expression in relation to the 16°C sample of day 0 from female 1. The expression at specific developmental stages (1: hatching, 2: teeth formation, 3: first-feeding) is displayed for *mhc2* (A-B) and *tlr2* (H-I), whilst expression measured in real time is displayed in C for *mhc2* and J-K for *tlr2*. D-G and L-O show the effect of age on *mhc2* and *tlr2*, respectively. Relationships between age and *mhc2* expression can be explained by linear regressions at 16 or 18°C and quadratic regressions at 20 or 22°C ($P < 0.0001$; $R^2 > 0.36$). The relationship between age and *tlr2* expression can be explained by a cubic regression at 20°C and linear regressions at 16, 18 or 22°C ($P < 0.002$; $R^2 > 0.66$). Data points with an asterisk (*) were not included in the statistical model due to insufficient sample size. Values represent means (± SEM) among four crosses at each temperature and treatments with the same letters are not significantly different ($P > 0.05$).

Type I IFNs are cytokines, which are expressed in response to the detection of viral antigens and elicit an antiviral immune response. The expression of type I IFNs is regulated with the Interferon regulating factors 3 and 7 (*irf3* and *7*) and in the present study, we demonstrate that their

expression is dependent on larval stage (Fig. 5A, B, I, J; $P < 0.0001$), and age (Fig. 5D, K; $P < 0.0001$) as well as temperature when measured in real time (Fig. 5C, K; $P < 0.029$). Gene expression of *irf3* was significantly elevated (6.66 ± 0.69-fold) at 22°C compared to (4.59 ± 0.45-fold) 16°C (Fig. 5C) and significantly increased throughout ontogeny with increasing larval age (Fig. 5D). Here, general expression was 2.24 ± 0.25-fold ($\Delta ct(\overline{T})$: 11.29 ± 0.16) on day 0 and increased in a linear manner to reach a maximum of 8.26 ± 0.78-fold ($\Delta ct(\overline{T})$: 9.19 ± 0.14) at 14 dph. *Irf7* was significantly affected by the temperature × age interaction ($P = 0.013$). Significant differences in mRNA levels of *irf7* among temperatures occurred on 4 dph ($P = 0.045$) where expression at 22°C was about 40% higher than the expression at 16°C (Fig. 5K). Additionally, *irf7* levels increased steadily throughout development in all tested temperatures (16 – 22°C; $P \leq 0.007$; Fig. 5L-O).

