Marine larval hatchery technology

Microbial management and immune system ontogeny in European eel

Bandara, Kasun Anuruddha

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PhD Thesis

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By
A M Kasun Anuruddha Bandara

Cover photo: A European eel larva swimming in a Kreisel tank.
Credit: Sune Riis Sørensen, DTU Aqua

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Preface

The present thesis was submitted as a partial fulfilment of the requirements for obtaining the Doctor of Philosophy (PhD) degree to the National Institute of Aquatic Research (DTU Aqua), Technical University of Denmark (DTU) and Department of Biotechnology and Food Science, Norwegian University of Science and Technology (NTNU).

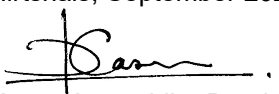
This PhD was funded by the Nordic Five Tech Alliance and funds offered by the DTU Aqua and the Department of Biotechnology and Food Science at NTNU. All the experiments were conducted as a part of the project, “Improve Technology and Scale-up Production of Offspring for European Eel Aquaculture” (ITS-EEL) funded by Innovation Fund Denmark and ENV - “fonden”. The PhD study, running from March 2020 to September 2023, pursued a double degree granted by both DTU and NTNU and was supervised by Dr. Jonna Tomkiewicz (DTU Aqua), serving as the main supervisor, along with two co-supervisors: Prof. Olav Vadstein (NTNU) and Dr. Sebastian Nikitas Politis (DTU Aqua). Experiments related to the different studies that will come across in this thesis were run at EEL-HATCH, an experimental facility belonging to DTU Aqua, Hirtshals, Denmark. Laboratory work related to gene expression analysis was carried out at the DTU Aqua, Lyngby. As a part of the external research stay, laboratory work and analysis related to the bacterial community structure was performed during a one-year research stay at the laboratories of the Department of Biotechnology and Food Science, NTNU, Trondheim, Norway.

This PhD thesis delves into the microbial interactions between early life history stages and the ambient environment and molecular ontogeny of the immune system during the captive production and culture of offspring of one of the most enigmatic fish species, the European eel. Accordingly, four studies were carried out to encompass various crucial facets of the hatchery phase, including egg and larval stocking density, first-feeding diets, food quantity, and immunostimulation. The journey undertaken within these pages embarks from the embryonic stage, progressing through yolk-sac larvae to free-feeding larvae, uncovering the intricate microbial associations that influence development, growth, and survival. Furthermore, the thesis delves into the ontogeny of the immune system, shedding light on the mechanisms that shape the immunocompetence of European eel larvae as they progress through their early developmental phases.

In its entirety, this doctoral program granted me an invaluable chance to cultivate expertise in designing and executing experimental research. I gained proficiency in employing molecular techniques to investigate both bacterial community structure and the development of the immune system. Moreover, I enhanced my skills in data analysis, interpretation, and the effective communication of acquired knowledge. Furthermore, being a participant in the global academic communities during my PhD journey at both DTU and NTNU has been an engaging and thrilling encounter.

As we navigate the uncharted waters towards establishing the hatchery technology for the European eel, which is challenging yet interesting, I invite readers to join me on this voyage of discovery and exploration.

Hirtshals, September 2023



Kasun Anuruddha Bandara

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English Summary

The European eel (*Anguilla anguilla*) was once a common fish in streams, lakes and fjords with considerable economic and social importance. It has a high market value and has been a dedicated target for both commercial fisheries and aquaculture in Europe. Since the turn of the century, however, fishing has been in decline, and the same applies to aquaculture, which is based on wild-caught glass eels. The glass eels, which are in a juvenile stage, are caught in targeted fisheries to supply farms with fry and assist in restocking. Today, the European eel population is under pressure due to historically low recruitment levels of glass eels and designated as "Critically Endangered" on the IUCN Red List. In response, ICES recommends a complete cessation of eel fishing at all stages to facilitate stock recovery. If we are to preserve the eel as a food fish, a sustainable solution would therefore be to develop hatchery techniques and close the eel's life cycle in captivity. Hatchery production of glass eels also has the potential to contribute to the management of European eel in the long term. However, establishing hatchery technology for breeding European eel in captivity is challenging due to the eel's complex life cycle and a distinct lack of knowledge about the reproductive stages and early life history in nature. The eel has life stages in the sea as well as in continental waters. The reproduction of European eel involves a migration over a long distance to the spawning area in the Sargasso Sea, where the earliest larval stages occur. From here, their larvae, the so-called leptocephalus larvae, are transported by ocean currents to the coasts of Europe and North Africa, where the leaf-shaped larvae transform into a juvenile stage, the glass eel. Glass eels, which are small and transparent, find their way into freshwater systems such as lakes, marshes and rivers or remain in coastal waters. Further on, they become yellow eels and later silver eels, which migrate to the Sargasso Sea to spawn. By the time they start the migration, eels are not sexually mature but held in a pre-pubescent stage through hormonal inhibition.

Due to the lack of insight regarding the eel's reproductive biology in nature and the needs of the early life stages, experimental research is the driving force in the effort to close the life cycle in captivity. Research over several decades for both the European and Japanese eel has created knowledge and progress in broodstock rearing, assisted reproduction, artificial fertilisation and egg quality, as well as the culture of the early life stages, which includes optimisation of culture systems, biophysical conditions and larval nutrition. For the European eel, this in the past decade led to a stable production of offspring that reach the feeding stage. Now, work on the establishment of larval culture, including the development of suitable larval feeds, is in focus but challenged by high mortality during early life stages. It is essential to solve this problem to close the life cycle and scale up production, but it remains unclear which factors or which combination of factors are responsible for the high mortality. In this context, the current PhD project focuses on the interaction between the bacteria in culture systems and offspring, concurrently investigating how the immune system of the offspring develops and functions under culture conditions. The project includes four separate studies based on controlled experiments, which together investigated how different treatments and culture conditions in captivity affected the composition of bacterial communities and the performance of offspring (e.g., survival, growth and development), while molecular studies of gene expression have provided new knowledge about the immune system development in the early stages of European eels.

In the first study, we investigated the importance of egg stocking density during incubation (varying from 500 eggs/L to 4000 eggs/L) for the survival of embryos and newly hatched larvae, and how it affected the bacteriome in the rearing water but also in the embryos and larvae, as

well as their immune system responses. The study covered embryonic development from 0 hours post-fertilisation (hpf) until hatching (approx. 55 hpf), as well as the subsequent larval stages from hatching to 3 days post-hatch (dph). Hatching success was also recorded for the different incubation densities. The results showed higher survival at the lowest incubation density (500 eggs/L), but no effect on either hatching success or expression of genes related to immune or stress/repair responses. Mortality was highest during the embryonic incubation phase, while mortality decreased in newly hatched larvae. The high mortality identified at the highest incubation densities during the embryonic phase was associated with the presence of potentially detrimental bacterial taxa. Molecular studies of the expression of immune related genes indicated that the relatively undeveloped immune system in the eel's early life stages is probably unable to counteract the unfavourable microbial environment that occurs at high incubation densities. Although the lowest incubation density significantly improved offspring survival, it was a marginal improvement of 5% compared to the highest density. Coupled with the unaffected hatching success and molecular stress response at high incubation densities, it brings up practical considerations such as space requirements and workload in a hatchery. While mortality during the egg stage may be affected by several factors, the results indicate that effective management of bacterial communities during incubation and in the recirculating systems for larval culture is essential to prevent the selection for and proliferation of potentially harmful bacteria, especially in connection with larval feeding, which is initiated at the end of the yolk sac phase (approx. 10 dph).

It is particularly necessary to be able to control the r-selection in bacterial communities during larval cultures when initiating feeding. Research towards identifying the nutritional needs of European eel larvae struggles with a bottleneck in terms of larval survival especially from day 20 to 24, when high mortality indicates that the larvae have not ingested, digested and assimilated food in time. At the same time, the liquid diets used affect the bacterial communities. Therefore, the following study was conducted to investigate changes in water and larval bacteriomes as well as the larval immune response in a feeding experiment. The feeding trial included three different experimental diets (*Diet 1*, *Diet 2* and *Diet 3*) offered to larvae aged 9 to 28 dph. Samples were collected for analysis of the bacteriome structure of water and larvae, as well as for analysis of the larvae's gene expression related to immune and stress response. Regardless of the diet, an increased expression of the gene *hsp90 was found* during the critical period from 20 to 24 dph, which indicates activated stress/repair mechanisms. At the same time, all diet groups showed a change towards a bacterial community in the larvae with reduced *evenness* (homogeneity) and increased occurrence of potentially harmful and opportunistic bacterial groups. This suggests that, in addition to food intake and nutrient quality, deleterious interactions between larvae and bacteria contribute to the marked mortality observed. Beyond the critical period, the highest survival was seen in larvae fed with *Diet 3*. In this group, on 22 dph, an upregulation of the genes *tlr18* and *c1qc*, which code for actors in the recognition of pathogens and complement system, respectively, was registered. This upregulation suggests a molecularly more mature immune system with functional immuno-competence, probably enabling greater resistance to dominant harmful bacteria.

The third study was carried out as part of a subsequent trial, which aimed to improve the protocol for the first feeding of European eel larvae. Here, the focus was on optimising the amount of feed offered during larval culture, using the most promising diet (*Diet 3*), identified in the previous study. While large quantities of feed are assumed to promote food intake and growth in eel larvae, it may simultaneously promote the growth of fast-growing, opportunistic bacteria, thereby causing dysbiosis (imbalance) in the bacterial communities. In the experiment, we offered eel larvae a low (0.5 mL food/L water) or high (1.5 mL food/L water) amount of *Diet 3*. For both experimental

groups, we investigated survival, food intake and growth of the eel larvae, but also the composition of larval and water bacteriomes, as well as the larvae's expression of genes related to food intake and immune and stress response between 9 and 30 dph. Despite a marginally lower survival at the beginning of the first-feeding period in the high-food group, survival was similar between the two groups at the end of the experiment. The group of larvae that received the high amount of food showed a higher degree of gut fullness, faster growth and a healthier bacteriome compared to the group that received the low amount. Analysis of bacterial communities indicated that bacteria associated with the diet affected the larval bacteriomes, especially in the group with high amount of food. Gene expression analysis showed that the gene encoding *ghrelin*, also called the hunger hormone, was upregulated on 30 dph in the low-food group, indicating that these larvae were starving compared to the high-food group. The expression profiles of immune and stress response genes showed no effect of the amount of food, indicating that feeding with high amounts of food should not negatively affect the larvae.

Research has previously pointed to a phase where the European eel larval immune system is molecularly still fairly undeveloped, which includes the period between hatching and ~8 dph when the larvae develop teeth. During this potentially immuno-compromised period, it is conceivable that they are vulnerable to interactions with harmful bacteria. Therefore, in the last study, *β-glucan* (BG), which is a known immunostimulant and modulator of bacterial communities, was tested for its prophylactic potential, especially during the yolk sac phase. Thus, yolk sac larvae were exposed to BG by adding a concentration of 5 mg/L to the water of the rearing tank. This was performed daily from 5 to 9 dph, after which the larvae entered the feeding stage and followed through the feeding period until 20 dph to assess any enduring effects. The larval survival, growth, development, expression of genes related to immunity and stress, and bacterial communities were tracked in comparison to a control group. The result showed no change in larval survival or growth, but for the BG-treated larvae a markedly lower rate of deformities was detected. Molecular analysis showed a downregulation of genes associated with anti-inflammatory response (*il10*) and stress (*hsp90*) in the BG-treated larvae. Furthermore, a positive alteration of the bacterial communities in both rearing water and larvae was seen in the BG-treated group. However, these positive effects were not long-lasting, as the difference evened out in 20-day-old larvae. The results indicate that BG treatment has the potential as an early prophylactic treatment in the culture of European eel larvae to reduce the incidence of deformities, modulate immune and stress response and positively alter the bacterial communities.

Altogether, this PhD project has generated new knowledge about bacterial community succession and changes in response to rearing practices and immune system functions as well as how these factors affect the offspring survival during the early life stages of cultured European eel. The eggs' maternal protective bacteriome appeared to be disrupted by common high-density rearing practices in hatcheries, causing a dysbiosis that in turn affects the survival of offspring. The early life stages of eel do not have fully matured immune-related mechanisms to deal with microbial challenges, but we found that the immune system can be strengthened in later stages. Through this, resistance to harmful bacteria can potentially be improved. Here, for example, the inclusion of whey protein in larval diets and BG treatment can contribute to managing the bacterial communities and promoting the development of the immune system, which is crucial for improving the survival and quality of hatchery-produced eel larvae. The gained insight contributes to the improvement of offspring-rearing protocols but also emphasises the need for further research that can help reduce mortality during the embryonic stage and further elucidate the microbial dynamics in feeding larval culture to close the life cycle of this valuable fish species in captivity.

Dansk Resume

Den europæiske ål (*Anguilla anguilla*) var engang en hyppigt forekommende fisk i vandløb, søer og fjorde med en betydelig økonomisk og social betydning. Den har en høj markedsværdi og har været et dedikeret mål for både kommercielt fiskeri og akvakultur i Europa. Siden århundredeskiftet har fiskeriet imidlertid været nedadgående, og det samme gælder akvakultur, som er baseret på vildtfangne glasål. Glasålene, som er et juvenilt stadium, fanges i målrettede fiskerier, og forsyner akvakulturerhvervet med sættefisk til opdræt og genudsætning. I dag er den europæiske ål som bestand presset med historisk lave rekrutteringsniveauer af glasål, og den betegnes som "Kritisk Truet" på rødlisten, der føres af IUCN (Den Internationale Union for Bevarelse af Naturen). I forlængelse af dette anbefaler ICES (Det Internationale Havundersøgelsesråd) at stoppe af fiskeri på af ål i alle stadier for at skabe de bedste forudsætninger for, at bestanden genoprettes. Skal vi bevare ålen som spisefisk, vil en bæredygtig løsning derfor være at lukke ålens livscyklus i kultur for sikre en produktion af glasål til opdræt i akvakultur. Klækkeriproduktion af glasål vil også på sigt have mulighed for at bidrage til forvaltningen af den europæiske ål.

Det er imidlertid en udfordring at etablere klækkeriteknologi til formering af europæisk ål i fangenskab på grund af ålens komplekse livscyklus og en udpræget mangel på viden om de reproduktive stadier og den tidlige livshistorie i naturen. Ålen har livsstadier i havet såvel som i kontinentale vandområder. Den europæiske ås formering involverer vandring over en lange afstand til gydeområdet i Sargassohavet, hvor de tidligste larvestadier forekommer. Herfra transporteres deres larver, de såkaldte leptocephallarver, med havstrømme til Europas og Nordafrikas kyster. Her forvandler de bladformede larver sig til det juvenile stadium, glasål. Glasålene, som er små og gennemsigtige, søger ind i ferskvandssystemer til søer, moser eller lignende eller bliver i kystnære farvande. Gennem deres vækst bliver de til gulål og senere til blankål. Det er blankålene, der vandrer til Sargassohavet for at gyde, men på det tidspunkt, hvor de starter vandringen, er ålene ikke kønsmodne, men fastholdt i et stadium før puberteten gennem en hormonal hæmning.

På grund af den manglende indsigt i ålens reproduktionsbiologi i naturen og de tidlige livsstadiers behov er eksperimentel forskning drivkraften i arbejdet med at lukke livscyklus i fangenskab. Forskning gennem flere årtier inden for både den europæiske og den japanske ål har skabt viden og fremskridt relateret til hold af stamfisk, assisteret reproduktion, kunstig befrugtning og æg- og larvekvalitet samt kultur af de tidlige livsstadier, hvilket omfatter optimering af kultursystemer, biofysiske forhold og larveernæring. For den europæiske ål har dette i det seneste årti ført til en stabil produktion af afkom, der når det fødesøgende stadie. Arbejdet med etableringen af larvekultur inklusive udviklingen af egnet larvefoder er nu i fokus men udfordret af høj dødelighed i de tidlige livsstadier. Det er essentielt at løse dette problem for at lukke livscyklus og opskalere produktionen, men det er fortsat uklart hvilke faktorer eller hvilken kombination af faktorer, der er årsag til den høje dødelighed. I denne sammenhæng fokuserer nærværende PhD-projekt på samspillet mellem de bakterier, der findes i kultursystemerne, og larverne, samt hvordan dette hænger sammen med udviklingen af deres immunsystem. Projektet omfatter fire separate studier baseret på kontrollerede eksperimenter, som tilsammen har undersøgt, hvordan forskellige behandlinger og kulturforhold i recirkuleringsanlæggene påvirkede bakteriesamfundenes sammensætning og ynglens overlevelse, mens molekylære studier af genekspression har givet ny viden om udviklingen af immunsystemet de tidlige stadier hos europæisk ål.

I det første studie undersøgte vi, hvilken betydning tætheden af æg under inkubationen (varierende fra 500 æg/L til 4000 æg/L) havde for overlevelsen af klækkede ålelarver, og hvordan det påvirkede bakteriomet i vandet og hos embryoer og larver samt udviklingen af deres immunsystem. Studiet dækkede embryonaludviklingen fra 0 timer efter befrugtning indtil klækning ca. 55 timer efter befrugtning, samt de efterfølgende larvestadier fra klækning til 3 dage efter klækning. Klækningssucces blev ligeledes registreret i forhold til de forskellige inkubationstætheder. Resultaterne viste en højere overlevelse ved laveste inkubationstæthed (500 æg/L), men ingen påvirkning af hverken klækningssucces eller ekspression af gener relateret til immunitet eller fysiologisk stress. Dødeligheden var højst blandt embryoer i inkubationsfasen, mens dødeligheden faldt hos de nyklækkede larver. Den høje dødelighed identificeret ved de højeste inkubationstætheder i embryonalfasen var associeret til forekomsten af potentielt patogene bakterier arter. Molekylære studier af ekspressionen af immunrelaterede gener indikerede, at det relativt uudviklede immunsystem i ålens tidlige livsstadier ikke formår at håndtere det ugunstige mikrobielle miljø, som opstår ved høje inkubationstætheder. Selv om den laveste inkubationstæthed signifikant forbedrede ynglens overlevelse, var tale om en marginal forbedring på 5% øget overlevelse i forhold til den højeste tæthed. Sammenholdt med den upåvirkede klækningssucces og molekylære stressrespons ved høje inkubationstætheder åbner det for praktiske overvejelser såsom pladsbehov og arbejdsmængden i et klækkeri. Mens dødeligheden i ægstadiet kan skyldes flere faktorer, peger resultaterne på, at en effektiv håndtering af bakteriesamfundene under inkubering og i recirkuleringssystemerne i larvekulturen er væsentlig for at forhindre efterfølgende vækst af potentielt patogene bakterier særligt i forbindelse med fodring af larverne, som initieres i slutningen af blommesækfasen ca. 10 dage efter klækning.

Det er især væsentligt at kunne kontrollere r-selektionen af bakterier i larvekulturer i forbindelse med fodringen. Arbejdet med at identificere ålelarvers ernæringsbehov kæmper med en flaskehals relateret til larvernes overlevelse fra dag 20 til 24, hvor høj dødelighed indikerer, at larverne ikke rettidigt har taget tilstrækkelig næring til sig. Samtidig påvirker den halvflydende næring, der anvendes, bakteriesamfundene. I følgende studie undersøgte vi derfor interaktioner mellem bakteriomet i vandet og hos ålelarverne samt deres immunrespons i et fodringsforsøg. Fodringsforsøget omfattede tre forskellige eksperimentelle diæter (*Diet 1*, *Diet 2* og *Diet 3*) og larver i alderen 9 til 28 dage efter klækning. Vi indsamlede prøver til analyse af vandets og larvernes bakteriesamfund samt til analyse af larvernes ekspression af gener relateret til immunitet og stress. Der blev uanset diæten fundet en øget ekspression af genet, *heat shock protein 90* i den kritiske periode fra dag 20 til 24, hvilket indikerer stress herunder igangsætning af reparationsmekanismer. Samtidig viste alle diætgrupper en ændring i retning af et potentielt patogent bakteriesamfund hos larverne med reduceret *Evenness* (homogenitet) og øget forekomst af potentielt patogene bakteriegrupper. Dette tyder på, at skadelige interaktioner mellem larver og bakterier foruden fødeindtag og næringskvalitet bidrager til den observerede markante dødelighed. Ved en sammenligning af forskelle mellem de tre diæter sås den højeste overlevelse hos larver fodret med *Diet 3*. I denne gruppe sås på dag 22 efter klækning en opregulering af generne receptor *TLR18* og komponent *C1QC*, der koder for genkendelse af patogener. Denne opregulering peger på en øget immunkompetence og sandsynligvis større modstandsdygtighed mod dominerende skadelige bakterier.