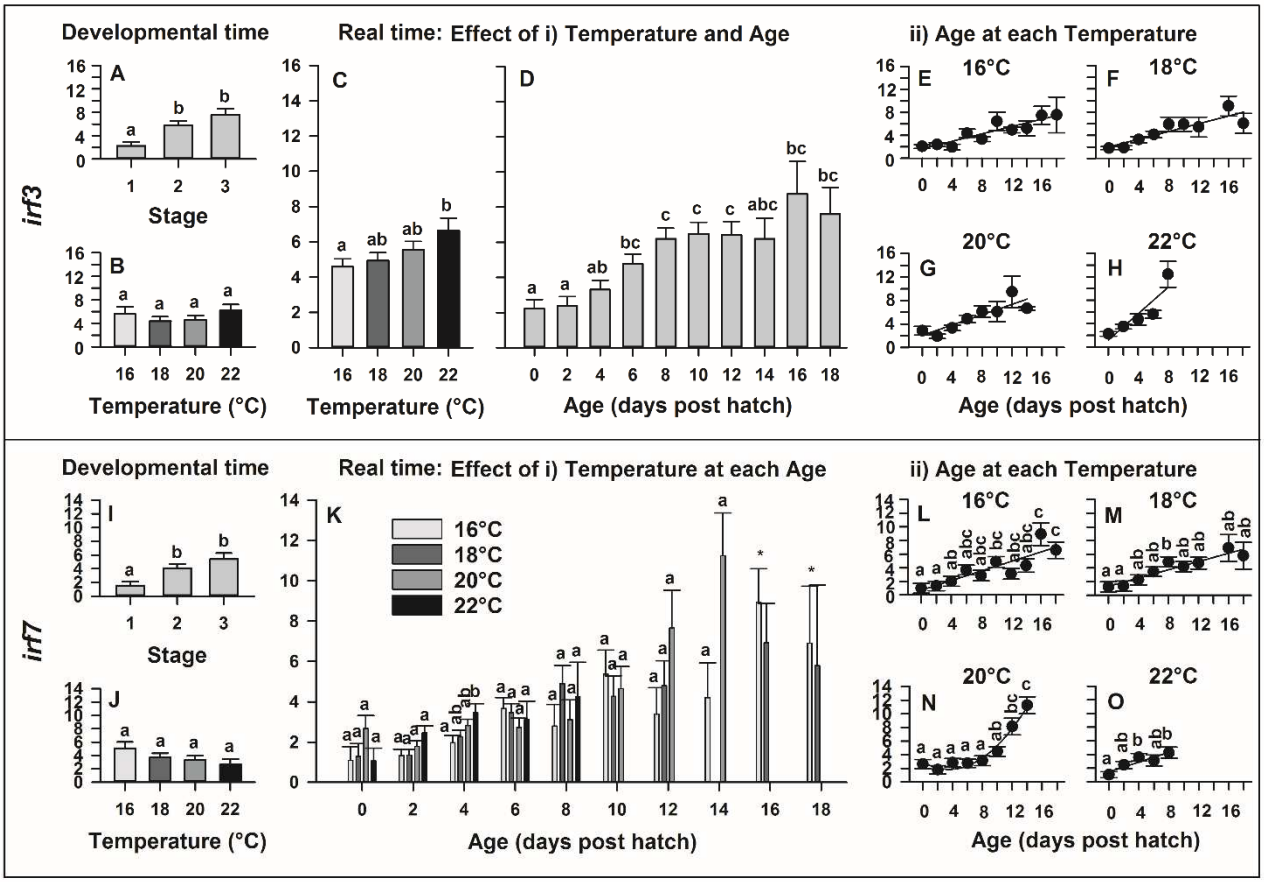


Fig. 5: Gene expression of *irf3* and *irf7* in European eel (*Anguilla anguilla*) larvae reared under four different temperatures. All y-axes display x-fold expression in relation to the 16°C sample of day 0 from female 1. The expression at specific developmental stages (1: hatching, 2: teeth formation, 3: first-feeding) is displayed for *irf3* (A-B) and *irf7* (I-J), whilst expression measured in real time is displayed in C-D for *irf* and K-L for *irf7*. E-H and M-P show the effect of

age on *irf3* and *irf7*, respectively. Relationships between age and *irf3* expression can be explained by linear regressions at all temperature treatments ($P < 0.0001$; $R^2 > 0.74$). The relationship between age and *irf7* expression can be explained by a quadratic regression at 20°C and linear regressions at 16, 18 or 22°C ($P < 0.002$; $R^2 > 0.72$). Data points with an asterisk (*) were not included in the statistical model due to insufficient sample size. Values represent means (± SEM) among four crosses at each temperature and treatments with the same letters are not significantly different ($P > 0.05$).

Gene expression of the pro-inflammatory cytokine Interleukin 1β (*il1β*) was only significantly increased at stage 3 (first-feeding; $P = 0.033$) but at this stage it was up-regulated approximately 3-fold (Fig. 6A). The real time analysis, however, revealed a significant temperature × age interaction (Fig. 6C; $P = 0.003$). Significant differences in gene expression of *il1β* among temperatures occurred on 6, 8 and 12 dph ($P < 0.02$). On 8 dph in specific, expression levels of *il1β* were approximately 6-fold (Δct: 5.96 ± 0.97) higher at 22°C compared to the other temperatures (Δct: 8.70 ± 0.15). Larval age led to a significant increase in *il1β* expression at all rearing temperatures ($P \leq 0.001$) except at 20°C (Fig. 6D-G), probably due to high levels of variation between individuals at this temperature. A linear increase of *il1β* expression was observed at 16°C and 18°C ($P < 0.0001$; $R^2 \geq 0.53$), leading to an approximately 4-fold increase within the sampling period. The relationships between age and *il1β* expression can be explained by a sigmoidal cubic regression at 22°C ($P < 0.001$; $R^2 = 0.95$) with low levels of expression during the first 6 dph and a sudden increase at 8 dph, resulting in the 6-fold up-regulation described above.

Expression of the antimicrobial c-type lysozyme (*lysc*) was also significantly up-regulated at the first-feeding stage (stage 3) by approx. 3-fold (Fig. 6H; $P = 0.0001$). Furthermore, expression of this gene was doubled at 22°C compared to 20°C (Fig. 6I; $P = 0.041$). In real time, the temperature × age interaction significantly influenced the gene expression of *lysc* (Fig. 6J; $P < 0.0001$). Here, significant differences in expression of *lysc* among temperatures occurred on 8, 12 and 14 dph ($P \leq 0.04$). More specifically, and in line with expression patterns of other genes at day 8 ph, a strong up-regulation (300 %) of *lysc* at 22°C compared to the other temperatures was observed. Additionally, a rearing temperature of 16°C led to a significant lower level of expression of *lysc* compared to 18°C (16°C: Δct: 12.93 ± 0.35, 18°C: Δct: 11.37 ± 0.45) on 12 dph and compared to 20°C (16°C: Δct: 13.11 ± 0.92, 20°C: Δct: 11.04 ± 0.19) on 14 dph. Expression of *lysc* was also significantly affected by larval age (Fig. 6K-N) at all rearing temperatures ($P \leq 0.01$). Similar to *il1β*, expression

of *lysc* linearly increased at 16°C ($P = 0.012$, $R^2 = 0.29$) and 18°C ($P < 0.001$, $R^2 = 0.31$) throughout larval development. The relationships between age and *lysc* expression can further be explained by a sigmoidal cubic regression at 20°C ($P < 0.0001$; $R^2 = 0.96$) leading to a 9-fold expression at 14 dph (Δct: 11.04 ± 0.19) compared to day 0 (Δct: 19.14 ± 0.58; Fig. 6M). As observed in *il1β*, rearing larvae at 22°C led to a constant level of *lysc* expression during the first 6 dph and a sharp increase in mRNA levels at 8 dph. This is best described by a parabolic quadratic regression at 22°C (Fig. 6N; $P = 0.0001$; $R^2 = 0.59$).

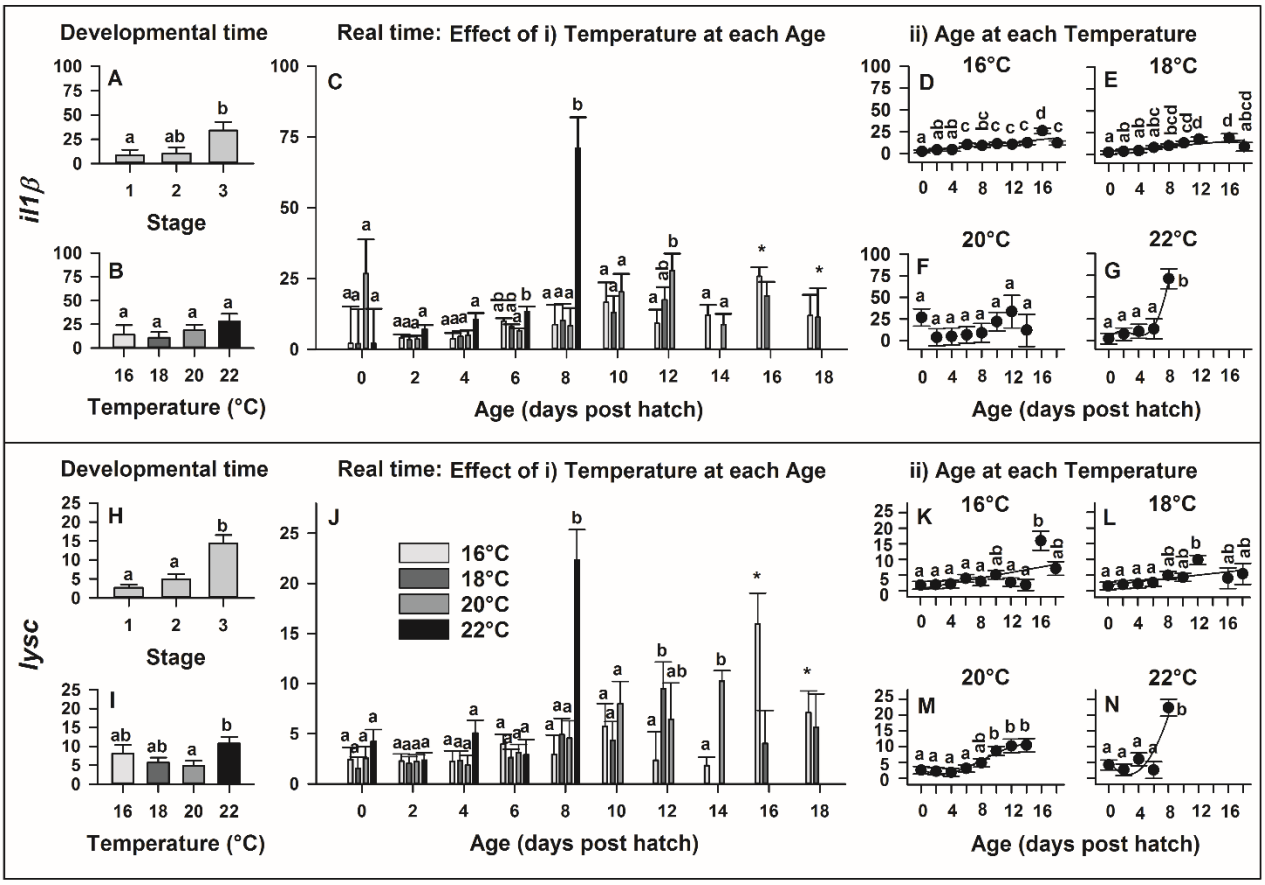


Fig. 6: Gene expression of *il1β* and *lysc* in European eel (*Anguilla anguilla*) larvae reared under four different temperatures. All y-axes display x-fold expression in relation to the 16°C sample of day 0 from female 1. The expression at specific developmental stages (1: hatching, 2: teeth formation, 3: first-feeding) is displayed for *il1β* (A-B) and *lysc* (H-I), whilst expression measured in real time is displayed in C for *il1β* and J for *lysc*. D-G and K-N show the effect of age on *il1β* and *lysc*, respectively. Relationships between age and *il1β* expression can be explained by linear regressions at 16 or 18°C and a cubic regression at 22°C ($P < 0.0001$; $R^2 > 0.53$). The relationship between age and *lysc* expression can be explained by linear regressions at 16 or 18°C, a cubic regression at 20°C and a quadratic regression at 22°C ($P < 0.002$; $R^2 > 0.29$). Data points with