Det tredje studie blev udført som en del af et efterfølgende forsøg, som sigtede mod at forbedre protokollen for startfodring af europæiske ålelarver. Her var fokus på at optimere mængden af foder som tilsættes i larvekultturen ved anvendelse af det mest lovende foder *Diet 3*. Mens store fodermængder antages at fremme fødeindtaget og væksten hos ålelarverne, kan det have samtidig fremme væksten af hurtigvoksende, opportunistiske bakterier, og herigennem forårsager

dysbiose (ubalance) i bakteriesamfundene. I forsøget gav vi ålelarver henholdsvis lav (0,5 mL føde/L vand) eller høj (1,5 mL føde/L vand) mængde af *Diet 3*. For de to forsøgsgrupper undersøgte vi overlevelse, fødeindtag og vækst hos ålelarverne, bakteriesamfundets sammensætning i larver og vand, samt larvernes ekspression af gener relateret til immunrespons og stress hos larver mellem dag 9 og dag 30 efter klækning. På trods af en marginalt lavere overlevelse i starten af perioden hos gruppen, som modtog høj fodermængde, var overlevelsen ens mellem de to grupper ved forsøgets afslutning. Gruppen af larver, som modtog den høje mængde foder, viste en højere grad af tarmfyldning, bedre vækst og et sundere bakteriom sammenlignet med gruppen, som modtog den lave mængde. Analyse af bakteriesamfundene indikerede, at bakterier associeret med foderet, påvirkede larvebakteriomet, særligt i gruppen med højt foder niveau. Genekspressionsanalyser viste, at genet, der koder for *ghrelin*, også kaldet sult-hormonet, var opreguleret på dag 30 efter klækning, i gruppen med lavt foderniveau, hvilket indikerer at disse larver sultede sammenlignet med gruppen med høj fodermængde. Ekspressionen af immun- og stressrespons gener viste ingen effekt af fodermængden, hvilket indikerer, at fodring med høje fodermængder ikke påvirkede larverne negativt.

Forskningsresultater har tidligere peget på, at larvernes immunsystem er relativt uudviklet i den tidlige fase, som omfatter perioden mellem dag 0 ved klækning og dag 8, hvor larverne har tydeligt udviklede tænder. I denne periode kan det tænkes, at de er sårbare overfor interaktioner med skadelige bakterier. I det sidste studie undersøgte vi derfor brugen af *β-glucan* (BG), som er en kendt immunstimulant og modulator af bakteriesamfund og således potentielt en faktor, som kan beskytte larverne særligt i blommesækfasen. Vi eksponerede således blommesæklarver for BG ved at tilsætte det i en koncentration på 5 mg/L til vandet i kultursystemet. Det blev gjort dagligt fra dag 5 til 9 efter klækning, Efter perioden med BG-tilsætning, blev larverne fodret, og vi fulgte larvernes overlevelse indtil dag 20 for at vurdere effekten af BG sammenlignet med en kontrolgruppe. Vi fokuserede her på at undersøge larvernes overlevelse, vækst, udvikling og genekspression relateret til immun- og stressrespons samt bakteriesamfundets sammensætning. Resultatet viste ingen ændring i larvernes overlevelse eller vækst, men for de BG-behandlede larver blev der påvist en markant lavere rate af deformiteter. Molekylære analyser viste en nedregulering af gener tilknyttet antiinflammatorisk respons (f.eks. *interleukin 10*) og stress (*heat shock protein 90*) hos de BG-behandlede larver. Desuden sås en påvirkning af bakteriesamfundene i både opdrætsvand og hos larver som følge af BG-behandlingen. Disse positive effekter var dog ikke langvarige, idet forskellen var udlignet hos 20 dage gamle larver. Resultaterne indikerer, at BG-behandling har potentiale som tidlig profylaktisk behandling i kultur af larver af europæiske ål i form af reduktion i forekomsten af deformiteter, immun- og stressreaktioner gennem en positiv indflydelse på bakteriesamfundet.

Samlet set har dette ph.d.-projekt genereret ny viden om dynamikken og udviklingen i bakteriesamfund og larvernes mikrobiom, larveoverlevelse og funktioner i immunsystemet hos de tidlige livsstadier af europæiske ål i kultursystemer. Æggenes oprindelige bakteriom synes at blive påvirket af høje tætheder, som er almindelig praksis i klækkerier, gennem en ubalance (dysbiose) i bakteriesamfundene, hvilket igen påvirker afkommets sundhed. De tidlige livsstadier hos ålen har endnu ikke opnået immunrelaterede mekanismer til håndtering af mikrobielle udfordringer, men immunsystemet kan styrkes i senere stadier ved hjælp af tilsætning af præbiotika. Herigennem kan modstandsdygtighed overfor skadelige bakterier forbedres. Vi fandt at BG bidrog til at styre bakteriesamfundet og fremme af udviklingen af immunsystemet, hvilket afgørende for at forbedre ålelarvernes overlevelse i opdræt. Den ny indsigt kan bidrage til forbedring af opdrætsprotokoller, men der er fortsat behov for forskning, der kan bidrage til at nedsætte mortaliteten i ægstadiet samt til yderligere at belyse den mikrobielle dynamik i larvekulturer med fodertilsætning med det formål at lukke livscyklussen for denne værdifulde fiskeart i opdræt.

Synthesis

1 Aquaculture

1.1 Importance of fish aquaculture for global food security and nutrition

Fisheries and aquaculture sectors play an important role in ensuring global food security and nutrition in the twenty-first century, strengthening the targets of the United Nations' Sustainable Development Goal 2; end hunger, achieve food security, improve nutrition, and promote sustainable agriculture. In this context, fish is a rich source of protein, omega-3 fatty acids and minerals (Borgstrom, 2012; Holub and Holub, 2004). However, due to overfishing, pollution, and poor management among other, fisheries production has stagnated for several decades generally fluctuating between 86 million tonnes and 93 million tonnes per year since the late 1980s (Figure 1). In total, fisheries and aquaculture production of aquatic animals (excluding aquatic mammals and reptiles) reached 178 million tonnes in 2020, for which the contribution of aquaculture alone was 87.5 million tonnes (Figure 1) (FAO, 2022). Here, aquaculture is expected to overtake capture fisheries in 2027 and account for 52% of all fish production by 2030. World per capita apparent fish consumption is projected to reach 21.2 kg in 2030, up from an average of 20.5 kg in 2018-2020. Therefore, an increase of 12.8% (+1.2% p.a.) in global fish production (supplied by capture fisheries and aquaculture) is expected, targeting production of 201 million tonnes in 2030 (FAO, 2022). Since capture fisheries production is forecasted to decline further as a result of climate change and increased degradation of wild fisheries habitats, this increase in fish production is expected to be driven, primarily by the continued growth in aquaculture production (OECD, 2022).

1.2 Diversification of species: An essential requirement to ensure the sustainable progress of the aquaculture sector

The rapid expansion of aquaculture, compared to terrestrial livestock farming, is partly attributed to its diverse range of species (Troell et al., 2014). Globally, more than 600 aquatic species have been cultured since 1950, with more than 400 species presently cultivated in the aquaculture sector (Cai et al., 2019). This diversification of species in aquaculture enables increased farming efficiency through, e.g., polyculture of species in the same rearing system, farming the most suitable species according to local conditions, or seasonal variation of species. It also helps the aquaculture sector to overcome market satiation and broaden its market base. Last, but not least, species diversification strengthens the resilience against the challenges to the long-term sustainability of the sector (e.g., climate change, disease outbreaks, market fluctuations) as reviewed in Cai et al. (2023). Even though the global picture of aquaculture seems diverse, the diversity of the species cultured nationally, generally remains low (Cai et al., 2022; Metian et al., 2020). While diversification of species is a prerequisite to ensure the resilience and sustainability of the aquaculture industry, both, at the national and global scales (Le François et al., 2010), the establishment of commercial aquaculture for new species requires overcoming different challenges.

According to Olsen et al. (2008), the challenges faced when establishing the aquaculture of a species can be grouped mainly into three clusters:

Resource cluster - availability of resources (e.g., juveniles, space, energy),
Attitude cluster - public view on ethics, environment and health, and
Innovation cluster - knowledge generation and innovative technical development.

Among these challenges, the availability of juvenile stages that are suitable for stocking in grow-out systems is a prerequisite for commercial farming but is often a bottleneck. Here, closing the life cycle in captivity (or domestication) plays a vital role in meeting this challenge and improving aquaculture production, while ensuring sustainability. Such domestication, achieving a full life cycle in captivity over several generations of a new species, requires specific skills, knowledge and technology related to broodstock management, rearing of early developmental stages (e.g., incubation of eggs and rearing of larvae) and juveniles (Teletchea, 2015).

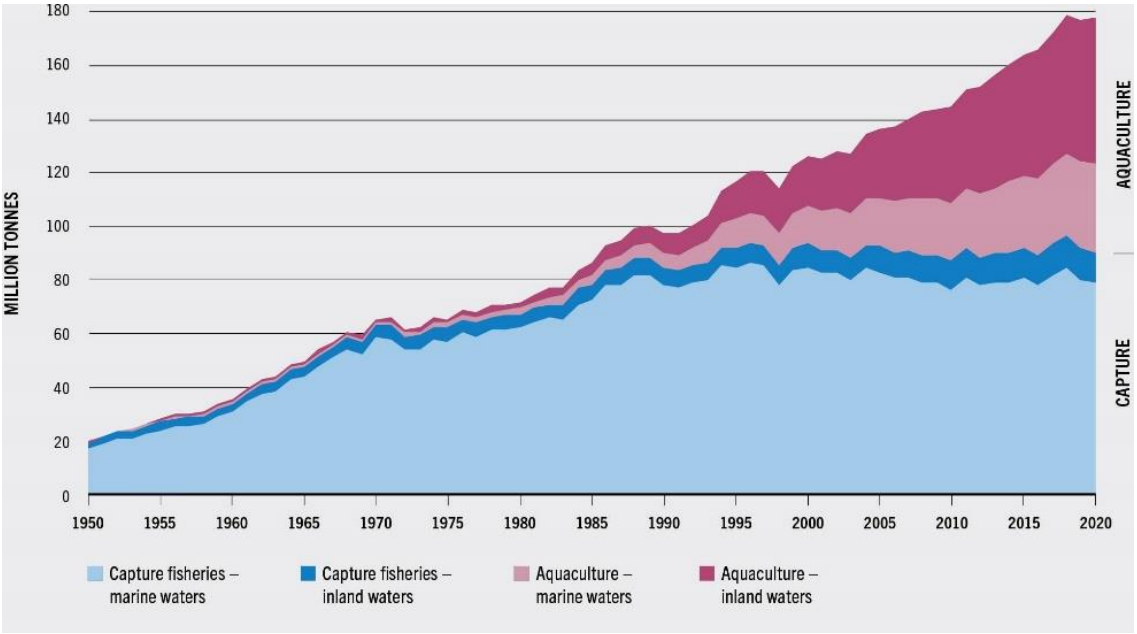

Figure 1: World capture fisheries and aquaculture production (Notes: Excluding aquatic mammals, crocodiles, alligators, caimans, and algae. Data expressed in live weight equivalent) (Source: Food and Agriculture Organization of the United Nations (2022))

In this context, the establishment of hatcheries for juvenile production for many species has promoted and ensured high productivity. In Europe, this is for example the case for species such as salmon (*Salmo salar*), trout (*Oncorhynchus mykiss*), and seabass (*Dicentrarchus labrax*) while for other, researchers and producers strive to close the cycle as for example for the European eel (*Anguilla anguilla*). On that account, it is important to mention that the present aquaculture practice of this species still entirely depends on wild glass eels that are caught in targeted fisheries (Aarestrup et al., 2010). As such, the establishment of hatchery techniques and technology, enabling captive production of glass eels, would directly resolve the aquaculture sustainability issues and alleviate the fishing pressure on the natural population.

2 European eel: A high-value species for commercial aquaculture in Europe

2.1 Aquaculture of European eel

The history of intensive aquaculture of European eel (*Anguilla anguilla*) goes back some 40 years (Nielsen and Prouzet, 2008). Today, cultured eels are destined either for human consumption or restocking purposes. Until recent years, the main producers engaging in intensive farming using recirculation systems include the Netherlands, Germany, and Denmark. Their production still entirely relies on wild-caught juvenile eels (so-called glass eels) imported from France, Portugal, Spain, and the UK (FAO, 2023). Eel aquaculture experienced growth until the late 1990s, after which it entered a downward trajectory starting in the mid-2000s and continuing to the present day. This decline amounted to an approximate 50% reduction, resulting in a 2020 production of 4628 tonnes (Figure 2) (ICES, 2021a) and associated with low glass eel recruitment rendering the species critically endangered (Pike et al., 2020).

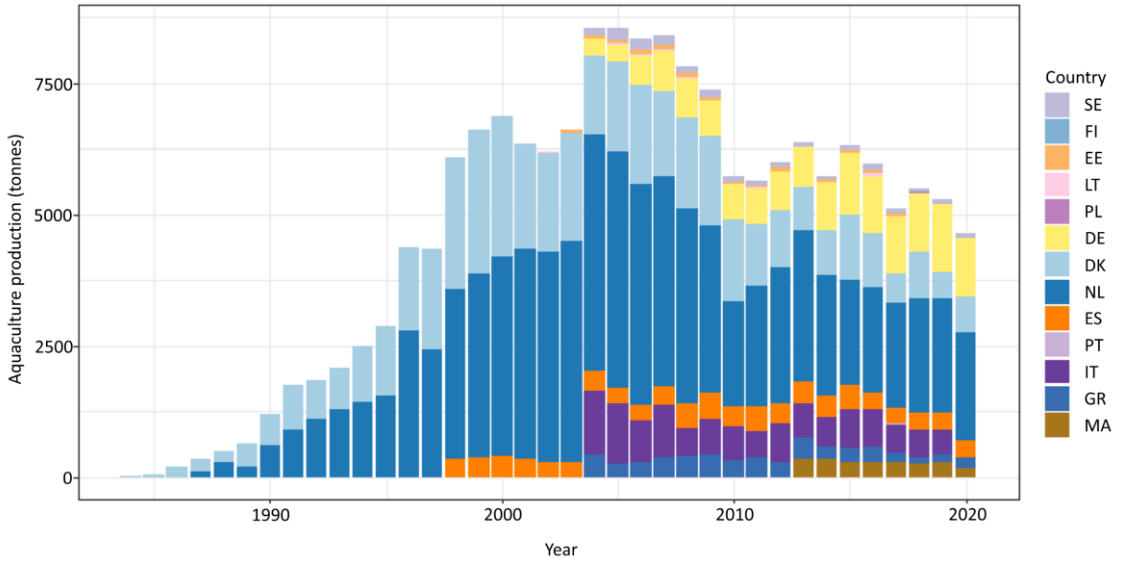

Figure 2: Reported aquaculture production of European eel in Europe from 1984 onwards, in tonnes, in Sweden (SE), Finland (FI), Estonia (EE), Lithuania (LT), Poland (PL), Germany (DE), Denmark (DK), Netherlands (NL), Spain (ES), Portugal (PT), Italy (IT), Greece (GR) and Morocco (MA). (Source: ICES (2021a))

2.2 Recruitment dynamics and threats

Even though the declining trend in landings, which has received much less attention than that of recruitment, was reported as early as 1975, while the drop in glass eel recruitment was first remarked in 1985 (OSPAR, 2010). Glass eel recruitment has remained low for the last two decades and the stock status of the European eel continues to be concorded as critical, due to the very low level of glass eel recruitment and no clear sign of an upturn (Figure 3) (ICES, 2021b). Besides the reproductive potential, the success and extent of the glass eel recruitment are determined by global (e.g., oceanic and climate changes) and local (e.g., local hydrological and/or climatic factors such as moonlight, river flow, tidal cycle) factors (reviewed in Arribas et al. (2012)). An analysis of the stock dynamics predicted that the recovery of the European eel stock can be expected within 80 years with zero fishing pressure or extended more than 200 years at a fishing pressure of just 10% of the current rate of fishing mortality (Åström and Dekker, 2007).

Due to the continuously low stock replenishment, the European eel is still listed as “Critically Endangered” in the IUCN Red List assessment (Pike et al., 2020). The survivorship of the European eel is threatened generally at all its habitat ranges including freshwater, marine coastal and oceanic habitats due to a combination of natural and anthropogenic causes. Natural causes affect mainly the oceanic phase of the life cycle, whereas the continental phase appears to be affected mainly by anthropogenic causes. Natural causes include mainly the consequences of oceanic and climate change such as changes in North Atlantic Oscillation, river flow changes, warming of sea surface temperature, and predation. Threats that are directly related to human activities include overfishing, migration obstacles (e.g., dams, water abstraction structures or other barriers to river flow), habitat loss, water pollution and the spread of the invasive parasitic nematode (*Anguillicola crassus*). Among the anthropogenic causes, overfishing represents one of the major threats because all the continental life stages (from glass eels to silver eels) are subjected to fishing activities. Currently, all commercial production of European eel, both from fisheries and aquaculture, depends solely on the exploitation of the wild stock (reviewed in Podda et al. (2021)). On the other hand, the recommendation of the International Council for the Exploration of the Sea (ICES) to achieve a fast recovery of European eel stock is to ensure zero anthropogenic mortality, including fishing for aquaculture purposes (ICES, 2021b), leading to unpredictable markets related to NGO campaigns against unsustainable eel fisheries. Thus, the lack of sustainably produced glass eels and related market constraints hinder the enhancement of aquaculture of this commercially high-value species. As such, sustainable growth of European eel aquaculture exclusively relies on closing the life cycle in captivity.

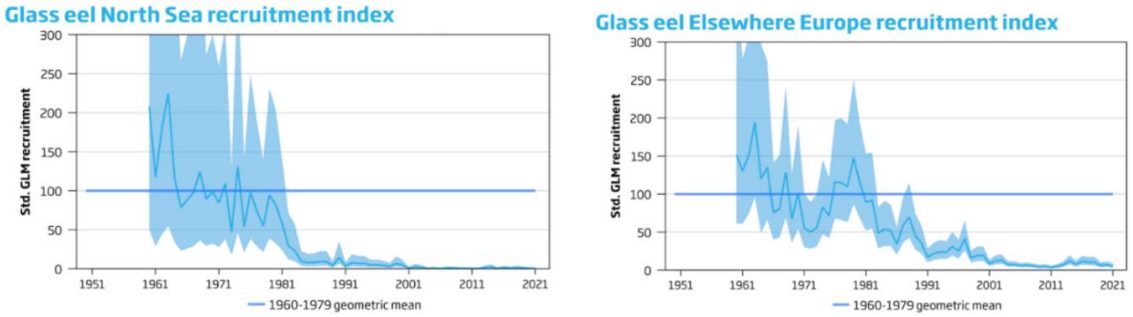

Figure 3: Glass eel recruitment indices for the continental “North Sea” (top left panel) and “Elsewhere Europe” (top right panel) for the European eel. The “North Sea” series is from Denmark, Norway, Sweden, Germany, Netherlands, and Belgium; the “Elsewhere” series is from the UK, Ireland, France, Spain, Portugal, and Italy (Source: ICES (2021b)).

In this context, the urge to develop a hatchery protocol for European eel is indispensable (Violi et al., 2015). Additionally, self-sustaining aquaculture production might support management and conservation measures of this critically endangered species by reducing fishing pressure on continental life history stages and assisting in restocking programs. However, reconstruction of their life cycle in captivity remains challenging due to the complex and enigmatic life cycle of European eel due to their complex life cycle (Tomkiewicz et al., 2019).

2.3 Life cycle: in nature *vs*. captivity

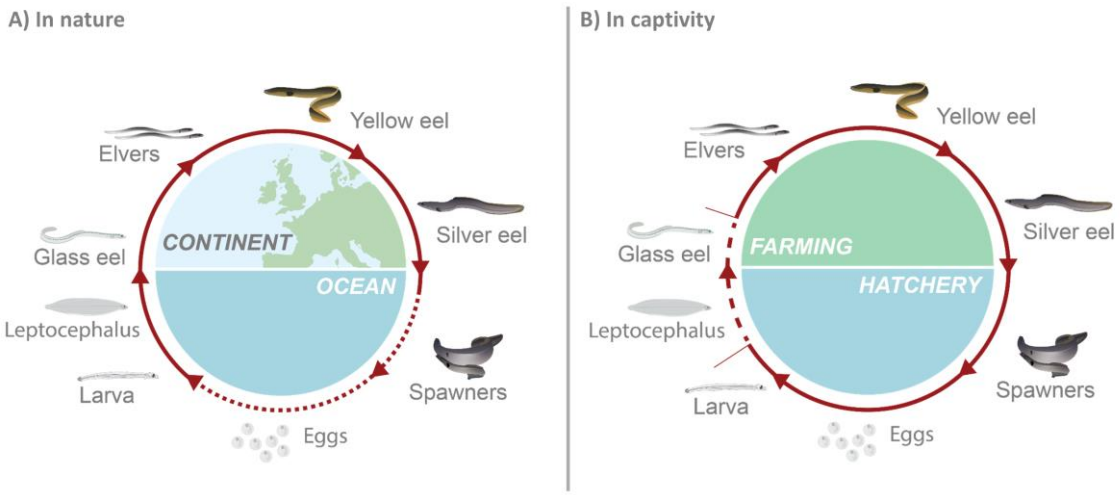

Figure 4: Life cycle stages of European eel in nature (A) and captivity (B). Solid and broken lines indicate the known and unknown phases of the life cycle in nature and in captivity, respectively (Source: Tomkiewicz et al. (2019)).

The European eel is a catadromous fish with a life cycle that includes oceanic reproductive, embryonic and larval phases, and a continental growth phase (McDowall, 1988) (Figure 4A). The continental phase includes four stages: a transparent juvenile stage that enters continental areas (glass eel), pigmented juvenile stages (elver and yellow eel), and an adolescent stage that leaves the continental areas (silver eel) as described in Tesch and Thorpe, (2003) and Van Ginneken and Maes, (2005). These continental stages show a wide geographical distribution in coastal, brackish and freshwater habitats of Europe and the Atlantic coast of North Africa (ICES, 2020). The oceanic stages include spawners (migrating silver eels, adult females and males), eggs and larvae (pre-leptocephalus and leptocephalus stages). Although neither spawning adults nor eggs and earliest larval stages have been found in nature, the feeding larval stage, called leptocephalus was discovered in the Sargasso Sea about a century ago for the first time (Schmidt, 1923) and during recent surveys (e.g., Miller et al., 2019b), identifying the Sargasso Sea as the breeding area for the European eel. Interestingly, Wright et al. (2022) recently tagged migrating adults and provided the first direct evidence of European eel reaching the Sargasso Sea. On the other hand, the leaf-shaped leptocephali drift with the ocean currents towards the continental areas of Europe and North Africa to metamorphose into glass eels before entering the estuaries (Tesch and Thorpe, 2003).

The complexity of the life cycle and lack of knowledge about certain stages in nature make it challenging to reproduce and close the life cycle of European eel in captivity (Figure 4B). Present success in applying assisted reproduction has led to steady production of viable offspring and culture of larvae that enter the feeding stage (Mordenti et al., 2019; Tomkiewicz et al., 2019). The present focus is now on establishing the first feeding and leptocephalus larval culture (Benini et al., 2022; Benini et al., 2023b), thereby closing the life cycle through captive production of glass eels. Promising in this context is that the life cycle has been closed for the closely related Japanese eel, for which upscaling to commercial production is now in focus (Tanaka et al., 2003).

2.4 Utilising Recirculating Aquaculture Systems: An eco-friendly approach for hatchery culture of European eel

Recirculating aquaculture systems (RAS) have gained increasing attention for intensive fish production due to their benefits over the flow-through systems. These benefits include reduced water consumption (Verdegem et al., 2006), low ecological impact due to enhanced waste management and nutrient recycling (Piedrahita, 2003), better control over the physiochemical parameters of the rearing environment, improved hygiene and biosecurity (Summerfelt et al., 2009; Tal et al., 2009), improved fish welfare due to the possibility to maintain a constant water quality (d'Orbcastel et al., 2009), and reduced risk of escapees (Zohar et al., 2005). However, RAS technology also possesses drawbacks such as high capital and operational costs or increased technical complexity (Schneider et al., 2006).

Interestingly, most eel farms in northern Europe have been and still are based on intensive recirculation technology (introduced in the late 1980s) (Aarestrup et al., 2010). Currently, the prototype hatchery known as EEL-HATCH, affiliated with DTU Aqua in Hirtshals, is conducting research on the feasibility of utilising RAS for establishing land-based hatchery technology for European eel propagation. Here, saltwater RAS is being utilised for broodstock rearing and larval culture. Ongoing efforts are dedicated to further refining RAS technology for rearing feeding larvae. In this regard, RAS holds significant potential as a sustainable method for the captive production of European eel offspring.

The life cycle of European eel in nature (Figure 4A) is characterised by a long larval phase, which is associated with their long-distance migration (>6000 km) to the continental areas. During this landward migration, larvae were passively transported by ocean currents to coastal waters, where they metamorphose into glass eels. The duration of the larval phase using Lagrangian simulations was estimated as 313 days, when corrected to the temperatures that larvae experience during the migration (from 13.0°C in the spawning areas to 22.5°C in the recruitment areas) (Zenimoto et al., 2011). After effectively closing the life cycle of the European eel in captivity, the culture systems must facilitate this extended larval phase until the larvae undergo metamorphosis into glass eels. These glass eels can then be employed in grow-out farms. Considering the reduced water consumption and improved control over rearing conditions, both crucial aspects during the extended larval rearing phase, the investigation into the viability of RAS technology for effectively closing the life cycle of European eel holds significant potential. Given the significant role that bacterial communities play in the operation of RAS, it is imperative to conduct comprehensive examinations of bacterial interference throughout the rearing process of European eel offspring. Moreover, the immunologic capacity of these offspring (comprising eggs and larvae) is pivotal in shaping their interaction with the bacterial community. As such, this doctoral research project is dedicated to investigating and addressing these dual aspects.

3 Hatchery production and challenges for larval culture

Every developmental stage from gametes to adults is crucial to ensure the success and the sustainability of commercial aquaculture of a fish species, where the failure to establish any of these stages in captivity collapses the whole production cycle. Since the development of all organs and biological systems occurs at the embryonic and larval phases, the performance of fish (e.g., growth and survival) at later stages of life is directly associated with how these systems are established during early life cycle stages. Therefore, the way for both, the production of high-quality and robust juveniles, as well as the enhancement of survival in captivity must be paved during the earlier developmental stages (Helvik, et al., 2009). As such, similar to many other animal breeding programs, the success of aquaculture hatchery production also depends largely on the gamete quality (Migaud et al., 2013). Gamete quality can be defined as the competence of the gametes to ensure fertilisation and subsequent development into a healthy embryo (Bobe and Labbé, 2010). The term emphasises other features related to early developmental success such as embryonic survival or the lack of abnormal development (Brooks et al., 1997; Kjørsvik et al., 1990). Gamete quality can be affected by a variety of intrinsic (e.g., genetics) and extrinsic factors (e.g., broodstock nutrition, environmental factors, husbandry practices) (Bobe and Labbé, 2010) and subsequently determines the viability of embryos and larvae (Valdebenito et al., 2015).

Among the different developmental stages, the early life stages (embryos and larvae) are known to be the most sensitive and vulnerable stages, both, in nature and captivity (Yúfera, 2018). The fish larva is a transitional life form that develops from the embryo through various developmental stages, including pre-feeding (i.e., dependent on the yolk as the sole source of nutrients and energy) and free feeding (i.e., on exogenous food) stages until metamorphosis into juveniles. In contrast to the early life stages (e.g., embryos) of farmed mammalians, which develop inside the mother under stable conditions with a continual supply of nutrients and constant temperature, the development of the early life stages of most fish takes place in a fluctuating and harsh environment (Helvik, et al., 2009). Therefore, in most fish captive breeding programs, early life stage rearing remains the main challenge, hindering commercial production.

The hatchery establishment experiences different challenges that constrain the production of high-quality juveniles, required for stocking in grow-out facilities. These challenges include lack of knowledge regarding optimal rearing (physical, chemical and microbial) conditions and larval feeding behaviour, unknown nutritional requirements and species-specific differences in husbandry and feeding practices (Planas and Cunha, 1999; Yúfera, 2018). In particular, the larval culture of most marine fish species is characterised by poor offspring performances and the lack of reproducibility and predictability in quality (Vadstein et al., 2018). Therefore, larval rearing is considered the major bottleneck that slows down the commercialisation of aquaculture of new marine species. Thus, to overcome this bottleneck, optimisation of zootechnics concerning nutrition, physiochemical and microbial issues is indispensable (Helvik, et al., 2009). While suboptimal nutrition and physiochemical parameters undoubtedly cause poor larval performance, they do not explain the lack of reproducibility in larval performance between full sibling groups grown under similar nutritional and environmental conditions. Thus, the lack of reproducibility during larval rearing trials has been hypothesised to also be driven by microbial interference (Vadstein et al., 1993).

4 Microbial interference during the hatchery phase

According to the “hologenome theory”, all animals perpetuate symbiotic relationships with bacteria. Therefore, host-microbiota interactions in vertebrates, including fish, gained much attention recently. Bacteria contribute to host health and well-being in different ways: assisting in the development and function of the gut system (Cheesman and Guillemin, 2007), stimulating of immune system and functioning as a barrier against potential pathogens (Hiippala et al., 2018; Lazado et al., 2011; Lazado and Caipang, 2014), or supporting development and maturation of epithelial tissues (Kanther and Rawls, 2010; Naito et al., 2017). While most of the bacteria associated with fish larvae are beneficial or neutral towards their host (Ringo, 1999; Vadstein et al., 2013), specific pathogens and opportunistic bacteria also exist (Olafsen, 2001; Vadstein et al., 2004). However, many of the infections that are associated with the mortality of marine fish larvae in captivity are often caused by environmental bacteria, which become infective when host-defence mechanisms are weakened due to different reasons (e.g., nutritional deficiencies, poor water quality, chronic stress) (Toranzo et al., 2005). In the hatchery, bacterial interactions play important roles both at the egg and larval stages of fish (Hansen and Olafsen, 1999).

4.1 Fish egg-microbiota interactions

The eggshell is composed of a thick lamellar inner layer (zona radiata), and a thinner outer layer (chorion or zona pellucida) (Lønning, 1972; Lønning et al., 1988). The chorion and zona radiata are made of a highly hydrophobic protein aggregate and glycoproteins (Oppen-Berntsen et al., 1990). Due to its glycoproteinaceous nature, the eggshell can be easily adhered to and colonised by bacteria (Riis-Vestergaard, 1982). The developing embryo secretes inorganic and low molecular weight organic compounds, which diffuse through the eggshell creating a chemical gradient. This gradient may attract the bacteria that can utilise the secreted compounds leading to primary bacterial establishment on the eggshell (Brooks et al., 1997). The primary bacterial colonisation likely occurs in a selective manner, which is reflected to some extent by the species-specific differences in the primary eggshell bacterial communities and the large variation in egg surface receptors, which provide adhesion sites for a variety of bacteria (Hansen and Olafsen, 1989). It has been suggested that the primary eggshell microbiome acts as part of the first line of defence and intercepts the adhesion of potentially pathogenic bacteria. However, the intensive incubation of eggs during the hatchery phase often results in bacterial over-growth on the eggshell and may interfere with the commensal relationship between indigenous bacteria and opportunists (Hansen and Olafsen, 1999).

Uncontrolled bacterial colonisation and growth cause serious problems in hatcheries such as hampering embryonic development, and hatching, or affecting larval health and development negatively (Hansen and Olafsen, 1999). For instance, colonisation by pathogens or opportunists can cause disease conditions, such as surface ulceration of eggs (Hansen et al., 1992). Additionally, excessive overgrowth of the obligate aerobic bacteria may cause hypoxia in the developing embryo. Even though embryos are unlikely to be affected by the anoxic conditions at the early stages of development, demand for oxygen increases towards hatching, where oxygen deficiencies may lead to lactic acid accumulation, retarded development, and possibly neural injuries. Both, induced and delayed hatching were reported in fish as a result of hypoxic conditions (Hansen and Olafsen, 1999). Moreover, egg surface bacteria release exotoxins and toxic metabolites (e.g., NH_3 and H_2S), which may be deleterious to developing embryos (Barker et al., 1989). Interestingly, vertical transmission and intraovum infection have been demonstrated for fish (Cipriano, 2005; Kohara et al., 2012; Sauter et al., 1987), while members of some genera

(e.g., *Listeria*, *Corynebacterium*, and *Staphylococcus*) has been frequently reported in high-mortality egg groups (Sauter et al., 1987).

4.2 Fish larval-microbiota interactions

Understanding the bacteriome dynamics during the larval stage, which is among the most susceptible stages for microbial infections, is necessary to improve growth and survival during the hatchery phase (Borges et al., 2021). While commensal bacteria benefit the larvae (by e.g., stimulating epithelium differentiation and maturation, promoting feed utilisation efficiency and growth, and promoting host immune response) (Borges et al., 2021), dysbiosis of the host-associated microbiomes is often associated with microbial infections (Vonaesch et al., 2018). The establishment of larval microbiota appears to occur in a non-selective manner, where host preferences and characteristics of microbes (e.g., competitive ability) also seem to play an important role (Makridis et al., 2000; Verschuere et al., 2000). Unlike terrestrial animals, which are exposed to the maternal microbiome during birth (Mändar and Mikelsaar, 1996), the influence of the maternal microbiome on the establishment of fish offspring microbiota might be less significant compared to the influence of environmental microbiota (Hansen & Olafsen, 1999). Important microbial sources that interact with mucosal surfaces of fish larvae (e.g., skin, gastrointestinal tract) include rearing water and feed. The bacterial community of the skin is colonised mainly by the bacteria in the rearing water, while bacteria sourced from both water (i.e., via active uptake) (Reitan et al., 1998) and feed (Skjermo and Vadstein, 1999) are important for colonisation of the gastrointestinal bacterial community. In this regard, since fish larvae are in close contact with their environment (i.e., rearing water), changes in bacterial communities in water might influence the larval bacterial community as well. Therefore, maintaining a healthy bacterial community in the rearing environment is crucial to establishing a healthy larval bacterial community.

Bacteria associated with the host and their environment (e.g., rearing water) can be beneficial, neutral, pathogenic, or opportunistic on their host, fish in the case of aquaculture. Initially, pathogenic bacteria and their role in diseases gained more attention. However, mortalities observed during early larval culture often have not been associated with the obligate pathogenic bacteria, but rather have been attributed to the proliferation of opportunistic bacteria (Skjermo and Vadstein, 1999; Vadstein et al., 1993). In contrast to obligate pathogens, which are specialised organisms that are not capable of surviving by any other means than infecting their host, opportunists do not depend on their host for survival and possess a myriad of other survival strategies. Opportunists are generally a part of the natural microbial environment and as the name implies, capable of being detrimental to their host by taking advantage of weakened host immunity or environmental changes favourable to opportunists over their host. In this context, the majority of pathogens associated with fish are opportunistic (Vadstein et al., 2004). Hence, understanding and potentially steering the factors that favour opportunistic bacteria in the community is essential to establishing a healthy bacterial community.

4.2.1 The hostile first-feeding environment of fish larvae

The typical first-feeding environment of marine fish larvae is a complex ecosystem with interactions among four functional ecological groups (fish larvae, zooplankton as live feed, phytoplankton, and bacteria) (Figure 5). In contrast to the terrestrial environment, aquatic organisms are exposed to higher bacterial densities in their environment (e.g., seawater), which typically contains 10^6 bacteria per mL (Verschuere et al., 2000). In intensive larval rearing tanks, these numbers can be even greater because of the high availability of dissolved organic matter

(DOM), which promotes bacterial proliferation. Specifically, food added to the system, sources DOM directly as uneaten food and indirectly as faeces defaecated by fish and live feed (e.g., in a typical first-feeding tank of marine fish larvae). Defaecation by both, fish and live feed on the other hand, sources bacteria into the rearing water. Additionally, exudation by the phytoplankton also contributes to the DOM content in the water (Vadstein et al., 2013). As such, interactions among different ecological groups in the first-feeding tank together with the operational parameters (e.g., hydraulic retention time) of the system determine the bacterial density and composition of the bacterial communities in the rearing system. Thereafter, supply of bacteria from internal (e.g., rearing water, larvae), and external (e.g., food / live feed) sources, growth of the heterotrophic bacteria that consume DOM within the system and predation (i.e., by larvae, zooplankton), determine the density of heterotrophic bacteria in the system (Figure 5).

Bacterial density is an important factor, though, larval performances seem to be affected more by the composition of the bacterial community, provided that the numbers are not extreme (Attramadal et al., 2012; Munro et al., 1995; Verner-Jeffreys et al., 2004). Here, the identification of the bacteria that are present and dominant in the community is more important than quantitative aspects (Vadstein et al., 2004). Therefore, our understanding of what is a healthy bacterial community and the driving forces that determine the bacterial community composition is essential to orchestrate an optimum microbial environment in the first feeding environment of fish larvae. Here, it is important to consider that the bacterial community composition of the system is determined by the composition of the different sources, differences in the growth potential of bacterial species and selective forces acting on the bacterial communities within the system (Vadstein et al., 2018).

In the first-feeding environment, all organisms (e.g., larvae, live feed, phytoplankton) release DOM into the rearing water (Figure 5). Additionally, faeces and uneaten food can be converted into DOM. This increase in the availability of DOM, which acts as a substrate for heterotrophic bacteria, increases the carrying capacity (cc) for bacteria in the system, allowing the regrowth of bacteria within the system. Depending on the differences in the growth potential of bacteria within the system and the magnitude of change in cc, regrowth within the system can significantly alter the species composition in the rearing water bacterial community, to which larvae are exposed (Vadstein et al., 2018, 2004).

Given that the bacterial community in the larval rearing system is highly diverse and rich in species (Bakke et al., 2015; Giatsis et al., 2015), and considering the inadequacy of our knowledge on the interactions at the species level and the outcome of competition in microbial communities (Larsen et al., 2012), an alternative approach has been suggested by Vadstein et al. (2018) to predict the species selection and succession of bacterial communities in larval rearing tanks. This approach is based on the general ecological theory based on selection (i.e., r/K selection) and succession (i.e., Pioneer-Mature community succession). The r/K selection theory hypothesises that selection pressure (e.g., substrate/ DOM availability) drives selection towards one of two growth strategies: r- or K-selected. The r-selected species grow faster to rapidly colonise unexploited environments when the availability of resources (e.g., DOM) per bacterium is high, but are characterised by lower competitiveness and specialisation abilities compared to the K-selected species. On the other hand, K-selected species show a high affinity to resources and thus, can outcompete the r-selected species at low resource availability conditions. When the bacterial densities are closer to cc, K-strategists dominate the bacterial community because they are competition specialists (Vadstein et al., 2018). As such, conditions in the intensive larval first-feeding tank, where substrate availability per bacterium is high due to enrichment with DOM (i.e., thus, increased cc), favour and select for r-strategists. The r-selected species are known to be

opportunistic and most pathogens show characteristics similar to r-strategists (Andrews and Rouse, 1982).

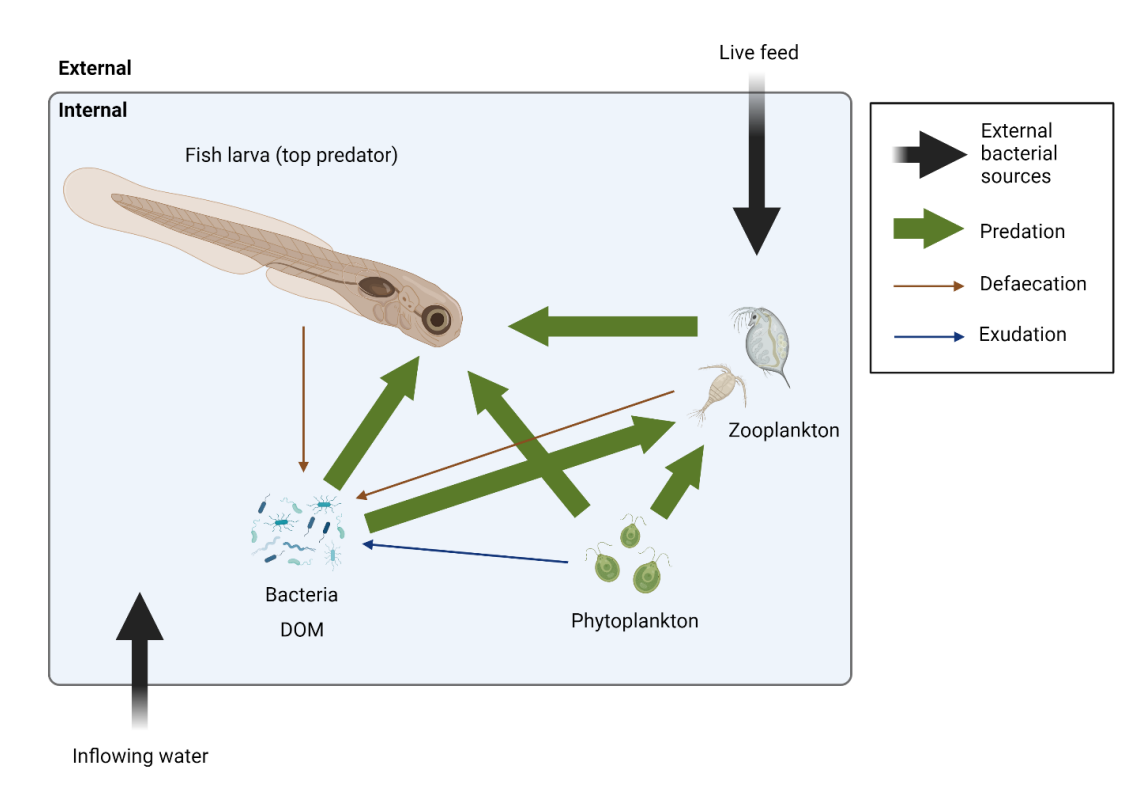

Figure 5: Typical first-feeding environment of fish larvae (modified from Vadstein et al. (2018)). Different external sources of bacteria (black arrows) such as inflowing water, live feed, and microalgae. Fish larva, live feed, microalgae, and bacteria interact through predation (green arrows, with the indication of the direction of energy flow). Fish larvae and zooplankton internally supplied bacteria and DOM through defaecation (brown arrows) and DOM by phytoplankton through exudation (blue arrow) (Created with BioRender.com).