an asterisk (*) were not included in the statistical model due to insufficient sample size. Values represent means (± SEM) among four crosses at each temperature and treatments with the same letters are not significantly different (P > 0.05).

4. Discussion:

The ultimate aim of this study was to elucidate the expression patterns of key immune genes during *A. anguilla* development and investigate how temperature influences these patterns. As no molecular tools were available to analyse immune-related genes in this fish species, we first set out to develop primers and characterize tissue specific expression of immune-related genes in healthy on-grown farmed individuals. Subsequently, the usability of this tool was evaluated during AngHV-1 exposure of tail explant cultures. Once their usability was established, we applied these new tools to study the 'critical' early life stages of eel across a broad thermal regime.

4.1 Tissue specific expression & AngHV-1 infection

Complement activation leads to the production of activated protein fragments that play an important role in innate immune responses [26]. In mammals, and possibly in fish, C3 is the central complement molecule of the three pathways [26]. It is known that complement proteins are mainly synthesized in the liver in mammals and fish [45]. The tissue specific expression pattern for *c3* (highest expression in liver) in the present study is therefore in line with previous findings. Similar results have been observed in other fish species, such as yellow croaker (*Larimichthys crocea*), Indian major carp (*Labeo rohita*), and orange spotted grouper (*Epinephelus coioides*) [46–48]. The gene complement C1q C chain (*c1qc*) encodes the large subunit (C1q) of the complement component C1. C1q acts as a pathogen sensor and binds directly to their surface or to antibodies bound to pathogens, which leads to the activation of the complement system via the classical pathway [28]. In mammals, C1q is mainly synthesized in macrophages and dendritic cells and not by hepatocytes unlike the other complement components [49]. This is reflected in the expression pattern observed in the analysed healthy eel tissue where *c1qc* expression was very low in the liver but high in spleen, kidney, and head kidney. This pattern seems to be conserved across many fish species and orders as it was also observed in Siberian sturgeon (*Acipenser baerii*), mandarin fish (*Siniperca chuatsi*), and zebrafish (*Danio rerio*) [50–52]. Both investigated complement factors *c3* and *c1qc* were not significantly up-regulated during AngHV-1, which is in line with results from common carp gills infected with cyprinid herpesvirus 3 (CyHV-3), which has been shown to be able

to modulate the immune response of the host [53]. However, targeted studies are needed to investigate the immunuomodulatory potential of AngHV-1.

Toll-like receptors (TLR) were the first receptors identified that recognize pathogen associated molecular patterns (PAMPs) [54]. In European eel, TLR2 is suggested to recognize bacterial and fungal pathogens [30]. This is in line with our results where the expression was not regulated during AngHV-1 infection. The *tlr2* expression in the present study was especially low in the spleen but high in hind-gut. Throughout the literature, it is clear that tissue specific *tlr2* expression varies among species [44,55–57]. As pathogens are often ingested with food or water, it was expected that *tlr2* is highly expressed in the hind-gut. However, the lack of expression in the spleen was not expected as this is a major immune organ and future studies should address this phenomenon.

Cytokines include interleukins, tumor necrosis factors, interferons and chemokines. In this study, we investigated *il10*, *il1β* and *tnfα* as well as the type 1 interferon inducing factors *irf3* and *irf7*. In humans, IL-10 is mainly produced by monocytes [28], whilst in fish the functional role of IL-10 is still under investigation. It has been shown that, similar to the expression observed in eel, sea bass (*Dicentrarchus labrax*) and carp (*Cyprinus carpio*) produce this cytokine intensely in head kidney [58,59]. This indicates that the head kidney is a major side of monocytes in eel. IL-1β is produced by macrophages in response to signalling via toll-like receptors (TLRs) and induces an inflammatory immune response. For the eels used in this study, *il1β* was constitutively expressed in all studied organs with the highest expression in gills and skin. The latter is contrary to other fish species, such as brown trout (*Salmo trutta*) and rohu (*Labeo rohita*), where *il1β* expression was always low in skin [60,61]. Interleukin-1β has been shown to be up-regulated in skin of fish following infection or injury as reviewed by [62]. Thus, it has to be investigated further if the high expression in skin is an eel specific characteristic or if it is due to an undetected infection or injury. Due to the low variability between the three tested individuals, this might be an eel specific trait attributed to their rudimentary scales and high mucus production [63]. Interestingly, *il1β* was also one of the genes up-regulated during the AngHV-1 infection. This is in line with the response in carp to CyHV-3, which also induces pro-inflammatory responses in skin [64].

The main role of TNF-α is the control and local restriction of infection and the here observed tissue specific expression corresponds to a study in rainbow trout (*Oncorhynchus mykiss*) [65] but differs from expression patterns in other fish species, such as mandarin fish (*Siniperca chuatsi*), rohu, and crucian carp (*Carassius carassius*) [61,66–68]. This indicates, as previously discussed by Kajungiro and colleagues, that constitutive expression of *tnfα* varies with fish species and tissues

[67]. During exposure of the explants to AngHV-1 in our *in vitro* experiments, the up-regulation was noticed, however due to high variation it was not significant.

The interferon regulating factors 3 and 7 have been previously characterised in European eel [29]. In healthy organisms, IRF3 is constitutively expressed in a variety of tissues and is present in a latent inactive form in the cytoplasm [69]. On the other hand, IRF7 is expressed at low levels in the cytoplasm of lymphoid cells [70]. Both are strongly induced upon viral infection. Surprisingly, these two type I IFN response markers were not up-regulated during AngHV-1 infection, which should be further investigated in the future. However, the group of fish herpes viruses is known to have strong anti-interferon abilities [71]. The present study aimed, amongst others, to complement the knowledge from a previous *A. anguilla* study [29] by adding the tissue specific expression of these two genes. *Irf3* was constitutively expressed at a low level in all analysed tissues whilst *irf7* displayed higher expression levels in organs involved in the immune response except for head kidney. The latter not only concurs with the spatial pattern found in PolyI:C treated eels [29] but also resembles the pattern observed in crucian carp and Japanese flounder (*Paralichthys olivaceus*) [72,73].

Immunoglobulin M (IgM) is, like all classes of immunoglobulins, produced by B-cells and therefore its expression gives an indication of the localisation of these immune cells. The observed tissue specific expression pattern of *igm* concurs with the pattern observed in turbot (*Scophthalmus maximus*) [74] whilst higher spleen expression levels were observed in pufferfish (*Takifugu rubripes*) [75]. The high expressions in head kidney and trunk kidney indicate the haematopoetic function of these organs in *A. anguilla* and confirm previous findings in New Zealand freshwater eels (*A. australis schmidtii* and *A. dieffenbachii*) [76]. The low expression of *igm* in spleen, similar to the expression of *tlr2* in this organ, is however unexpected and should be addressed in future studies. In respect to the AngHV-1 infection, *igm* up-regulation was not expected due to the early sampling point, which precedes activation of the adaptive immune response in teleosts [77].

Another member of the immunoglobulin superfamily is the clusters of differentiation (CD). In humans, CD3 is initially expressed in the cytoplasm of pro-thymocytes and migrates to the cell membrane when T-cells mature. Hence, CD3 is only expressed on T-cells and can therefore be used as a marker for the presence of this cell type. Our study showed that *cd3* was ubiquitously expressed in all tested immune organs of on-grown farmed European eel. Unfortunately, due to the involution of thymus in eels [76], it was not possible to test expression in thymus tissue. In fish, mature T-cells

565 have been shown to be abundant in lymphoid tissues such as thymus, kidney, and spleen and in
566 mucosal tissues (intestine and gills) [78].
567 The major histocompatibility complex (MHC) binds peptide fragments of pathogens and
568 presents them on the cell surface for recognition by appropriate T-cells. In mammals, MHC II class
569 molecules can be found on antigen-presenting cells, i.e. B-cells, macrophages, and dendritic cells
570 [28]. A detailed characterization of MHC II in eel was beyond the scope of this study and thus we
571 concentrated on the tissue specific expression. The *mhc2* expression pattern observed in the present
572 analysis is similar to tissue expression recorded in swamp eel where hardly any expression was
573 detected in heart and muscle, whilst the highest expression was observed in the stomach, spleen and
574 skin [79]. Certain genotypes of MHC class II were shown to be associated with higher survival of
575 carp to CyHV-3 [80]. Therefore, the up-regulation of *mhc2* gene could be considered important for
576 the response to AngHV-1 as this might enable disease prevention methods for eel aquaculture in the
577 future.
578 Taken together, our *in vitro* viral model infection induced pro-inflammatory responses
579 marked with up-regulation of *il1β*. Furthermore, up-regulation of *mhc2* links the responses with the
580 adaptive arm of immunity. Interestingly, the antiviral responses markers (*irf 3* and *7*) were not up-
581 regulated which could be related with anti-IFN activities of alloherpesviruses. Considering the time-
582 point at which the samples were collected the findings fit to the initial phase of mucosa responses to
583 the alloherpesviral pathogen [81].
584
585 As pointed out by [30], the phylogenetic distance to more common fish species and their
586 status as non-model fish hampered the immune-related research of European eel in the past.
587 However, advancement in the understanding of their immune system is vital to improve health and
588 survival under rearing conditions [24]. The present study now provides new molecular tools to
589 characterize the immune system of European eel in more depth and, as described below, to
590 understand the development of the immune system and its response to environmental factors. We
591 therefore applied these tools to elucidate expression of key immune genes during development of
592 the immune system from hatch to the first-feeding stage.
593
594 **4.2 Ontogeny at 18°C**