Two characteristics of intensive larval rearing: disinfection and addition of feed, lead to perturbations in the bacterial community and tend to select for r-selected and opportunistic bacteria. While disinfection of water (e.g., UV treatment, ozonation) is a useful tool of preventing the introduction of obligatory pathogenic bacteria into the rearing tank, it also makes DOM more bioavailable by reducing the bacterial numbers in in-flowing water (Hess-Erga et al., 2010). Consequently, competition in the rearing water bacterial community is reduced favouring subsequent proliferation of r-strategist, except if hydraulic retention time (HRT) is very short. Since the cc is not affected by disinfection, bacterial densities remain unchanged. The addition of feed (e.g., live feed or formulated feed) increases the availability of DOM and thus, the cc in the rearing tank, favouring the growth of r-selected opportunists. Here, HRT is an important operational parameter that determines the type of community selected within the rearing tank. A shorter HRT might not retain water inside the tank long enough for bacteria to proliferate. On the other hand, a long HRT allows the establishment of K-selection. However, at HRTs that are commonly used during typical larval rearing, the rearing water bacterial community is at a stage, which is dominated by r-selected species (Vadstein et al., 2018). Thus, this typical practice of larval rearing leads to dysbiosis in the commensal microbiota resulting in a bacterial community dominated by opportunistic bacteria creating a “hostile environment” for fish larvae from a microbial perspective.

In this context, the larval immune system must play an important role in assisting larvae to thrive in this hostile environment.

5 The immune system of fish larvae: structure and development

5.1 Teleostei immune system

The immune system is an assortment of cellular and humoral components that function together to defend the body against foreign substances (e.g., microorganisms, toxins, or malignant cells) by identifying and responding to endogenous and exogenous stimuli (e.g., Pathogen-associated molecular patterns – PAMPs, danger-associated molecular patterns - DAMPs). The vertebrate immune system is composed of two arms: the innate and the adaptive arm (Figure 6). The Teleostei fish are the earliest class of vertebrates possessing the elements of both arms of the immune system (Biller-Takahashi and Urbinati, 2014). The innate arm, which is evolutionarily older than the adaptive arm is the first and fastest to respond to a stimulus and does not retain memory of previous responses. The adaptive arm, if the pathogen persists, assists the defence process with specificity and memory (Smith et al., 2019). Despite the differences, today it is known that the two arms work together to eliminate the invaders or to trigger the defence mechanism (Biller-Takahashi and Urbinati, 2014).

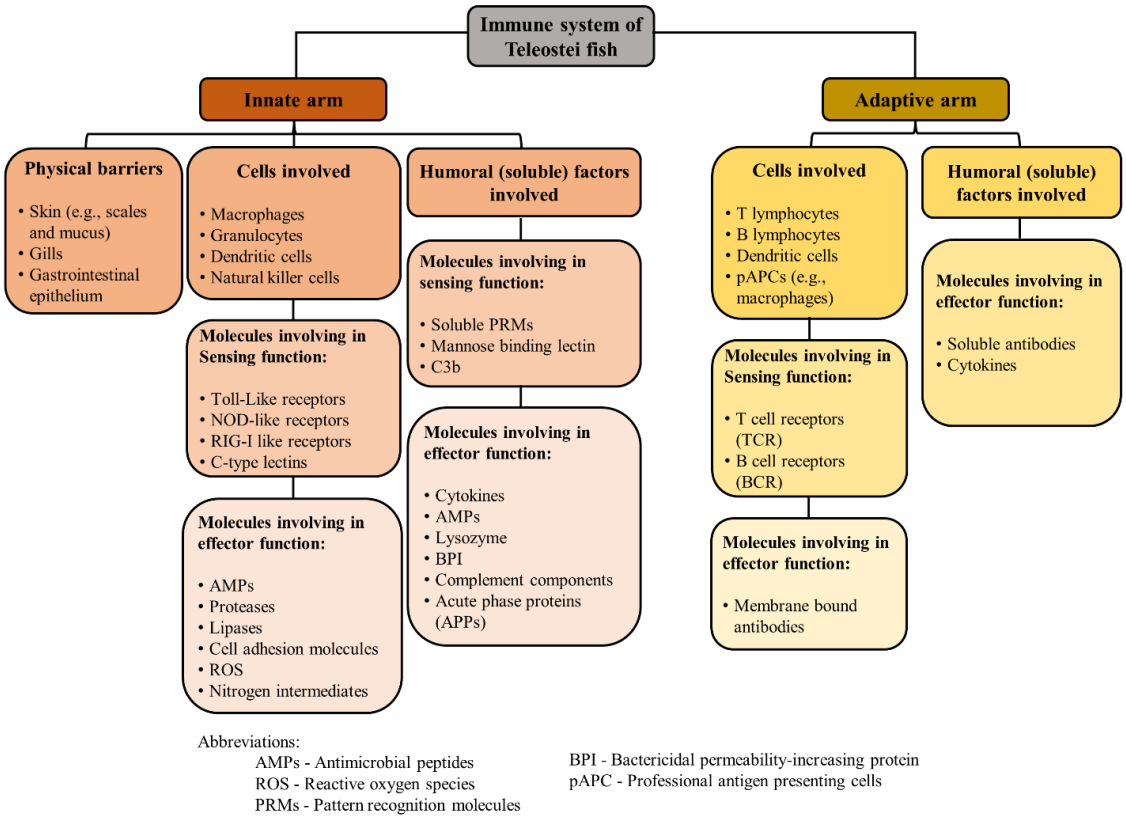

Figure 6. Different components of the innate and adaptive arms of the immune system of Teleostei fish involved in sensing and effector functions during an immune response.

The innate arm of the immune system includes three components: (1) physical barriers, (2) cellular components, and (3) humoral components (Figure 6). Physical barriers, which include skin (e.g., scales and mucus), epithelial layer of gills and the gastrointestinal tract act as the first line of defence in the innate arm (Magnadóttir, 2006). A pathogen that passes through the physical barriers encounters the cellular and humoral components of the innate system. The cells involved in the innate immune response in Teleostei fish include monocytes/macrophages, granulocytes

(i.e., such as mast/eosinophilic granule cells and neutrophils), dendritic cells, and natural killer cells (Smith et al., 2014). The innate immune system is equipped with a range of cell membrane-associated and soluble pattern recognition receptors (PRRs) [e.g., Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors, C-type lectins (CLRs)] that recognise and bind to PAMPs (e.g., bacteria-derived LPS, viral RNA, bacterial DNA) or DAMPs found on proteins or other biomolecules that are released from stressed or injured cells. The domains of all PRRs that recognise the PAMPs/DAMPs are coupled to a domain that transduces a downstream cellular signalling cascade to initiate an immune response (Smith et al., 2019). As a result, activated immune cells will be involved in several responses depending on their cell subtype. For example, neutrophils are involved in various intracellular and extracellular defence mechanisms such as the release of granules containing cytotoxic and antimicrobial enzymes, the release of neutrophil extracellular traps (NETs), phagocytosis and the production of reactive oxygen species (ROS) and nitrogen intermediates (e.g., NO). Macrophages destroy pathogens through phagocytosis, production of ROS and nitrogen intermediates and release various cytokines and chemokines (reviewed in Havixbeck and Barreda (2015); Neumann et al. (2001)). Moreover, macrophages act as a bridge between the two arms (i.e., innate, and adaptive) of the immune system by acting as one type of professional antigen-presenting cell (pAPC). Macrophages present the phagocytosed materials (i.e., antigens) to the T lymphocytes, a component of the adaptive arm through the process called “antigen presentation” (McKinney and Flajnik, 1997).

In addition to the cell-mediated immune response, the innate immune system can also induce an immune response that is mediated by the humoral components, which are macromolecules produced by cells and released into the extracellular fluids following infection by a pathogen. Among the most studied humoral effector molecules in fish, there are complement components, lysozymes, antimicrobial peptides (AMPs), and acute phase proteins (APPs) (Smith et al., 2019). Complement components are serum proteins of a cascade reaction, which leads to the removal of the pathogen or foreign particle through opsonisation and phagocytosis and through the promotion of the inflammatory response (Boshra et al., 2006). Lysozymes are lytic enzymes that are involved in antibacterial defence functions such as lysis of bacterial cell walls, opsonisation and phagocytosis and activation of the complement system (Saurabh and Sahoo, 2008). AMPs (or host defence peptides) are generally positively charged oligopeptides with a varying number of amino acids. They bind to bacterial cell surfaces that normally possess net negative electrostatic charge and disrupt the bacterial cell wall through pore-forming action (Cuesta et al., 2011). APPs are produced and released by the hepatocytes in response to the secretion of various cytokines into the bloodstream by immune cells, such as macrophages (Roy et al., 2017). APPs include coagulation factors (e.g., fibrinogen and prothrombin), transport proteins (e.g., ferritin), complement components, C-reactive protein (CRP) and serum amyloid proteins (SAP) and are involved in a diverse range of defence activities such as inflammatory response, activation of the complement system and clearance of apoptotic cells (reviewed in Bayne and Gerwick (2001)).

Like the innate immune system, the adaptive immune system includes both, humoral and cellular components (Figure 6). B lymphocytes are the key elements responsible for the humoral adaptive immune response, whereas T lymphocytes are involved in the cellular adaptive immune response. One of the key roles of the B lymphocytes is the production of high-affinity antibodies (Abs) against foreign antigens. Abs occur in two forms: a soluble (or free) form that is secreted from B lymphocytes and a membrane-bound form, which binds with the signalling molecules Igα/Igβ to form the B cell receptors (BCR). Abs recognise and bind specific pathogens to activate phagocytosis. Also, Abs bind to extracellular antigens, in addition, to complement components to promote opsonisation (or agent neutralisation). B lymphocytes act also as pAPC to present

processed antigens to activate the T lymphocytes. T cell receptor (TCR), which is always membrane-bound recognises the antigen presented by pAPCs (e.g., B lymphocytes, macrophages, and dendritic cells) only if the antigen is bound to a major histocompatibility complex (MHC I or MHC II), cell surface proteins found on pAPCs. Once stimulated by the processed antigen presented by the pAPCs, T lymphocytes can be activated to function as a helper (CD4+) T cell, a regulatory (CD4+) T cell or a cytotoxic (CD8+) T cell. CD4+ T cells, when activated, release cytokines, which activate and regulate the immune responses to eliminate the invading agent. Cytotoxic (CD8+) T cells induce the apoptosis of the target cells if the antigen is established in the intercellular compartment (reviewed in Biller-Takahashi and Urbinati (2014); Smith et al. (2019)).

5.2 Ontogeny of the immune system

The immunological arrangement and the chronology of the immune system ontogeny depend largely on the evolution and ecology of a certain fish species (Buchmann and Secombes, 2022). The major immune organs of teleosts include the thymus and kidney, while the spleen, which is erythropoietic and lymphopoietic acts as a secondary immune organ. In freshwater teleosts, even though the kidney is the first organ to contain haematopoietic progenitor cells, the thymus is the first to become a lymphoid organ. In marine teleosts, however, the order that the immune organs develop is kidney (e.g., anterior/ head, and middle), spleen, and then thymus. Since fish do not possess bone marrow and lymph nodes, the head kidney plays a major role in haematopoiesis as well as direct antimicrobial activity. The undifferentiated cells are found to be migrating from the kidney to the thymus, where they differentiate into lymphocytes. The spleen plays a major secondary role in the teleost immune system by the clearance of blood-borne antigens and immune complexes and by the antigen presentation to initiate the adaptive immune response (reviewed in Sujata Sahoo et al. (2021)).

In teleosts, three sites of primitive haematopoiesis have been described: 1) exclusively in the yolk sac blood islands (e.g., angelfish), 2) in intra-embryonic intermediate cell mass (ICM) (e.g., zebrafish) and 3) in the yolk sac for a short time and, then in the ICM (e.g., killifish and rainbow trout). Generally, the cellular immune response develops slightly earlier than the humoral response. However, the exact timing of the appearance of the lymphocytes varies among the different fish species. Even though both lymphoid organs and lymphocytes appear early, acquisition of the full immunocompetence in fish is quite late (reviewed in Zapata et al. (2006)).

At the egg stage, effector molecules (e.g., Abs, complement components, AMPs) of maternal origin have been identified. Shortly after fertilisation, the development of the associated organs, tissues, cells, and molecules of the immune system has been recognised. Therefore, the early stages (e.g., developing embryos and newly hatched larvae) achieve immune protection through the innate components and adaptive components of maternal origin (Buchmann and Secombes, 2022). Maternally transferred factors, however, persist only for a short period and usually disappear completely when the adaptive arm has developed enough to function correctly (Hanif et al., 2004). At the same time, a delay in the development of adaptive immune components was observed in many cultured marine fish species. For instance, in Mediterranean gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*), delayed maturation of lymphomyeloid organs is observed (Hanif et al., 2004; Scapigliati et al., 1995). Moreover, production of thymocytes is delayed in fish, indicating that fish are unable to synthesise specific Abs until several weeks after hatching (Swain and Nayak, 2009). Thus, to overcome this immunological limitation, evolutionarily the innate arm of the immune system is equipped with a wide range of capacities and mechanisms to protect the early stages of fish (e.g., developing

embryos and early larval stages) against the hostile environment by mounting a quick immune response.

6 Interaction between microbiota and larval immune system

As fish live in close contact with their environmental microbiome, the health status of the fish is closely associated with their environmental microbiome (Ellis, 2001). The establishment of “normal” or protective microbiota promotes the health status by excluding the invaders (i.e., through competitive exclusion) and facilitating the development and maturation of the immune system (Balcázar et al., 2006). The normal or protective microbiota achieve the competitive exclusion of harmful microorganisms through different mechanisms such as secretion of antimicrobial substances, competition for attachment sites and nutrition or inhibition of virulence gene expression (reviewed in Gómez and Balcázar (2008)).

The ability of microbiota to stimulate the host immune response has been demonstrated in several fish species, specifically in pursuit of probiotic strains for aquaculture. For instance, feeding rainbow trout (*Oncorhynchus mykiss*) with mixed-species probiotics stimulated both humoral and cellular innate immune responses as detected by increased lysozyme activity and an increased number of immune cells (Irianto and Austin, 2002). In this regard, the immuno-modulatory effect of *Lactobacillus* probiotic strains has been shown for rainbow trout (*O. mykiss*) (Panigrahi et al., 2004) and brown trout (Balcázar et al., 2007), demonstrated by increased levels of serum lysozyme and complement activity, as well as for tilapia (*Oreochromis niloticus*), demonstrated by increased phagocytic activity (Pirarat et al., 2006). Similarly, the ability of probiotic strains of the *Carnobacterium* genus to regulate inflammatory response has been shown for rainbow trout (*O. mykiss*), where significantly higher expression of anti-inflammatory cytokines (IL-1β and TNF-α) was observed in the head kidney (Kim and Austin, 2006).

The development of protocols for obtaining germ-free and gnotobiotic larvae for different fish species has allowed us to compare the immune system development of the host, reared in a germ-free environment and with (known) bacteria. Several such studies have shown that microbiota play an important role in stimulating the host's immune response. Such studies for cod (*Gadus morhua*) larvae demonstrated that the transcription of genes involved in immune response and nutrient digestion was regulated by bacteria. Moreover, the host responses varied depending on whether the bacteria were dead or alive, where higher transcript levels were investigated in the larvae that encountered live bacteria (Forberg et al., 2012). On the other hand, bacteria downregulated certain immune responses in cod (*G. morhua*) larvae, which allowed their colonisation of the larval mucosal surfaces (Vestrum et al., 2021). Similarly, DNA microarray comparisons of gene expression in the digestive tracts of 6 days post fertilisation zebrafish revealed that expression of certain genes related to innate immune response was regulated by the microbiota (Rawls et al., 2004).

7 European eel offspring culture: State-of-the-art

Knowledge regarding spawning adults or embryonic and early larval stages of European eel from nature is scarce. Thus, while the development of hatchery protocols for this species becomes challenging, it relies on the augmentation of the knowledge base purely through experimentation. This requires knowledge of broodstock management and assisted reproduction, fertilisation and embryonic development as well as zootechnics for larval rearing to obtain the captively produced glass eels (Tomkiewicz et al., 2019). As the hormonal control of the gonadal maturation is associated with their long spawning migration, European eel do not spawn naturally in captivity (Dufour et al., 2003; Vidal et al., 2004). Instead, spawning in captivity is achieved through assisted reproduction via hormonal induction of gonadal maturation (Palstra et al., 2005; Pedersen, 2004). Here, induction of vitellogenesis in females is achieved through weekly intramuscular injections with pituitary extract (of salmon or carp) for 11–21 weeks (Da Silva et al., 2018; Pedersen, 2004; Tomkiewicz, 2012). To induce follicular maturation and ovulation, a priming dose of pituitary extract and 17α,20ß-dihydroxy-4-pregnen-3-one (DHP) is administered to females (Da Silva et al., 2018; Ohta et al., 1996; Palstra et al., 2005). On the other hand, male broodstock receives weekly injections of human chorionic gonadotropin (hCG) (Asturiano et al., 2006; Tomkiewicz, 2012). It has been demonstrated that, upon hormonal-assisted gonadal maturation, fertilisation of gametes can be obtained either by spontaneous or strip spawning approaches (Di Biase et al., 2016). While spontaneous spawning may yield higher-quality eggs, strip spawning and in vitro fertilization add controllability to the process. Several studies have focused on the standardisation of in vitro fertilisation protocols for the European eel (Asturiano et al., 2006; Butts et al., 2014; Peñaranda et al., 2008; Sørensen et al., 2013, 2016a), which together with studies on broodstock diets and feeding regimes e.g., Kottmann et al. (2020b), have led to improvements in egg quality and fertilisation success. Assisted reproduction protocols were further improved through research that explored the impact of pituitary extracts of different origins (carp *vs*. salmon) (E. Benini et al., 2022; Kottmann et al., 2020a), and the effects of strip-spawning timing or the necessity of a primer dose for sperm production and quality (Koumpiadis et al., 2021; Tomkiewicz et al., 2011).

With the success of assisted reproduction of European eel, research also oriented towards the establishment of protocols for embryonic incubation and early larval rearing. For the first time, Sørensen et al. (2016b) described the ontogeny and growth of early life stages of captive-bred European eel throughout the yolk sac stage. Here, it was described that embryonic development of European eel spans for ~ 46–48 hours post fertilisation (hpf) at 20°C and the typical development of eggs and embryos during this period is depicted in Figure 7A (Sørensen et al., 2016b). The newly hatched European eel (at 0 days post-hatching - dph) larva (Figure 7B) is characterised by a relatively large yolk reserve and prominent oil globule compared to the size of the larvae (Sørensen et al., 2016b). Eel larvae depend on the yolk and oil reserves during the pre-feeding period, which usually lasts from hatch until 9 dph (at 20°C) (Figure 7B). Optimisation of the bio-physical conditions and stage-specific requirements during early larval rearing was addressed in relation to light (Politis et al., 2014), microbial interference (Sørensen et al., 2014), temperature (Politis et al., 2017), salinity (Politis et al., 2021, 2018), and pH (Sganga et al., 2022). A better understanding of environmental requirements by the early developmental stages, together with the technological advancement of rearing systems, have led to a stable production of European eel larvae that enter the feeding stage. However, establishing a protocol for culturing feeding larvae remains challenging, due to limited knowledge about their feeding ecology in nature during the first feeding (preleptocephalus) phase and long-lasting migratory (leptocephalus) stage.

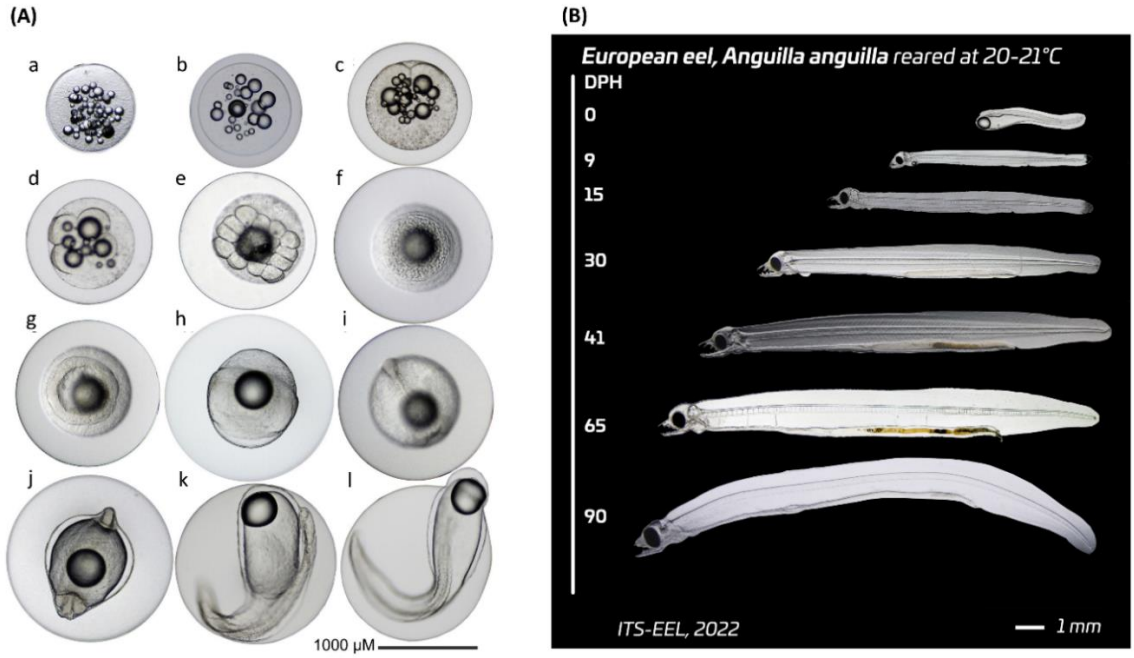

Figure 7: Embryonic development (at 20 °C) (A) (Source: Sørensen et al. (2016b)) and larval development (at 20-21°C) (B) of European eel in captivity (Graphics by S.R. Sørensen and S.N. Politis).