Our findings showed that the studied immune genes could be clustered into three groups, which were differently affected by temperature: low-level age independent expression and expression positively or negatively correlated with age.

In a further analysis of the same larvae [34], 18 - 20°C was found to be the optimal temperature in regard to survival, growth, and development. In order to elucidate regular ontogeny of the immune system of European eel larvae, we therefore focused on the 18°C treatment to discuss temperature influences. Both complement component *c3* and immunoglobulin M (*igm*) seem to have been already expressed during the embryonic stage and might have been transferred maternally to the larvae as seen in various other fish species [45,82–85]. However, to confirm this, further studies are needed. It has previously been suggested that at this developmental stage, *c3* and *igm* work together to facilitate binding of opsonized bacteria to complement receptors on phagocytes [26,85]. In carp, it was demonstrated that *c3* gene expression peaked around the time of hatching and that it is produced in the yolk syncytial layer [45]. During eel ontogeny, *c3* gene expression was also linked to a shrinking yolk sac area [34], probably indicating that C3 is important for innate immune function shortly after hatch. Similar to *c3* and in line with the present study, it was shown in sea bass that IgM is transferred maternally through the yolk. Its persistence only lasted a few days and got exhausted with yolk absorption [86] to then completely disappear during later larval stages [87]. In the European eel larvae studied here, *igm* levels peaked at hatch and decreased rapidly to basal levels afterwards. Interestingly, studies on Japanese eel demonstrated that whilst *igm* and T-cell-related *lck* genes were expressed already 3 days post fertilisation, the development of lymphoid tissues were delayed and neither spleen, thymus nor lymphocytes were observed in larvae of similar sizes [25,88]. This is in line with the observed absence of *cd3* expression in the present study and it can therefore be assumed that adaptive immune protection is still underdeveloped in very early larval stages (i.e. pre-leptocephalus stage). Overall, this would have implications for the use of vaccines during early life stages, thus further studies are needed to link the present findings to the functionality of the adaptive immune system.

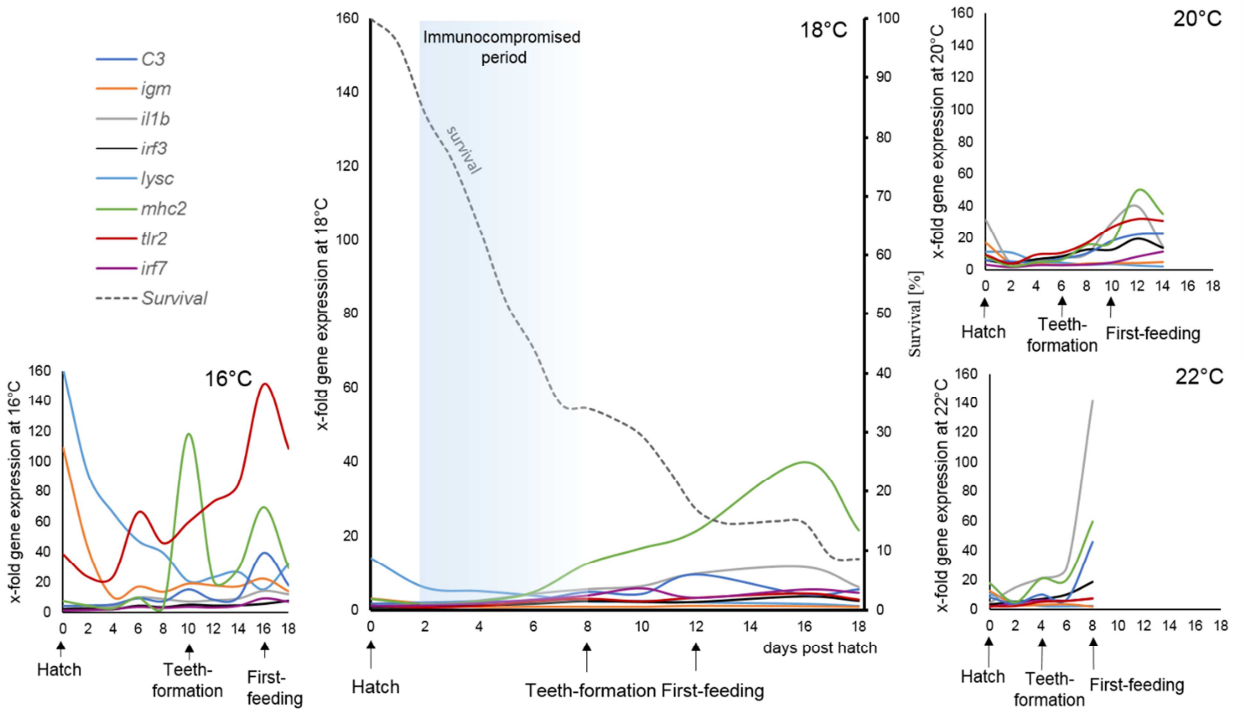
Toll like receptor 2 (TLR2) is involved in the recognition of bacterial and parasitic ligands. In this study, *tlr2* expression increased throughout larval development in a temperature independent manner. To our knowledge, this is the first study investigating *tlr2* expression during larval development of fish. However, it was previously shown that toll like receptors are already expressed before hatch and that their expression is temperature independent in zebrafish [89]. Our results therefore indicate that eel larvae were probably already able to detect pathogens and that this