Recent studies of leptocephali gut contents are still inconclusive but suggest that their natural diet consists of amorphous material of different origins, such as larvacean houses, faecal pellets, gelatinous zooplankton, and materials associated with marine snow (bacteria, protists, fungi, and other microorganisms) (Ayala et al., 2018; Miller et al., 2019a). These materials are difficult to obtain in sufficient quantities for mass rearing of eel larvae, but the first-feeding diets that have been tested and proven successful for eel larvae in captivity are in sharp contrast to their suggested natural diets. For Japanese eel (*Anguilla japonica*), a species closely related to European eel, the first successful rearing of feeding larvae was reported when the larvae were fed with a slurry-type diet based on egg yolk of spiny dogfish (*Squalus acanthias*) (Tanaka et al., 2001). Gradual modifications and improvements in the diet, together with improved rearing techniques, have enabled the closure of the life cycle of Japanese eel and thus, the production of further generations of captive propagated offspring (Tanaka, 2015). For European eel, pioneering studies tested different first-feeding diets such as a paste-like-diet based on rotifers (*Brachionus plicatilis*) (Butts et al., 2016, Politis et al., 2018) and a slurry-type diet based on pasteurised egg yolk from thornback ray (*Raja clavata*) (Elisa Benini et al., 2022). Although these diets did not support the growth and survival of the larvae beyond the so-called “point of no return”, these studies improved knowledge of feeding biology with insights into molecular mechanisms. Furthermore, the essentiality of the inclusion of long-chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in first feeding diets for European eel has been demonstrated by Lund et al., 2021. Rearing of European eel beyond the point-of-no-return was first reported when larvae were fed with a slurry-type diet based on pasteurised spiny dogfish (*S. acanthias*) egg yolk (Benini et al., 2023b). While all these research findings have contributed immensely to the progress development of a hatchery protocol for the European eel, closure of their life cycle in captivity still remains a challenge, indicating the need for further optimisation of the current protocols.

8 Gaps in knowledge

Protocols for obtaining viable offspring in captivity were developed relatively recently for the European eel, opening a new arena for a variety of research possibilities. At the present state, progress in hatchery technology requires experimental work to fill gaps in knowledge throughout early life history to ensure the production of healthy offspring that can survive through the long larval phase until they metamorphose into glass eels. While existing research has explored various aspects such as assisted reproduction and the influence of biophysical conditions on quality, development, and physiology of European eel offspring, significant mortality during their early life stages pose a challenge to achieving a complete life cycle in captivity. These early mortalities can be attributed to several factors, including poor gamete quality, inappropriate first-feeding larval diets, and suboptimal rearing conditions. However, the negative impacts of host-microbial interactions and the underdeveloped host immune system have not received adequate attention. To date, only one study (Sørensen et al., 2014) has addressed bacterial interference during the captive rearing of early life stages of European eel. Furthermore, no studies have investigated the connection between host, surrounding bacterial community structure, host immune response, and overall performance (e.g., survival, growth, and development) of European eel under captive rearing conditions. Therefore, this PhD research project focused on examining bacterial interference during the culture of eel offspring and the development of early stages of European eel in captivity. The goal was to uncover the relationships between these factors and the survival and healthy development of embryonic and larval stages. As such, the conducted research aimed to provide valuable insights regarding the factors that determine the success or failure of eel offspring in captivity, with the scope of overcoming the current challenges, hindering offspring survival and development. Accordingly, four different studies were conducted to address different hatchery-related aspects, spanning from eggs to first-feeding larvae.

So far, the effect of egg incubation density has not been investigated for any eel species with aquaculture importance. However, the optimum stocking density of eggs during incubation is an important operational parameter for a hatchery to maximise the utilisation of resources (e.g., space, labour) without compromising the offspring development, survival, and (microbial) water quality. Thus, Study 1 of this PhD tested the effect of egg stocking density on survival during embryonic incubation and early larval raring (until 3 dph) on bacterial community composition of offspring and rearing water as well as expression of immune and stress related genes of offspring.

Moreover, testing larval diets and feeding regimes for cultured eel species has gained significant attention as feeding the eel larvae in captivity has been a major bottleneck. Currently, progress in hatchery protocols for European eel larvae has led to increased production of larvae that enter the feeding stage, enabling testing first-feeding diets. So far, changes in the bacterial community composition of larvae and rearing water as well as larval immune responses in relation to first-feeding diets have not been addressed for any captively reared eel species. In contrast to the typical first feeding environment of marine fish larvae (Figure 5), major differences in the first feeding environment of European eel include the absence of live feed and algae, as well as lack of predation on bacteria by live feed (Figure 8). Eel larvae feed on slurry-type food and most probably on bacteria (intentionally or unintentionally), which has not been demonstrated experimentally, while bacteria receive DOM from eel larvae and the food. Unlike live feed, the formulated liquid food used during eel larval culture contains ingredients that can easily serve as substrate available for bacteria. Thus, the conditions in the first-feeding tank might favour an r-selected bacterial community dominated by potentially harmful bacteria. On the other hand, the influence of the food-associated bacterial community might be higher on the larval bacterial

community of eel due to their feeding behaviour in captivity, during which they swim through a puddle of food. However, bacterial interference and molecular immunologic development as well as their link to larval performance have not previously been investigated in the “atypical” first feeding environment of eel larvae in captivity. As such, this PhD project aimed to fill this gap in knowledge in studies 2 and 3. Here, Study 2 followed changes in the larval bacterial communities and expression of immune-related genes in connection to three different prototype first-feeding diets in an attempt to relate the observed pattern of larval survival to changes in bacterial communities and to the molecular immune system ontogeny.

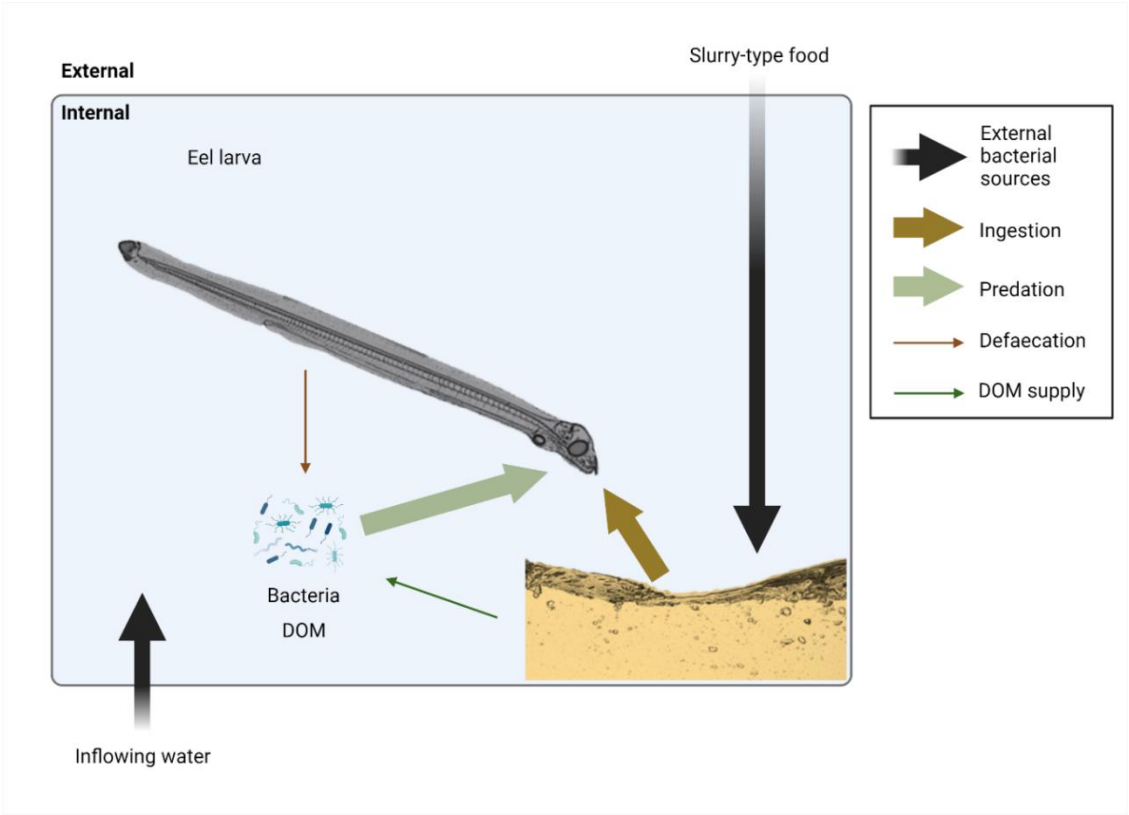

Figure 8: First feeding environment of European eel. Different external sources of bacteria (black arrows) include inflowing water and food added to the tank. Fish larvae ingest food and most probably the bacteria (light-green arrow), while contributing to the bacteria-DOM pool through defaecation. Formulated slurry-type food added to the system is an important source of DOM (dark-green arrow) (Created with BioRender.com).

Furthermore, while the life cycle of Japanese eel has been closed in captivity using a slurry-type diet, establishing a first feeding larval culture protocol is at a pioneering stage for European eel. At that time, the larval rearing protocol for European eel larvae suggested feeding with 0.5 mL of food per L of water (Benini et al., 2023a), which is three times less than the amount fed to Japanese eel (1.5 mL of food per L of water) (Okamura et al., 2019). Therefore, we hypothesized that increasing the amount of food given to European eel larvae would increase the feeding success in terms of feeding incidence (i.e., the fraction of larvae that ingest the food) and gut fullness (i.e., the fraction of gut-containing food). However, since the knowledge regarding the influence of food amounts on bacterial communities of larvae and rearing water has been lacking, increasing the food amount seemed to pose a risk as this might select for detrimental and opportunistic bacteria due to the high availability of substrate (DOM) for bacterial growth. Accordingly, Study 3 investigated whether the food amount can be increased to gain potential

benefits (e.g., improved feeding success and growth), without compromising the healthy larval-bacterial interactions and the associated larval wellbeing. For this study, two amounts of slurry-type food (Low = 0.5 mL of food per L of water and High = 1.5 mL of food per L of water) were tested to evaluate the effects on feeding success, growth, and survival of European eel larvae. Moreover, the bacterial community composition of water, larvae, and food, as well as the changes in the larval bacterial community over time were studied. Finally, the expression of genes related to immunity, stress, and food ingestion was investigated in response to both food amounts.

Lastly, newly hatched European eel larvae might be susceptible to negative larval-bacteria interactions due to a potentially immuno-compromised period, which might be one of the reasons behind the observed mortalities (Miest et al., 2019). This emphasises the importance of maturing the larval immune system as early as possible through incorporating relevant tools such as improving maternally transferred immunity in offspring or applying immuno-stimulants in the current larval culture protocol. Here, first studies have revealed the potential for gut-priming or immuno-stimulating agents in European eel offspring, but with no direct recommendations, suggesting the need for further research (Politis et al., 2023). Therefore, the effect of β-glucan, which is a yeast cell wall compound with reported immunostimulatory properties and prebiotic properties (reviewed in Meena et al. (2013)) has been tested in Study 4. Accordingly, yolk sac larvae were exposed from 5 dph to β-glucan, which was added to the rearing water until they reached the feeding stage (9 dph) to study the effect of β-glucan exposure on larval survival, growth, development (e.g., deformities), molecular ontogeny of the immune system and bacterial community composition of larvae and rearing water during and after (until 20 dph) the exposure period.

9 Objectives

The overall objective of the present PhD was to investigate the bacterial interference and immune system ontogeny at the molecular level at different developmental stages of fish offspring (e.g., eggs/embryos, yolk sac larvae, and free-feeding larvae) during culture and relate to offspring performance (e.g., growth, development, and survival), using European eel as a model. Accordingly, four studies, which address important hatchery-related parameters have been designed and conducted. The specific objectives of each study are outlined below.

Firstly, Study 1 aimed to explore the impact of egg stocking density on both, the bacterial community composition of offspring and the surrounding water. Concurrently, this study investigated the expression of genes related to immune and stress responses in the offspring. Moreover, it sought to establish the potential association of bacterial communities and the immune and stress responses to the observed patterns of embryonic and larval survival as well as hatch success. Overall, this study aimed to contribute to the improvement of current egg incubation protocols by providing stocking density recommendations.

Then, Study 2 had two primary objectives. First, it aimed to unveil the influence of three different slurry-type diets on the succession of bacterial communities both, during and after the first feeding period. Second, the study sought to reveal the intricate connections between bacterial communities, expression of genes related to immune and stress/repair responses, and key indicators of larval performance, such as survival rates. By addressing these objectives, the study aimed to provide valuable insights regarding the relationships between diet, bacterial communities, gene expression, and larval well-being.

Study 3 sought to examine the impact of increasing the quantity of food provided to first-feeding larvae on several critical factors, including larval performance metrics such as growth and survival, the composition of bacterial communities of both, the larvae and the rearing water, and the expression of genes associated with food intake, immunity, and stress responses. Overall, the study aimed to contribute to the ongoing refinement of the larval culture protocol for European eel by optimising the feeding regimes currently employed.

Last, Study 4 encompassed several key objectives. Firstly, it aimed to evaluate the effectiveness of β-glucan as an immuno-stimulant for yolk sac larvae of European eel, assessing its potential to enhance their molecular immune responses. Secondly, the study examined the impact of introducing β-glucan into the rearing water on the composition of bacterial communities of both the offspring and the rearing water itself. Finally, the study investigated how exposure to β-glucan influenced various aspects of larval development and performance, including the occurrence of deformities, survival, and growth.

10 Approaches

This PhD project applied a multi- and inter-disciplinary approach combining controlled experiments, analyses of experimental records, image analyses (e.g., morphometrics and categorisation of deformities), and molecular analyses, targeting expression levels of specific genes and bacterial community composition analyses through 16S rRNA gene sequencing (Figure 9). Experiments, image analysis and molecular work were carried out at the experimental facilities of DTU, while library preparation and sequence data analysis related to bacterial community composition were carried out at laboratories and computational facilities at NTNU.

NIS-Elements D analysis software (Nikon Corporation, Japan, Version 3.2) was used for digital imaging of the larvae and analysing the images for different important parameters. These parameters included larval growth (in terms of larval body area), deformities, and feeding success (in terms of feeding incidence and gut fullness) (Figure 9).

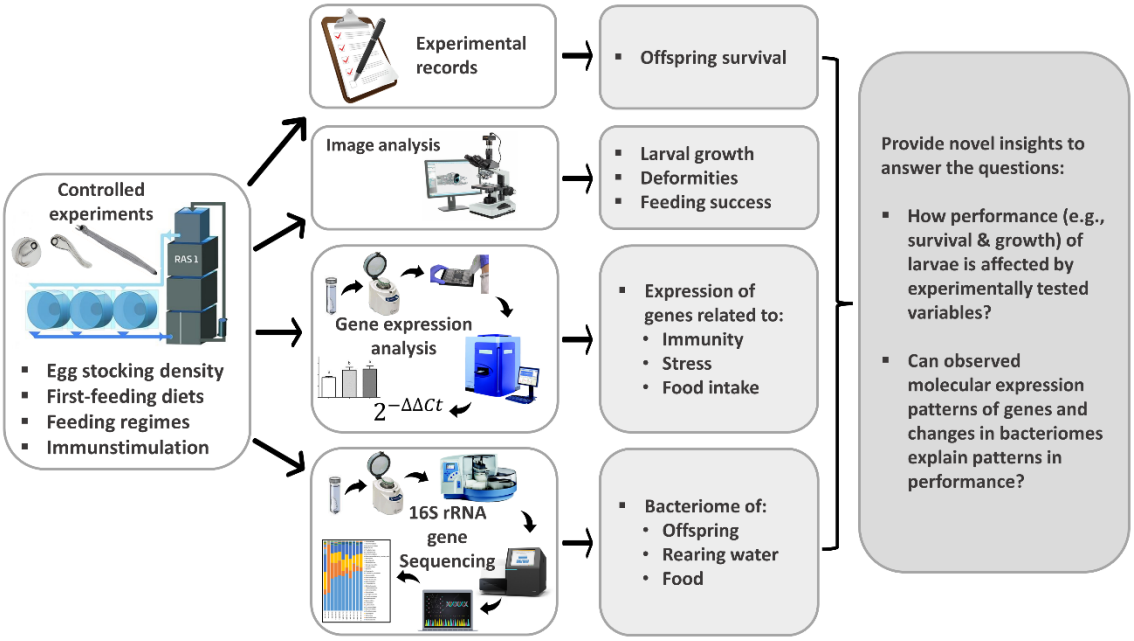

Figure 9: Multi- and inter-disciplinary approaches utilised during this PhD project to address selected issues during the experimental hatchery phase of European eel.

Molecular tools were utilised to study the expression of genes with different functions in response to the different hatchery-related variables tested. In studies 1, 2 and 4, the focus was on the expression of genes related to immunity and stress. In addition to those genes, for study 3, the expression of genes related to food intake was also studied. The availability of the recently developed molecular toolbox for European eel (Miest et al., 2019), which includes reference genes and genes from innate and adaptive arms of the immune system was an advantage. Cycle threshold (Ct) data obtained from Biomark™ HD qPCR system (Fluidigm) (for studies 1 and 2) or QuantStudio5 (Thermo Fisher Scientific) (for studies 3 and 4) were processed using the delta-delta Ct method (Livak and Schmittgen, 2001) to get relative gene expression values, which were statistically analysed to measure differences in expression of genes of interest for different samples (Figure 9).

Bacterial community composition in different samples (e.g., offspring, water, and food) was evaluated through amplicon sequencing of the hypervariable region of the bacterial 16S rRNA

gene with the Illumina platform. The Illumina sequencing data were processed using the USEARCH utility (version 11) (https://www.drive5. com/usearch/) and amplicon sequence variants (ASVs) were generated with the Unoise3 command (Edgar, 2016). Bacterial community composition analysis (e.g., alpha and beta diversity, differential abundance testing) was performed with the aid of different packages developed for R statistical software.

In the end, based on the knowledge generated through these multi- and inter-disciplinary approaches, efforts were taken to address two questions: how larval performance is affected by the variables tested during the experiment and if observed patterns in performances can be explained with the knowledge gained from molecular analyses and bacterial 16S rRNA gene sequencing data.

11 Findings of my PhD

Study 1: Effects of European eel egg and larval stocking density on rearing water and offspring bacteriome and derived immune response

During the early hatchery phase of European eel, a substantial loss (~75%) of offspring due to mortality occurs at the embryonic incubation phase. The observed mortalities might partly be attributed to negative host-microbial interactions because the environment inside the incubators, where eggs are stocked at high densities and contain DOM at high concentrations is favourable for the growth of r-strategic (thus probably opportunistic) bacteria. Egg stocking density is an important factor that determines the DOM concentration in the incubation environment. Therefore, one strategy to improve bacterial water quality during incubation is to simply reduce the initial stocking densities of eggs. However, lower egg stocking densities require more space (e.g., more incubators) and labour during the hatchery operation. Thus, Study 1 was conducted to determine a practically sound egg stocking density that can be used to refine the current hatchery protocol for European eel. Accordingly, we explored the offspring survival, hatching success, succession of bacterial composition in rearing water and offspring and expression of immune and stress related genes in offspring of European eel, reared from fertilisation until 3 dph, in response to different initial stocking densities (500, 1000, 2000 and 4000 eggs/L) (Figure 10).

According to the results of this study, survival was highest in the lowest stocking density (500 eggs/L) and significantly decreased over time, where the largest drop was observed during the embryonic phase, reaching a survival of ~26 % at 48 hpf. However, neither the hatching success nor the expression of immune and stress related genes were affected by the stocking density within the range tested. Alpha diversity analysis indicated that the bacteriome of the unadulterated eggs had the lowest ASV richness and the highest evenness of all the developmental stages studied. Interestingly, we observed a strong maternal effect on initial egg bacterial communities as indicated by a bacteriome unique to each batch. Moreover, the observed patterns of survival in response to stocking density and age can be explained by the differences observed in bacterial community composition. Density-dependant differences in the bacterial communities were detected in embryos and incubation water (at 48 hpf), wherein the highest stocking density, all the ASVs that were significantly abundant compared to the lowest stocking density belonged to the phylum Proteobacteria including several ASVs belonging to the Vibrionales order. This indicates that the bacterial communities of embryos and water in the highest density were dominated by potentially harmful bacteria. This was further supported by the difference in the pattern of how the ASV richness changed over time between the lowest and highest stocking densities. In the lowest stocking density, ASV richness increased with age, while in the highest stocking density, ASV richness increased from 0 to 48 hpf and remained stable until 3 dph. This might indicate that the embryos in the highest stocking density were quickly (within 48 hpf) colonised by r-strategists (and probably opportunistic) bacteria, owing to their fast-growing ability in response to the high availability of DOM, without leaving space for further colonisation later during the development.

When comparing the bacterial communities over the age, we observed that the bacterial community of the embryos (at 48 hpf) were dominated by the ASVs of phylum Proteobacteria (including members of potentially harmful orders Vibrionales, Rhodobacterales, Oceanospirillales and Alteromonadales), whereas several additional ASVs belonging to other phyla (such as Actinobacteria, Bacteroidetes, and Planctomycetes) were also detected at higher abundances in the bacterial communities of initial eggs (0 hpf) and 3 dph larvae. Thus, the sharp drop in survival

that was observed during the embryonic incubation phase compared to the larval rearing phase, can be attributed to the embryonic bacteriome that was dominated by potentially harmful bacteria.

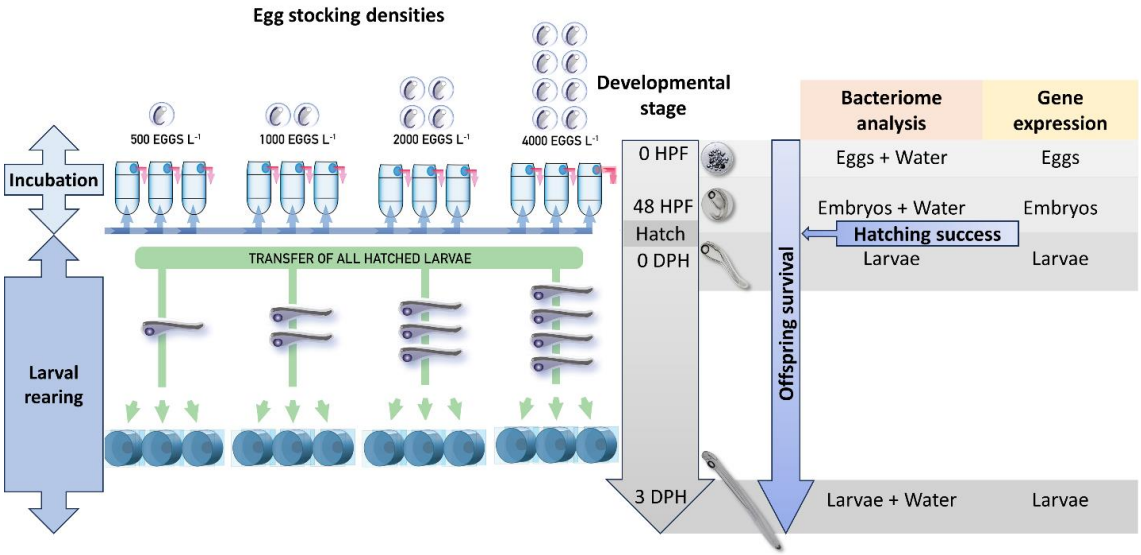

Figure 10: Schematic representation (not to scale) of experimental design of Study 1, where offspring survival, hatching success, bacterial community composition of offspring and water and expression of immune and stress related genes were explored during a period spanned from hatch (0 hpf) until 3 dph, in response to four different initial stocking densities (500, 1000, 2000 and 4000 eggs/L).

When comparing bacterial communities of offspring to that of their rearing environment, they were different all the time for both lowest and highest stocking densities and at both embryonic and larval rearing phases. Moreover, ASV richness was always higher in the bacterial community of rearing water than in the offspring, indicating the existence of selection mechanisms in the host (i.e., host preferences) to determine which bacteria colonise them independently of their environment. Interestingly, we found density-dependant differences in the evenness between the bacterial communities of offspring and their rearing environment, where evenness was comparable in the lowest stocking density, while it was higher in rearing water than in embryos in the highest stocking density. However, the evenness of the rearing water bacterial communities between the lowest and highest densities were similar. This indicates the presence of stronger selection pressure (most probably the availability of DOM) in the highest stocking density, which led to the selection in favour of certain bacterial groups (e.g., competitive exclusion), thus reducing the evenness in the bacterial community.

This study, while documenting the succession of bacterial communities in offspring and the rearing environment during embryonic incubation and early larval rearing phase of the European eel, revealed that the major early mortalities were associated with negative bacterial interactions. On the other hand, as expression of stress and immune-related genes were not affected by density, the molecular immune system does not appear to be fully matured to handle such a challenging microbial environment during this early life stage, which could partly be due to an evolutionary adaptation to the oligotrophic environment they would naturally thrive in at this stage and only aggravates the challenges faced during hatchery production.

In conclusion, our findings suggest that using a low-density approach (500 eggs/L) during incubation can effectively enhance survival rates. However, it is worth considering the possibility of using higher stocking densities (up to 4000 eggs/L), as a reasonable compromise. This higher

density results in only a modest reduction in survival of approximately 5%, with no adverse effects on hatching success or cellular stress response. Additionally, it offers the advantage of optimising infrastructure and labour resources. Nonetheless, it is crucial to implement measures for managing bacterial communities during high-density incubation to prevent mortality associated with bacteria through the avoidance of r-selection. Furthermore, the results of this study have inspired further exploration into the realms of bacterial interference and the molecular immune competence of larvae, with a particular focus on later developmental stages, especially during the feeding phase.

Study 2: Exploring bacterial community composition and immune gene expression of European eel larvae in relation to first-feeding diets

During the current larval culture practice for the European eel, larvae are fed with a slurry-type diet, which is poured on the bottom as a puddle of food. This diet contains nutrients that can easily go into solution and act as a substrate for bacteria. Most probably the conditions inside the rearing tank during the feeding are favourable for r-strategic and opportunistic bacteria leading to negative larvae-bacteria interactions and thus, to poor larval performance (e.g., survival). The larval immune system must play an important function in such a hostile microbial environment. Thus, Study 2 was conducted to uncover the succession of the bacterial communities and molecular development of the larval immune system in response to prototype first-feeding diets and to relate them to larval survival (Figure 11). This study formed a component of a feeding trial, wherein diverse facets of larval development and rearing circumstances were explored, to understand their correlation with the survival and growth of the larvae. The current investigation centres on the progression of bacterial communities and the expression of immune genes in the larvae. Meanwhile, a separate study directed its attention towards appraising how different diets impacted larval appetite, feeding outcomes, growth rates, and the associated genetic factors (Benini et al., 2023a).

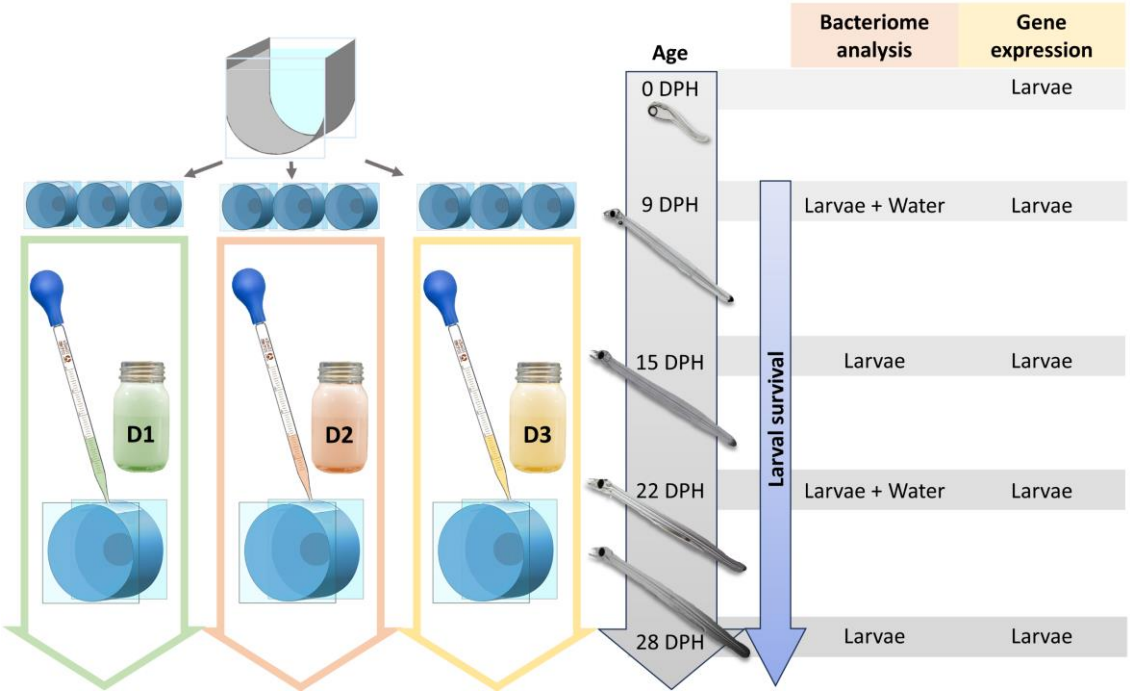

Figure 11: Schematic representation (not to scale) of experimental design of Study 3, in which the succession of the bacterial communities (in larvae and rearing water) and molecular

development of the larval immune system in response to prototype first-feeding diets and to relate them to the larval survival (D1 = Diet 1, D2 = Diet 2 and D3 = Diet 3).

The first feeding stage of European eel in captivity is generally characterised by a critical period between 20 to 24 dph, which is associated with mortalities, indicating the point of no return (Benini et al., 2023a). Results of the present study revealed that the larval stress/repair mechanism was activated during this critical period as indicated by an upregulation of the gene encoding heat shock protein 90 (HSP 90), independent of the diet. At the same time, a shift towards a potentially detrimental larval bacterial community was observed in all dietary groups. This was marked by a significant reduction in evenness of the larval bacterial community where several ASVs belonging to potentially harmful bacterial genera (e.g., *Vibrio*, *Enterococcus*, and *Pseudomonas* etc.) were significantly more abundant in the bacterial community during this critical period. This indicates that negative larvae-bacteria interactions were most likely associated with the larval mortality observed.

Beyond the point of no return, the highest survival was registered for larvae fed Diet 3. Molecular analysis of the immune related genes revealed that genes encoding for pathogen recognition receptor TLR18 and complement component C1qC were upregulated in this group, compared to the other two groups. Similarly, expression of gene encoding for the pro-inflammatory cytokine, IL-1β was higher in the larvae fed Diet 3 compared to Diet 1. As such, in the larvae fed with Diet 3, the immune components related to pathogen recognition, cellular signalling pathway and complement proteins important for opsonisation, phagocytosis and eventual lysis of harmful microorganisms were expressed at higher levels on 22 dph. This suggests a higher immunocompetency in this group, which probably assisted in handling the potentially harmful bacteria that dominated the larval bacterial community at this stage, leading to better performance in terms of survival. This study was the first to reveal that the negative larvae-bacterial interactions are associated with the mortalities during rearing of first-feeding European eel larvae and offered motivation for further investigation into bacterial interference during the first feeding phase of European eel culture.

Study 3: Effect of food amounts on larval performance, bacteriome and molecular immunologic development during first-feeding culture of European eel

Facilitating the access of many larvae to nourishment is vital to the triumph of cultivating first-feeding larvae, with the adequate provision of food serving a pivotal function. On the other hand, the food added into the tanks provides substrates (DOM) for heterotrophic bacteria. High substrate availability generally selects for r-strategists possibly leading to an unhealthy bacterial community dominated by opportunistic bacteria. Therefore, Study 3 was conducted to find out whether the food amount can be increased without compromising the healthy larvae-bacterial interactions and larval wellbeing, to gain the potential benefits (e.g., improved feeding success and growth). Here, two amounts (Low = 0.5 mL of food per L of water and High = 1.5 mL of food per L of water) of the Diet 3, which was identified as an appropriate diet from the Study 2 were tested for the effects on feeding success, growth, and survival of the European eel larvae. Moreover, the bacterial community composition of water, larvae, and food, as well as the changes in the larval bacterial community over time were studied. Also, the expression of genes related to immunity, stress, and food ingestion was investigated in response to food amounts (Figure 12).

The results of this study suggest that the High amount of food improved the efficiency of food intake, indicated by the higher gut fullness in the larvae. This further was confirmed by the results of molecular analysis, which showed that the expression levels of the *ghrl* gene, which encodes ghrelin or the “hunger hormone”, were higher in the Low food treatment by the end of the

experiment (30 dph), indicating that they were starved compared to the larvae fed with High amount of food. Furthermore, the larvae exhibited greater gut fullness at 25 dph in comparison to 15 dph, suggesting that the larvae needed a certain duration to acclimate and adapt to the food and feeding schedule. Biometric analysis revealed that the eel larvae fed with High food amount grew faster (in terms of body area) compared to larvae fed Low food amount. Interestingly, on 30 dph, compared to the Low food treatment, the larval bacterial community of High food treatment contained a higher abundance of the ASVs belonging to genera *Bacillus*, *Lactobacillus* and *Streptococcus*, which include the most tested probiotic strains in aquaculture. Therefore, apart from the larvae consuming a greater amount of food in the High-food treatment, the enhanced growth observed in comparison to the Low-food treatment could potentially be linked to the existence of ASVs from these beneficial bacterial genera. Notably, the genus *Bacillus*, known for its capacity to promote fish growth, might play a significant role in this regard. The feeding incidence (representing the proportion of larvae with food in their digestive tracts) remained consistent at approximately 75% across all age groups. This consistency indicates that the provided food was generally accepted by the larvae and was unaffected by variations in the quantity of food offered.

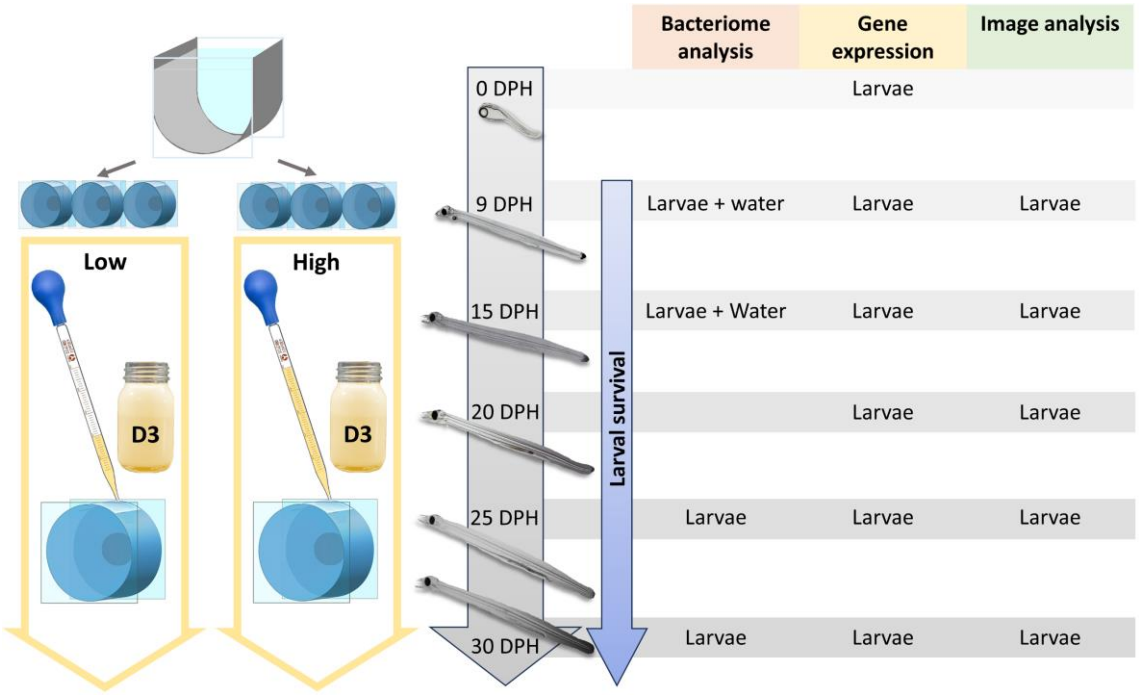

Figure 12: Schematic representation (not to scale) of experimental design of Study 4, where the effect of different amounts (Low = 0.5 ml/L and High = 1.5 ml/L) of Diet 3 (D3) on larval performance (e.g., growth and survival), feeding success (e.g., feeding incidence and gut fullness), bacterial community composition (e.g., in rearing water and larvae) and larval gene expression were investigated.

Molecular analysis did not find evidence of the effect of food amount on the expression of immune and stress related genes, indicating that feeding the larvae with High food amount did not act as an additional stressor on the larvae. An upregulation of *hsp90*, a gene related to cellular stress response and repair mechanism, was noticed from the end of the first-feeding window (25 dph onward), probably implying the larval diet used during the current experiment was suboptimal (e.g., deficient in nutrients). This was verified through the halt in larval growth, assessed by no changes in body area after 15 dph. Despite the initially lower survival in the larvae fed High

amount of food (from 12 to 23 dph), survival was similar in the two treatments from 24 to 30 dph, reaching a ~10% survival at the end of the experiment (30 dph). During Study 2, we observed a significant phase marked by larval mortality and activated stress/repair mechanisms. This phase was linked to a prevalence of potentially harmful bacteria within the larval bacterial community. However, in the current study, we did not observe as distinct a critical period or a shift towards a bacterial community dominated by potentially harmful or opportunistic bacteria as was evident in Study 2. This reaffirms our previous conclusion that detrimental bacteria contribute to the decline in larval survival. Nevertheless, the conflicting results between the two studies highlight the stochasticity of bacterial colonisation in larvae.

According to alpha diversity analysis, food amount did not affect the ASV richness, evenness, and exponential Shannon index (exp. Shannon) of the larval bacterial community. ASV richness and the exp. Shannon were lowest in the pre-feeding larvae (9 dph) and remained higher throughout the feeding period, indicating probably the opening of new niches that can be occupied by new bacteria sourced from external sources (e.g., water and food). Interestingly, when comparing the bacterial communities of the inflowing and outflowing water of the rearing tank on 15 dph, ASV richness remained unchanged in the Low food treatment, whereas it was reduced in the outflowing water compared to inflowing water in the High food treatment, probably implying that selective forces (e.g., availability of DOM) is stronger in the High food treatment leading to exclusion of certain ASVs. The outcome of this selection was noticed also in the larval bacterial community of High food treatment, where an increase in ASVs belonging to potentially harmful orders such as Rhodobacterales, Oceanospirillales and Flavobacteriales was observed on 15 dph compared to pre-feeding larvae. Thus, the presence of bacteria from these potentially harmful groups is likely linked to the lower survival rate observed initially in the High-food treatment after the commencement of feeding, when compared to the Low-food treatment.

On the other hand, feeding the larvae with High amount of food benefited them later during the experiment where a healthier larval bacterial community was observed in this group on 30 dph compared to the Low-food treatment. Feeding the larvae with High amount of food not only promoted the growth of ASVs of potentially beneficial orders Lactobacillales and Bacillales including sequence matches to probiotics (e.g., *Bacillus cereus*, *Leuconostoc mesenteroides* and *Staphylococcus haemolyticus*), but also excluded potentially harmful bacteria, including ASVs with high sequence similarity to *V*. *campbellii*, *V*. *harveyi* and *V*. *Fluvialis*, which are pathogens reported in aquaculture. The transition from an initially less healthy bacterial community to a relatively healthier one in the High-food treatment can be attributed to the bacterial composition of the food itself. The food primarily consisted of potentially beneficial orders like Lactobacillales and Bacillales, which likely contributed to this positive shift.

In conclusion, the High food amount benefited first-feeding European eel larvae as indicated by higher gut fullness, faster growth, and healthier larval bacterial community on 30 dph compared to larvae fed the Low food amount. Molecular analysis showed that most of the larvae fed the Low amount of food were starving on 30 dph as indicated by the upregulation of the *ghrl*, the gene encoding for ghrelin. Also, Feeding High amount of food did not trigger molecular mechanisms related to stress/ immune response. However, upregulation of *hsp90*, which was noticed from the end of the first-feeding window and cessation in larval growth emphasise the dietary imbalances of the food used during the present experiment. Thus, while further optimisation of the diet formulation is needed, the use of High food amount can be recommended to incorporate into the current rearing protocol of first-feeding larvae of European eel.

Study 4: β-Glucan enhances development and modulates the bacteriome and immuno-stress response of European eel larvae

The applicability of β-glucan, which is known for its immunostimulatory, and bacteriome-modulatory properties was evaluated in Study 4 as a tool to overcome the early hatchery phase issues associated with the undeveloped larval immune system and unhealthy bacterial communities. Accordingly, yolk sac larvae were exposed to Yeast b-1,3/1,6-glucan (MacroGard®) (BG) at a concentration of 5 mg/L by adding it into the rearing water for 5 days (from 5 to 9 dph), until they reached the feeding stage. Then, the larvae were fed with a slurry-type diet (Diet 3 of Study 2) based on spiny dogfish (*S. acanthias*) egg yolk as the major dietary component until they reached 20 dph. During the experimental period, larval survival was followed, larvae and rearing water were sampled for bacterial community composition analysis, and larvae were sampled for gene expression analysis and digitally imaged for biometrics and categorisation for deformities (Figure 13). The experiment was repeated with four batches of larvae.