ability increased with age, which confirms that these receptors play an important part in immune surveillance throughout the different life stages [6]. Additionally, anti-bacterial protection is provided through the expression of lysozyme. This protein is one of the maternal immune factors that are transferred to the egg and it has been detected in the early life stages (oocytes to larvae) of various fish species [90,91]. For example, in brown trout *lysc* was constitutively expressed from the unfertilized egg to three weeks post hatch [60]. In the present study, mRNA of c-type lysozyme (*lysc*) were detected from day 0 onwards, and expression increased constantly during development resulting in the highest expression at the stage of first-feeding. This, together with the above described results on *tlr2* expression and the presence of skin lectin at 8 dph in Japanese eel [25], indicates that innate antibacterial protection is an important factor in the immunological protection during early larval development in eel.

The cytokines TNF-α and IL1-β induce a cell signalling cascade leading to an inflammatory immune response after detection of pathogen and danger associated molecular patterns (PAMPs, DAMPs) by respective receptors [28]. During eel ontogeny, expression of *il1β* increased steadily indicating an increase in the potential to mount a pro-inflammatory immune response and also the presence of cytokine producing immune cells, such as macrophages. Another indicator for the presence of these cells is the increasing expression of *mhc2* throughout eel larval development. On the contrary, *tnfa* was not expressed during the first 18 dph. This is very dissimilar to patterns observed in brown trout and rohu [60,61]. The effect an absence of *tnfa* would have on the innate immune response however needs to be established in further infection studies. Both interferon regulating receptors studied, exhibited a steady increase throughout larval development. This is similar to a study on zebrafish where *irf3* also increased during the course of larval development [20]. As IRFs regulate the transcription of interferons (IFNs), they are involved in the innate immune response to viruses. It can therefore be assumed that the potential of the eel larvae to mount an anti-viral immune response increases throughout development and is already present during the very early life stages.

A previous study [35] indicated a stratified survival curve in European eel larvae, i.e. low initial mortality, followed by a steep decline in survival until mortality subsides. Similar patterns have also been observed in various fish species such as turbot [39], winter flounder (*Pseudopleuronectes americanus*) [92], Pacific herring (*Clupea pallasii*) [93], and cod (*Gadus morhua*) [94] larvae. A period of unusually high mortality during fish larval development has

659 previously been proposed under Hjort's critical period hypothesis and this critical period directly
660 influences recruitment [95]. Previous studies link this period of high mortality to feeding success,
661 size, predation, and life history strategy [95–97]. However, since such critical periods can also be
662 observed in culture, it could, in addition, be linked to pathogen sensitivity, meaning that eel larvae
663 are immunocompromised during early development. Our results indicate a sensitive phase (from ~1
664 dph to approximately the stage of teeth-formation) during which larvae most probably are
665 immunocompromised and hence highly susceptible to pathogens (conceptualised in Fig. 7).
666



667

668 **Fig. 7: Conceptual overview - Immunocompromised window during development of**
669 **European eel linked to survival.** The left axis displays gene expression calculated for the
670 respective rearing temperature only. Expression ($2^{-\Delta\Delta ct}$) was calculated in relation to the lowest
671 mRNA level for each temperature. For parameter variation see Fig. 1-6. Survival (grey dashed line)
672 is displayed on the right axis. For survival: Larvae from 12 parental combinations were stocked at
673 ~150 larvae/L in a recirculation aquaculture system equipped with ~45 L aquaria (50 x 30 x 35 cm),
674 UV sterilizer, protein skimmer, and biofilter. Larvae were reared at 18°C in 36 ppt filtered (1-10
675 µm) North Sea seawater supplemented with artificial salt. The number of surviving larvae was
676 estimated per aquarium by subjective estimate and recorded.
677

Thus, survival seems to be closely linked to immune ontogeny, which is not surprising as it is well established that during development, innate immunity provides quick protection against the hostile environment [6]. Obviously, the study of gene expression can only give an indication of functionality and hence these findings should be confirmed in the future through studies of immune organ development and pathogen challenge experiments. Additionally, the present study only provides a window into the ontogeny of key genes involved in the immune system and future extensive transcriptomic studies will have to be conducted to provide the complete picture. In order to increase survival rates and prevent high and unpredictable mortalities in aquaculture settings, it should thus be investigated if and how the onset of the innate immune system can be shifted earlier towards the time of hatch. Possible mechanisms would include maternal and larval immunostimulation as well as steering larval microbiota [39,98,99].

4.3 Temperature effects on ontogeny of the immune system

Temperature is an important factor influencing aquatic life and oviparous fish are directly exposed to it since early ontogeny. This study therefore did not only aim to elucidate some aspects of the European eel's development of the immune system but also how this is influenced by the ambient temperature. Together, this can provide insights into larval physiology, compulsory to defining optimal rearing conditions of European eel in aquaculture as well as identifying potential effects of global warming.

In the present study, immune gene expression was studied until larvae reached the first-feeding stage, where temperature influenced the age at which the larvae reach that developmental stage, i.e. 16 dph at 16°C, 12 dph at 18°C, 10 dph at 20°C and 8 dph at 22°C [34]. At the lower end of the thermal spectrum (16°C) immune defences appeared to be impaired as development is delayed and immune protein activity might be reduced [100].

To compensate for the thermal effect on development, we analysed the influence of temperature at specific larval stages as well as on real-time age (at specific dph). Expression of *igm* as well as of *tlr2* was temperature independent. This indicates that immunoglobulin protection against pathogens at hatch and pathogen detection via TLR2 will not be impaired within the thermal window for larvae. Similarly, there was no effect of rearing temperature on interferon regulating factors and interleukin β. Also, no statistically significant effect of rearing temperature was detected on *c3* expression. However, *c3* mRNA levels reflected a temperature dependent decrease of yolk-sac area (described in [34]). A temperature effect on initial protection especially during

temperatures close to the critical thermal maximum (i.e. $CT_{max} \geq 22°$) can therefore not be excluded. Furthermore, the expression of two studied genes (i.e. *mhc2*, *lysc*) was temperature dependent. This thermal influence on c-type lysozyme was surprising as especially innate immune parameters are seen as relatively temperature independent [101]. Immune-related gene expression was particularly affected at 22°C, which is close to the upper thermal limit of these larvae [34]. The observed overshoot of immune-related gene expression at 22°C at the first-feeding stage (8 dph) correlates with an increased expression of heat shock proteins 70 and 90 in the same larvae [34]. This is an indication of temperature induced cellular stress and an immune response towards damaged cells [102]. Protection against pathogens is thus probably impaired at temperatures close to the critical thermal maximum (CT_{max}), which in culture under sub-optimal rearing schemes can be crucial to survival, while in nature heat waves and rising sea surface temperatures could increase larval mortality and hence negatively impact glass eel recruitment.

To summarize, this study provides new molecular tools to study the immune system of eels in response to internal and external factors. Immune-related genes were shown to be influenced by developmental age, the environmental temperature, as well as the associated interactions. In conclusion, the observed ontogeny of the immune response has to be taken into account to optimise rearing conditions and disease prevention protocols (e.g. timing of vaccination, immunostimulation treatments) of *A. anguilla* in culture as it is linked to protection against pathogens and larval survival.

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Footnotes

Data availability

The data for this study is archived in the repository Mendeley Data following best practices [103] and is available under doi:10.17632/zdpj5vg3xv.1.

Competing interests

The authors declare that they have no competing or financial interests.

Author's contribution

Conceived, designed and carried out the tissue expression study: JJM. Conceived, designed and carried out the AngHV-1 infection study: MA. Conceived and designed the larval experiments: SNP, IAEB, JT. Primary funding acquisition: JT, IAEB. Provided eggs for the experiment, JT, IAEB. Performed the larval experiments: SNP, IAEB. Resources for the experimental work: JT, IAEB. Contributed reagents/materials/analysis tools: JJM, IAEB, JT. Performed molecular analysis: JJM, SNP. Analysed the data: JJM, SNP, IAEB. Writing - original draft preparation: JJM and SNP. Writing - Review and editing: MA, IAEB and JT. All authors gave final approval for publication.

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Highlights

Our main findings include:

- A molecular tool-box was developed to study immunity in European eel
- *Il1β* and *mhc2* were up-regulated in response to AngHV-1 infection.
- Elucidation of the molecular ontogeny of the immune system in European eels
- Temperature influenced expression of some immune genes during larval development.
- A potential immunocompromised period during larval development is discussed.