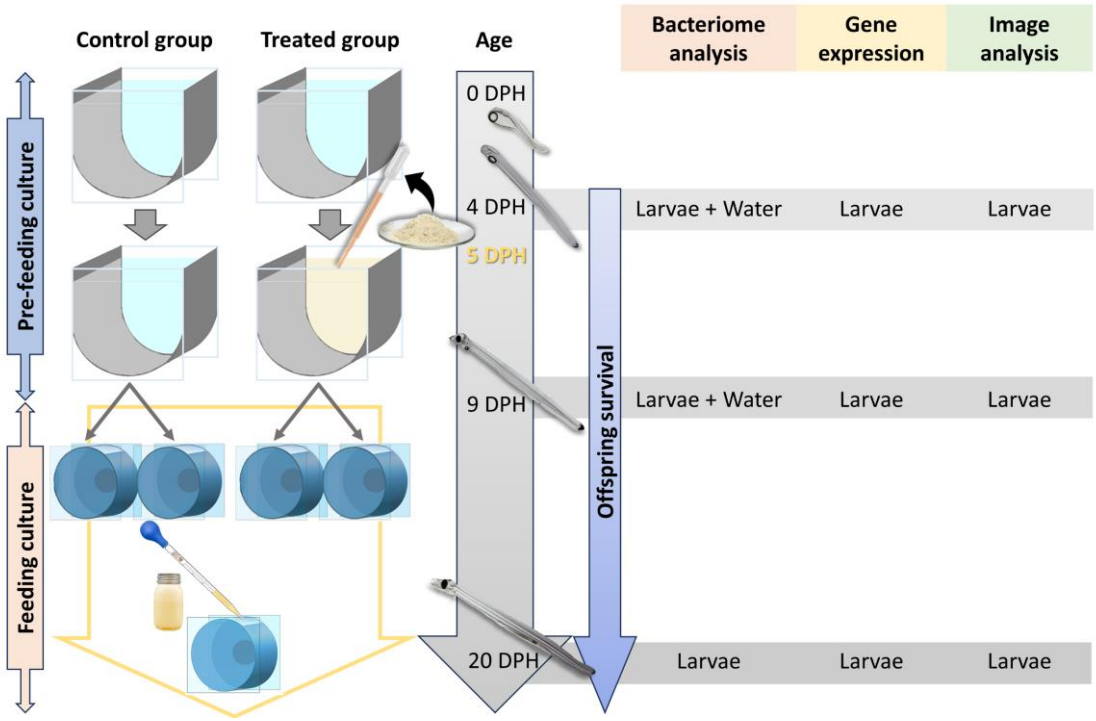

Figure 13: Schematic representation (not to scale) of experimental design of Study 2, which was carried out to study the effect of exposure to β-glucan during the pre-feeding period on offspring survival, bacterial community composition of larvae and water, expression of immune and stress related genes in larvae and on larval growth and deformities.

No effect of BG on survival and growth was found, neither at the end of the BG treatment nor during the feeding period, until 20 dph. However, we found beneficial effects of BG treatment on larval development, immune and stress response, and larval and water bacteriomes. Through analysis of a variety of deformities (pericardial edema, emaciation, deformed neurocranium, spinal curvature and deformed jaw), we demonstrated that BG treatment can reduce the occurrence of larval deformities in European eel larvae. Here, the occurrence of all deformity types analysed was significantly lower in the BG-treated group than in the control. Generally, detectability and/ or the occurrence of the deformities increased with age.

Examination at the molecular level of immune-related genes exposed the immunomodulatory characteristics of BG. This was highlighted by the decrease in the expression of the *il10* gene

within the BG-treated group compared to the control by the conclusion of the treatment period (9 dph). The *il10* gene is responsible for encoding the anti-inflammatory cytokine IL10, which plays a consistent role in mitigating inflammatory reactions. Furthermore, the *hsp90* gene, associated with the cellular stress response, was downregulated in the BG-treated group as opposed to the control. This suggests that BG can alleviate stress in the larvae. However, the other immune related genes studied were not affected by the BG treatment at the end of the treatment. Additionally, the modulatory effect of BG treatment on the molecular-level immune and stress response diminishes after a period of 10 days post-treatment (on 20 dph), highlighting the transient nature of this effect.

While examining the structure of the bacterial community, we found that the BG treatment caused changes in the bacterial populations. This resulted in significant differences in the bacterial communities present in both the larvae and the water by the conclusion of the treatment period (9 dph). The bacterial communities within the treated larvae displayed notably greater alpha diversity compared to the control larvae, primarily driven by heightened ASV richness. Conversely, we noted a distinct reduction in diversity within the water bacterial communities of the treatment group in comparison to the control. This discrepancy arises from decreased richness and evenness. Intriguingly, this highlights a pattern of selection occurring in the larvae, contingent on the ASV composition within the rearing water. A very important finding of this study was that BG treatment significantly reduced the abundance of ASVs of the genus *Vibrio*, especially in the rearing water. Reduction of ASVs of genus *Vibrio* in response to BG was also observed in the larval bacteriome but to a lesser extent. In addition to the reduced abundance of *Vibrio* spp., BG treatment led to a shift in the water and larval bacterial communities towards communities dominated by “Unassigned” taxa, reflecting most probably a K-selected community composed of diverse and specialised bacterial taxa.

Based on the findings of this study, it can be deduced that exposure of European eel yolk sac larvae to β-1,3/1,6-glucan within the rearing water yields benefits. This is evident through the significant reduction in deformities, enhancement of bacterial communities in both the rearing water and the larvae (notably fewer *Vibrio* spp. and increased prevalence of K-strategists), and decreased cellular stress response in the larvae treated with BG. However, the influence of β-1,3/1,6-glucan on bacterial communities and stress and immune responses wanes shortly after treatment cessation. The positive impacts on microbial and immune modulation do not correspond to larval growth and survival, suggesting the presence of other determinants influencing these factors. In conclusion, treatment of European eel larvae with β-1,3/1,6-glucan added to rearing water can serve as a prophylactic measure to consider during the rearing of pre-feeding larvae.

12 Conclusions and future perspectives

Until now, progress in research has made it possible to successfully reproduce European eel in captivity, producing viable offspring that reach the feeding stage. Nevertheless, challenges arise from low offspring survival rates and anticipated suboptimal growth during the initial stages of their development. These challenges impede the advancement of establishing the larval culture and developing hatchery protocols focused on achieving self-sustaining production of this commercially targeted but “Critically Endangered” fish species. Among the different factors behind high mortality and slow growth, bacterial interference and underdeveloped larval immune system were the main targets of this PhD project. Findings of the different studies of this PhD project significantly contributed to filling gaps in knowledge related to the succession of bacterial communities from eggs to different developmental stages until the preleptocephalus larvae, changes in the bacterial communities in response to different hatchery related paraments such as egg stocking density, different first-feeding diets, food amount, and β-glucan treatment. Furthermore, insights into the ontogeny of the immune system in response to those parameters were disclosed at the molecular level. Through the exploration of the convergence between analysis of bacterial community structure, molecular immune responses, and hatchery technology, the studies together have advanced our comprehension of bacterial interference within the culture of early developmental stages and shed light on the functioning of molecular immune mechanisms in these formative periods of European eel. These results have contributed to the progression of the European eel offspring rearing protocols.

A significant maternal influence on the bacterial communities of the pristine eggs was observed, revealing a distinct bacteriome for each family, generally with a high evenness in the bacterial communities. This unique "natural" bacteriome likely plays a safeguarding role against the establishment of harmful bacteria. However, specific operational factors related to the offspring culture contribute to a dysbiosis in the commensal microbiota, resulting in a bacterial community where opportunistic bacteria prevail, thus, generating an unfavourable r-selected bacterial setting for the offspring. This transition towards a hostile microbial environment, typically dominated by ASVs of orders Vibrionales, Oceanospirillales, Flavobacteriales, and Alteromonadales is generally linked to increased mortality rates and/or cellular stress responses. For example, the findings from Study 1 revealed that most mortalities (approximately 75%) occurred during the embryonic incubation stage rather than the larval rearing phase. Additionally, higher initial stocking densities that exceeded 500 eggs/L resulted in high mortalities. During both the embryonic incubation period and at the highest stocking density, the microbial communities showed an increased abundance of potentially harmful microorganisms. This suggested a potential link between these mortalities and unfavourable interactions between the offspring and bacteria. Likewise, the outcomes of Study 2 indicated a significant decline in larval survival close to the end of the first feeding window. This decline was attributed to harmful interactions between the larvae and bacteria, as well as the point of no return being reached by larvae that were unsuccessful in their first feeding attempts. Moreover, evident activation of larval stress and repair mechanisms in response to potentially harmful bacterial communities was observed through the upregulation of the *hsp90* gene. Similarly, in Study 4, the control group exhibited an upregulation of the *hsp90* gene in response to the presence of potentially harmful bacteria such as *Vibrio* spp. In addition to its response to hostile bacteriomes, the upregulation of *hsp90* seems to be linked to the unsatisfied nutritional needs of the larvae. This connection became apparent in both the findings of Study 2, where an upregulation of this gene was observed near the end of the first feeding window, and in the results of Study 3. In Study 3, this upregulation of this gene occurred towards the end of the experiment (25 and 30 dph), coinciding with a halt in larval growth.

Hence, our discoveries underscore the necessity of implementing bacterial community management strategies at various stages of the hatchery processes (e.g., embryonic rearing, pre-feeding, and feeding larval culture) to prevent the dominance of r-selected bacterial communities and associated mortalities. As exemplified in Study 4, β-1,3/1,6-glucan serves as an effective prophylactic measure to mitigate the presence of potentially harmful bacteria (e.g., *Vibrio* spp.), particularly within the bacterial communities of the rearing water. Additional potential approaches for achieving this goal encompass the utilisation of prebiotics and probiotics. The outcomes of Study 3 led us to deduce that dietarily incorporated whey protein can function as a prebiotic agent, steering the larval bacteriome towards favourable bacterial taxa (e.g., *Lactobacillus* sp.), ultimately benefiting larval growth. Notably, in this study, the presence of ASVs belonging to the genus *Bacteriovorax* correlated with a reduced occurrence of ASVs associated with potentially harmful bacterial genera (such as *Vibrio*). Consequently, the genus *Bacteriovorax* emerges as a viable candidate for isolating potential probiotic strains intended for the first feeding larval culture of the European eel. Furthermore, delving into the nature of the relationship between members of the genus Bacteriovorax and potentially detrimental bacterial genera (e.g., competition, predation, etc.) could yield valuable insights.

The immune system serves a crucial role in safeguarding the host from harmful bacteria. Conclusions drawn from Study 1 suggest that while European eel offspring possess certain components of both, innate and adaptive immune systems in their early embryonic stages, the molecular immune systems of the embryos and newly hatched larvae do not appear to be fully developed to handle a challenging microbial environment in captivity. This absence of a molecular immune response towards shifts in unhealthy bacterial communities might stem from an evolutionary adaptation to the oligotrophic environment they naturally inhabit at this stage, thereby exacerbating the challenges encountered during hatchery production. However, at later developmental phases, such as yolk sac larvae and feeding larval stages, adjustments to rearing techniques can enhance the immuno-competence of the larvae. For instance, as illustrated in Study 2, introducing whey protein (at 10%) into the larval diet enhanced the immune capability of the larvae, leading to improved survival rates after the first feeding window. Consequently, immuno-stimulation emerges as a viable strategy to address the underdeveloped immune system issue in European eel offspring. Whey protein inclusion in larval diets seems promising as an immunostimulant, though optimal inclusion levels need to be determined. Similarly, β-1,3/1,6-glucan displays promising potential as an immuno-modulator for yolk sac larvae, but further research is needed to fine-tune exposure protocols in terms of dosage and timing. The immuno-stimulation potential of β-glucan could also be explored for innate immune training in broodstock fish or gametes (eggs), thereby advancing the development and maturation of the offspring's immune system. Furthermore, assessing the effects of dietary inclusion of β-1,3/1,6-glucan or other products on enhancing the immunity of feeding larvae could prove valuable. Moreover, a crucial discovery from Study 4, namely the capacity of β-glucan to diminish the occurrence of larval deformities, warrants in-depth investigation to gain insights into the underlying molecular mechanisms.

Highlighting the impact of external bacterial sources (such as rearing water and feed) and selective pressures within the host (host preferences) and the rearing system (DOM concentration), we observed a distinct stage-specific pattern in the succession of bacterial communities from eggs to feeding larvae. The initial bacterial community of the eggs was typically marked by a minor number of bacterial taxa that exhibited uniform abundance. As the influence of the rearing water became apparent, there was an upward trend in ASV richness from embryos to newly hatched larvae. Throughout this phase, evenness maintained a lower level compared to the initial egg stage, signifying a selection process likely influenced by the elevated availability of

DOM in the rearing environment. Subsequently, the introduction of exogenous feeding led to an increase in ASV richness within the larval bacterial community compared to the pre-feeding larval stage. This increase is likely attributed to the colonisation of larvae by bacteria associated with the food. During the exogenous feeding period, evenness in the larval bacterial community tended to decrease or remain relatively stable. While the bacterial composition of the offspring was influenced by the bacterial content of the rearing water and the provided food, the bacterial communities of the offspring consistently differed from those of their environment (rearing water) and their food. This discrepancy suggests the presence of host-associated selective pressures, possibly involving host preferences that shape the selection of species from the available pool within their rearing environment. It is noteworthy considering that, unlike the norm for many other marine fish larvae that consume live feed organisms, the influence of liquid food on the eel larvae's bacterial community composition was substantial. This observation was likely attributed to the unorthodox feeding regimen adopted during captive rearing of eel larvae, involving the addition of formulated slurry-type (liquid) diets to the tank bottom, prompting larvae to submerge themselves into the food during feeding sessions. Nonetheless, our findings demonstrated that while the microbial management tools effectively guided the bacteriome towards a healthier state, the advantages did not always translate into improved larval survival. This suggests the presence of additional factors influencing early-life developmental competence. Consequently, there is a compelling need for further research to delve into these factors. Examples of areas deserving attention include the rearing and feeding conditions of broodstock, genetic (in)compatibility dynamics of gametes, the influence of offspring rearing techniques such as tank designs, and the critical aspect of larval nutrition.

Overall, the findings of the present PhD have gone a significant way towards enhancing our understanding of microbial interference and immune system development at the molecular level as well as the association of these two aspects to the mortalities observed during the early hatchery phase of the European eel. It is important to highlight the necessity of implementing microbial community management strategies during the cultivation of early life cycle stages to avoid r-selection. During the egg incubation phase, the use of RAS instead of the flow-through system can be explored as a potential tool for managing microbial communities. Furthermore, during the rearing of pre-feeding and feeding larvae, it is advisable to consider the integration of bacterial community management methods, such as probiotics, prebiotics, or synbiotics. To enhance larval immunity, it is recommended to explore immuno-stimulation techniques and incorporate immunity transferred from parental sources. While continuing further research in the fields covered by this PhD project, further advancement of research targeting the refinement of assisted reproduction protocols targeting quality improvement of offspring, rearing environment (e.g., tank design, flow dynamics), larval diets and feeding regimes are essential to close the loop for aquaculture for this commercially important yet critically endangered species.

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Manuscripts

Study 1:

Effects of European eel egg and larval stocking density on rearing water and offspring bacteriome and derived immune response

Kasun A. Bandara, Sebastian N. Politis, Daniela Eliana Sganga, Sune Riis Sørensen, Paraskevas Koumpiadis, Jonna Tomkiewicz, Olav Vadstein

Manuscript

Effects of European eel egg and larval stocking density on rearing water and offspring bacteriome and derived immune response

Authors:

Kasun A. Bandara[1,2]†, Sebastian N. Politis[1]†*, Daniela Eliana Sganga[1], Sune Riis Sørensen[1], Paraskevas Koumpiadis[1], Jonna Tomkiewicz[1], Olav Vadstein[2]

Affiliation:

[1]Technical University of Denmark, National Institute of Aquatic Resources, 2800 Kgs. Lyngby, Denmark

[2]Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, 7491 Trondheim, Norway

* Corresponding author

† Shared 1st authorship

Abstract

If human exploitation of the "critically endangered" European eel (*Anguilla anguilla*) is to continue, the eel's life cycle in captivity must be closed and techniques established to produce viable offspring in hatcheries. Despite stable offspring production, high mortality rates during early developmental stages have hindered progress in closing the lifecycle in captivity. We hypothesised that mortalities affecting embryos and newly hatched larvae, when the immune system is expected to play a vital role in protecting the offspring, could be linked to adverse microbial conditions arising in high-density rearing. We therefore set out to explore both the bacteriome changes in rearing water and offspring and the molecular immunologic development of offspring at different initial stocking densities (500, 1000, 2000, and 4000 eggs/L) from fertilisation to 3 days post-hatch (dph). The lowest density resulted in ~5% higher survival. Independent of density, most mortality (~75%) occurred during embryonic rather than larval development. The bacteriomes of both offspring and rearing water differed according to stocking density, with a potentially healthier bacteriome at the lowest density, marked by greater ASV richness and abundance from various phyla (Proteobacteria, Actinobacteria, Fusobacteria, and Firmicutes). In contrast, the bacteriome of the highest density was dominated by Proteobacteria ASVs, including the potentially harmful Vibrionales order. We observed stage-specific bacteriome changes in offspring, transitioning from an inherent and balanced bacteriome in unadulterated eggs to a dominance of rapid-growing opportunistic bacteria in embryos (48 hpf), and finally to a more diverse bacterial community in larvae (3 dph). Thus, host-microbe interactions appear to significantly impact the overall high mortality observed during embryonic development. As the expression of stress and immune related genes was not affected by density, it seems that the molecular immune system was incapable of handling microbial interference during these early stages, aggravating the challenges faced during hatchery production. We conclude that low-density (500 eggs/L) incubation is a valid strategy to improve survival. However, high stocking densities (up to 4000 eggs/L) might be an acceptable compromise allowing more efficient utilisation of infrastructure and labour. At 4000 eggs/L, survival was reduced by only ~5%, while there was no effect on hatching success and no cellular stress response. Nevertheless, bacterial community management tools should be in place during high-density incubation to avoid r-selection and thus counter bacteria-associated mortality.

Keywords: *Anguilla anguilla*, hatchery technology, early life history, microbial interference, immune gene expression.

1 Introduction

The European eel (*Anguilla anguilla*) is a high-value species for commercial aquaculture in Europe, where wild-caught glass eels (transparent juveniles) are stocked in grow-out facilities to be cultured for human consumption or restocking purposes (FAO, 2023). However, the species has been categorised as "critically endangered" with the natural population at a historical low (Pike et al., 2020). If eels are to remain a human food source, the lifecycle in captivity must be closed and healthy offspring produced, supporting in parallel sustainable aquaculture development, restocking measures, and conservation management. To this end, scientific inquiry has increasingly focused on closing the life cycle in captivity by developing hatchery techniques and technology (Mordenti et al., 2019; Tomkiewicz et al., 2019). Besides giving attention to assisted reproduction protocols and applying hormone therapy to the parent animals for the stable production of viable gametes (Benini et al., 2022; Kottmann et al., 2020b), research has focused on the development of culture methodology for offspring (Benini et al., 2023; Politis et al., 2021a). Nonetheless, offspring mortality remains a major challenge given the high sensitivity and susceptibility to ambient conditions of the European eel during its early life stages (Tomkiewicz et al., 2019).

In fish culture, early mortality and reduced offspring performance have often been associated with an uncontrolled succession of microbial communities and negative microbial interference, resulting in unfavourable water quality conditions and impacting the viability and quality of eggs, embryos and larvae (Olafsen, 2001; Vadstein et al., 2004). Moreover, the stocking density of eggs plays a pivotal role in determining the supply of dissolved organic matter (DOM), which in turn supports bacterial growth. This, in essence, shapes the dynamics of bacterial interactions within the community. High DOM availability favours the growth of fast-growing (thus r-selected) and opportunistic bacteria, leading to dysbiosis in the bacterial community (Vadstein et al., 2018). For example, as high-density incubation supports the growth of bacteria (Olafsen, 2001), bacterial colonisation can affect the relationship between indigenous microflora and opportunistic pathogens, potentially hampering offspring development and competence (Hansen and Olafsen, 1989). In some cases, interactions between microorganisms and offspring (eggs and larvae) have been associated with reduction in hatch success and post-hatch survival, with premature or delayed hatching (attributed to toxins secreted by certain bacterial colonies), and even with lethal effects arising from the physical prevention of chorion gas exchange (Hansen and Olafsen, 1999; Olafsen, 2001).

A simple strategy to combat water quality degradation during offspring rearing is to reduce initial stocking densities (Tucker, 1998), which reduces biological oxygen demand and waste production. Relatively low stocking densities reported for marine fishes range from 50 eggs/L for

California yellowtail, *Seriola lalandi* (Stuart and Drawbridge, 2013) to 500 eggs/L for cobia, *Rachycentron canadum* (Benetti et al., 2008). However, it has been found that in marine species such as North American burbot, *Lota lota maculosa* (Jensen et al., 2008) or striped bass, *Morone saxatilis* (Harper et al., 2010), stocking densities as high as ~10.000 eggs/L do not compromise survival and hatching success, provided biofilters or high water exchange rates are deployed. Similarly, the importance of microbial management during egg incubation and larval culture has previously been acknowledged as a counter to the negative impact of microbial interference on hatching success and larval longevity during the embryonic development of European eel (Sørensen et al., 2014).

Moreover, the natural spawning site and initial offspring habitat of European eels are believed to be in the Sargasso Sea, which is an oligotrophic environment with relatively low bacterial densities of app. 4.6–8.8 × 10^5 colony-forming units (CFU)/mL (Rowe et al., 2012). Consequently, their early life history stages may be challenged by the microbial environment experienced during high-density incubation and larval culture. In this context, the immune system assumes a crucial role in safeguarding the offspring from potentially harmful bacteria. Recent studies have elucidated the presence of maternally transferred immunity and the activation of own molecular immune components in the early development stages of European eel, but also the immature state of the immune system in the yolk sac larvae (Kottmann et al., 2020a; Miest et al., 2019). The management and optimisation of the rearing conditions of European eel would benefit from further insight into immune system ontogeny and its molecular response to changes in the microbial environment related to offspring density during incubation and larval culture.

In this context, the present study explores the succession of rearing water and offspring bacteriomes and examines the molecular ontogeny of the immune system in response to different initial stocking densities. The experiment was based on protocols previously developed for European eel offspring culture, where eggs fertilised in vitro (Butts et al., 2014) are stocked in incubators at 2 hours post-fertilisation (hpf). From fertilisation to larval hatch, embryonic development lasts ~ 48-56 hours at 18-20°C. The hatched larvae are little developed and have a prominent yolk sac (Sørensen et al., 2016b). Subsequently, early larval development is marked by a relatively short phase of yolk and oil absorption, spanning approximately 12 days post-hatch (dph) at 20°C (Sørensen et al., 2016b). In the present study, we primarily investigated how initial stocking density affects eggs, embryos, and larvae from fertilisation to 3 dph.

2 Materials and methods

2.1 Ethics statement

All fish were handled in accordance with the directives of the European Union on protecting animals used for scientific purposes (Dir 2010/63/EU). Experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit-Nr.: 2020-15-0201-00768). Efforts were made to minimise animal handling and stress. Adult fish were anaesthetised by submergence in an aqueous solution of benzocaine (saturated solution of ethyl p-aminobenzoate in ethanol, Sigma-Aldrich, Germany) at a concentration of 20 mg/L prior to initial morphometric measurements and PIT-tagging, ovarian biopsies, and strip spawning. Females were euthanised after stripping and males at the end of the experiment by prolonged anesthesia. The larvae used for this experiment were euthanised prior to sampling using tricaine methanesulfonate (MS-222, Sigma Aldrich, Germany) at a concentration of 15 mg/L.

2.2 Broodstock origin and rearing conditions

Wild-caught females from Saltbæk Vig (Jutland, Denmark) and farm-raised males obtained from a commercial Danish eel farm (Royal Danish Fish A/S) were transported to EEL-HATCH, an experimental hatchery of DTU Aqua located in Hirtshals, Denmark. Upon arrival, female eels were randomly distributed at a density of 13 fish per tank (8.57 ± 0.82 g/L) in black circular tanks (vol. 1150 L) connected to a recirculating aquaculture system (RAS). Male eels were held at a density of 25 fish per tank (7.22 ± 0.30 g/L) in similar black circular tanks (vol. 500 L) connected to a RAS. During the 2-3week acclimatisation period, the temperature was gradually adjusted to ~20°C and salinity to 36 practical salinity units (PSU) using North Sea water and artificial sea salt (Aquaforest aquaculture salt, Brzesko, Poland). The broodstock was reared under a 12 h light/12 h dark photoperiod with a 30 min graduated transition in the morning and evening to resemble natural conditions. To simulate the fasting period from the onset of the pre-pubertal silvering stage in nature, they were not fed during the artificial maturation period. Subsequently, in preparation for assisted reproduction, each eel was anaesthetised and tagged with a passive integrated transponder (PIT tag) in the dorsal muscle to distinguish its identity. The mean (± SD) initial standard length and body weight of the female eels (n = 5) used in this experiment were 66 ± 6 cm and 550 ± 100 g, respectively, and, in the case of males (n = 19), 43 ± 4 cm and 136 ± 9 g, respectively.

2.3 Assisted reproduction

Vitellogenesis in females was induced by weekly intramuscular injections of salmon pituitary extract (SPE; Argent Aquaculture LLC, Washington, USA) at 25 mg/kg of initial body weight (IBW) for 11–21 weeks. With appropriate body weight increase and with oocyte developmental

stage assessed by ovarian biopsies, females received an additional SPE injection as a priming dose. Subsequently, ~24h after the priming injection, females were injected intraperitoneally with a maturation-inducing steroid (MIS; 17α,20ß-dihydroxy-4-pregnen-3-one (DHP), Toronto Research Chemicals, Canada) at 2 mg/kg current body weight to induce follicular maturation and ovulation (Kottmann et al., 2020b; Ohta et al., 1997; Palstra et al., 2005). Ovulation occurred ~14-16 h after the MIS and eggs were collected on a sterile tray by applying pressure to the abdomen. Males received weekly injections of human chorionic gonadotropin (hCG; Sigma-Aldrich, Germany) at 1.5 IU/g IBW (Asturiano et al., 2006), and were then given an additional hCG injection ~12 h before they were strip spawned (Koumpiadis et al., 2021). Milt from 3-5 males was collected and kept in an immobilising medium until it was used for fertilisation within four hours after stripping (Butts et al., 2014). Only males with sperm motility of 75–90% were used for fertilisation (Asturiano et al., 2006; Butts et al., 2014).

Strip-spawned eggs were gently mixed with diluted milt according to a standardised sperm-to-egg ratio (Butts et al., 2014; Sørensen et al., 2016b). For fertilisation, reverse osmosis water was adjusted to 20°C and 39.9 PSU using Reef Salt (Aquaforest, Brzesko, Poland) and added to the gamete mix. After five minutes of gamete contact, eggs/embryos were transferred to 20 L buckets filled with ~15 L of the adjusted reverse osmosis water at 36 PSU to separate the buoyant viable eggs/embryos from the sinking unviable eggs (Politis et al., 2021b; Sørensen et al., 2016a). After 60 min, the floating layer of eggs/embryos was transferred to a second bucket for a further 60 min. Only the floating eggs/embryos from this second bucket were used for further experimentation.

2.4 Experimental setup

2.4.1 Egg incubation and larval rearing

Repeated five times using offspring from five different parental cross batches within the same spawning season (2020) at the EEL-HATCH facility, this experiment was designed to study the effect of four incubation densities: 500, 1000, 2000 and 4000 eggs/L (Figure 1). Replicated (n = 3) 2 L customised acrylic jars were randomly allocated for each treatment (n = 4) to incubate the eggs until hatching (Figure 1A). At 2 hpf, the floating layer of eggs/embryos was collected in a 1 L beaker, and density was determined by counting 3 × 0.1 mL subsamples. The required volumes were then distributed in each jar. Filtered and UV-treated North Sea water was adjusted to 36.3 ± 0.4 PSU with Reef-salt (Aquaforest Reef Salt, Brzesko, Poland) and supplied to each jar from a bottom inlet at a rate of 1.2 ± 0.1 mL/s. Water flowed out through a 250 µm mesh subsurface closed strainer (Politis et al., 2023) and was led to the drain on exiting the rearing jar. The water in each jar was gently aerated with filtered air (0.2 µm Ministart, Sartorius Stedium Biotech GmbH, Germany). For embryonic development, light was kept at a low intensity of ~10 lx (Politis

et al., 2014) and temperature at 19.2 ± 0.5°C. At 48 hpf, aeration was stopped, and the water flow was reduced to prevent mesh clogging and mechanical damage to newly hatched larvae. The larvae hatched at ~60 hpf and were transferred from each incubation jar to acrylic 8 L Kreisel tanks (Figure 1B), where they were reared until 3 dph. Each Kreisel tank was connected to a RAS and supplied with water at a flow rate of 1.5 ± 0.1 mL/s. Water temperature and salinity were maintained at 19.8 ± 0.1°C and 36.1 ± 0.3 PSU, respectively. Dead larvae were removed and counted every day to quantify survival. Yolk-sac larvae were not fed during the experiment, as this period corresponded to the endogenous feeding phase.

2.5 Sampling and analytical methods

2.5.1 *Sampling*

Eel offspring samples were taken from each replicate (n = 3), parental cross (n = 5) and density treatment (n = 4) for different analyses, at different stages. For gene expression analysis, ~30 eggs/embryos/larvae were randomly sampled at 0 hpf, 48 hpf, hatch (0 dph) and 3 dph. Sampled larvae were immediately euthanised through an anaesthetic overdose, preserved in RNAlater (Sigma-Aldrich, Germany) and stored at -20°C for subsequent analysis (Politis et al., 2017). Bacterial community composition analysis was carried out only for the offspring and water samples collected from the lowest (500 eggs/L) and highest (4000 eggs/L) density treatments. To characterise the bacterial community, ~10 eggs/embryos/larvae were sampled at 0 hpf, 48 hpf, hatch (0 dph) and 3 dph. As above, sampled larvae were immediately euthanised. All samples were stored at -20°C for later analysis. To investigate whether the rearing water sourced the bacteria for the bacterial community associated with embryos and larvae, samples (n = 3) were collected from water flowing into the incubation jars for each repeated experiment (n = 5). Collection occurred before the eggs/embryos were stocked, but also during culture from each replicate (n = 3) and parental cross (n = 5) at 48 hpf and 3 dph. In this process, 250 mL of water from each sample was vacuum filtered through 0.22 µm white gridded filters (diameter = 47 mm; Merck KGaA, Germany) using a Büchner funnel. Filters were then stored in sterile cryo-tubes at -20°C until processing (Bakke et al., 2013).

2.5.2 *Survival*

To determine rates of embryonic survival for each jar, dead eggs/embryos were counted at 24 and 48 hpf if they had settled to the bottom of each jar within 5 minutes after water flow and aeration had ceased. Larval survival was monitored daily through assessment of mortality, i.e., counting and removing dead larvae from all experimental units. Additionally, at the end of the experiment (3 dph), all larvae including those sampled from each experimental unit were enumerated and recorded. The survival percentage was then calculated for each day as a percentage of the total number of fertilised eggs stocked initially.

2.5.3 Bacterial community characterisation

DNA from the eggs/embryos, larvae and water was isolated using the MagAttract PowerSoil Pro DNA Kit (QIAGEN, Germany) and in accordance with the protocol developed by the supplier for automated high-throughput isolation of DNA with the KingFisher Flex platform (Thermo Fisher Scientific). Samples (pools of ~10 eggs/embryos, larvae, or filter papers) were homogenised in bead-beating tubes containing ~ 0.55 g of 0.1 mm glass beads (Bertin Technologies, France) and 800 µL of lysis buffer, using a Precellys 24 tissue homogeniser (Bertin Technologies, France). The homogeniser ran twice at 5500 rpm for 30 s with a 15 s break in between. The tubes containing the lysates were centrifuged at 15000 × g for 1 min, and the supernatants were transferred into 1.5 mL Eppendorf tubes. Then, 300 µL of CD2 solution was added to each Eppendorf tube, vortexed to mix, and centrifuged at 15000 × g for 1 min. Prepared lysates (i.e., supernatants from the previous step) were transferred to the KingFisher Flex platform (Thermo Fisher Scientific), where total genomic DNA was captured on specialised magnetic beads in the presence of buffers before being washed on the beads and then eluted.

Subsequently, the V3 and V4 regions of the bacterial 16S rRNA gene were amplified from the DNA isolates using the forward primer Ill-341F_K1: 5′- NNNNCCTACGGGNGGCWGCAG -3′ and the reverse primer Ill805R: 5′- NNNNGACTACNVGGGTATCTAAKCC -3′ (Klindworth et al., 2013). Each PCR reaction contained 0.02 U/µL Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific), 0.2 mM of each dNTP (VWR), 0.3 µM of each primer (SIGMA), 1x Phusion HF buffer (containing 7.5 mM $MgCl_2$) (Thermo Fisher Scientific) and PCR grade water (VWR) up to a total reaction volume of 25 µL and 1 µL of DNA extract as template. The PCR reactions were run with 35 cycles (T100™ Thermal Cycler, Bio-Rad Laboratories, CA, USA), following a previously described protocol (Bugten et al., 2022). The PCR amplicons were purified and normalised using SequalPrep Normalisation Plate (96) kit (Invitrogen, USA), following the protocol provided by the supplier. Using the Nextera®XT DNA Sample Preparation Kit (Illumina), a unique pair of index sequences that represented the PCR amplicons originating from each sample was added by an additional PCR step with 10 cycles. The indexed PCR products were purified and normalised using the SequalPrep Normalisation Plate (96) Kit (Invitrogen, USA). Finally, the samples were pooled and concentrated with AmiconUltra 5.0 Centrifugal Filters (Merck Millipore, Ireland) following the manufacturer's protocol. The amplicon library was sequenced in a MiSeq run (Illumina, CA, USA) with V4 reagents (Illumina) at the Norwegian Sequencing Centre (NSC), University of Oslo.

The Illumina sequencing data were processed using USEARCH utility (version 11) (https://www.drive5.com/usearch/). The command "Fastq_mergepairs" was used to merge the paired reads, trim off primer sequences and filter out reads shorter than 380 base pairs. The

"Fastq_filter" command (with an expected error threshold of 1) was used for further processing, which included demultiplexing, removal of singleton reads, and quality trimming. The “unoise3” command was used for chimera removal and for the generation of amplicon sequence variants (ASVs) (https://drive5.com/usearch/manual/cmd_unoise3.html), while taxonomy was assigned by applying the “SINTAX” script (Edgar, 2016) with a confidence value threshold of 0.8 against the RDP reference data set (v. 18). Before analysis of the data, ASVs representing eukaryotic amplicons (e.g., algae, fish DNA), Archaea and Cyanobacteria/Chloroplast were removed from the ASV table, as were ASVs with a total abundance less than 8.

2.5.4 *Gene expression analysis*

Total RNA from samples was extracted using the NucleoSpin (Mini) RNA isolation kit, following the protocol provided by the supplier (Macherey-Nagel GmbH & Co. KG, Germany). The extracted RNA concentration and purity were determined by spectrophotometry using NanoDrop™ One (Thermo Fisher Scientific), and RNA integrity was assessed by agarose gel electrophoresis. 600 ng of the resulting total RNA was transcribed using the qScript™ cDNA Synthesis Kit (Quantabio, Germany) according to the manufacturer’s instructions.

Expression levels of 14 genes (2 reference and 12 targeted) were determined by quantitative real-time PCR (RT-qPCR) using specific primers (Table 1). Primers were designed using primer 3 software v 0.4.0 (http://frodo.wi.mit.edu/primer3/) and on the basis of cDNA sequences available in GenBank databases. All primers were designed for an amplification size ranging from 75 to 200 nucleotides and an optimal Tm of 60°C. Transcribed RNA (cDNA) from two randomly selected replicate samples from each density treatment (n = 4), parental cross (n = 5) and sampling point (n = 4) was analysed with three technical replicates for each target gene, using the qPCR Biomark HD technology (Fluidigm) based on dynamic arrays (GE chips). A pre-amplification step was performed with a 500 nM primer pool of all primers using the TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.3 µL cDNA per sample for 10 min at 95°C and then 14 cycles of 15 s at 95°C and 4 min at 60°C. The PCR products obtained were diluted 1:10 with low EDTA-TE buffer. The pre-amplified products were loaded onto the chip, using SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, CA, USA) and DNA-Binding Dye Sample Loading Reagent (Fluidigm). Primers were loaded onto the chip at a concentration of 50 µM, and the chip was run according to the Fluidigm protocol with a Tm of 60°C. The relative quantity of target gene transcripts was normalised (ΔCT) to the geometric mean of the two most stable reference genes. Having previously been demonstrated to be the most reliable reference genes for fish larvae (McCurley and Callard, 2008), *ef1* and *rps18* were chosen as the reference genes. The coefficient of variation (CV) of technical replicates was calculated and checked. Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6 Statistical analysis

2.6.1 Survival, hatching success and expression of stress and immune related genes

Statistical analyses were performed in R (version 4.1.2; R Core Team, 2021) with alpha set at 0.05 for testing main effects and interactions. Data were analysed using generalised mixed-effects models, where the main model variables were stage and stocking density, but families were considered random. The model tested included an interaction effect between stocking density and stage whenever applicable. Post-hoc analyses were performed using the "emmeans" package for R (Lenth et al., 2022).

2.6.2 Measures of microbial diversity

Beta-diversity analyses were performed on an ASV table filtered to remove any ASVs that had less than two counts in at least two samples and rarefied at 11503 reads per sample (the choice of threshold was based on the sample with the lowest number of reads). Ordination by principal coordinate analysis (PCoA) based on Bray–Curtis (Bray and Curtis, 1957) and Sørensen–Dice dissimilarities was used to visualise differences in microbial community composition between different samples using the plot_ordination function within the phyloseq package (version 1.40.0). Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) based on the Bray-Curtis and Sørensen–Dice dissimilarity matrices was used to test for changes in community composition (beta diversity) as a function of stage and stocking density, and pairwise differences were tested using "pairwise.adonis2" in the vegan package (version 2.6.2). Alpha-diversity measures including ASV richness and evenness were calculated using the "vegan" community ecology package (version 2.6.2) developed for R statistical software, and they were then analysed as mentioned in section 2.6.1. The DESeq2 package (version 1.36.0) was used on the unrarefied ASV table to assess the differential abundances of ASVs between the samples that were found to be significantly different by PERMANOVA. DESeq2 includes a model based on the negative binomial distribution and a Wald's post hoc test for significance testing. The p values were adjusted using the Benjamin and Hochberg method (Benjamini and Hochberg, 1995) to deal with multiple comparisons.

3 Results

3.1 Offspring survival and hatching success

Embryonic and larval survival was affected significantly both by stage and by stocking density (Figure 2 A-B). However, no significant stage × stocking density interaction was detected. Over time, survival significantly decreased, but the largest drop was observed during the embryonic phase, reaching a mean (± SD) survival of 26.2 ± 4.7 % at 48 hpf. Later, larval survival decreased ($p < 0.0001$) from hatch to reach 13.9 ± 2.4 % on 3 dph. As is evident in Figure 2 B, survival was

highest ($p < 0.01$) at the lowest stocking density (500 eggs/L), but as Figure 2 C, shows, hatching success did not differ among the different stocking densities.

3.2 Bacterial community structure

3.2.1 Alpha diversity

Bacterial community composition analysis was conducted only for the samples collected from the lowest and highest stocking densities (500 eggs/L and 4000 eggs/L, respectively). A comparison of the Chao1 (estimated number of ASVs) with the observed ASV richness revealed that the sequencing depth covered approximately 83 and 88 % of the estimated total richness for water and larval samples, respectively.

In the bacterial community of the rearing water, ASV richness was significantly affected by both stage and stocking density, while no significant stage × stocking density interaction was detected. A significant increase in richness ($p < 0.05$) was observed from 0 hpf to 3 dph (Figure 3 A), while ASV richness in rearing water at the lowest stocking density was higher ($p < 0.05$) than at the highest stocking density (Figure 3 B). The evenness of ASVs significantly decreased from 0 to 48 hpf, while the evenness of ASVs in rearing water at 3 dph, was comparable to that at 0 and 48 hpf (Figure 3 C). On the other hand, the evenness of ASVs in rearing water was not affected by stocking density (Figure 3 D).

In offspring, on the other hand, a significant stage × stocking density interaction was found for ASV richness, but not for evenness. The lowest ($p < 0.05$) ASV richness across all developmental stages was found in eggs at 0 hpf (Figures 4 A, B). At the lowest stocking density, ASV richness increased ($p < 0.01$) with age (Figure 4 A), while at the highest stocking density, ASV richness increased ($p < 0.05$) from 0 to 48 hpf and remained stable until 3 dph (Figure 4 B). At 3 dph, ASV richness in larvae was higher ($p < 0.001$) in the lowest stocking density than in the highest stocking density (Figure 4 C). Moreover, a more even bacterial community was detected in the initial eggs than in the other developmental stages (Figure 4 D), while no effect on the evenness of bacterial communities was detected in relation to stocking densities (Figure 4 E).

When the bacterial communities of water and offspring were compared, both ASV richness (Figure 5 A) and evenness (Figure 5 F) were higher ($p < 0.05$) in the unadulterated inflowing water than in the initial eggs (0 hpf). Regardless of the stocking density, ASV richness was also higher ($p < 0.001$) in rearing water than in embryos at 48 hpf (Figures 5 B, C) and larvae at 3 dph (Figures 5 D, E). On the other hand, evenness of the bacterial community in the rearing water was comparable to that of embryos (at 48 hpf) and larvae (at 3 dph) at the lowest stocking density (Figures 5 G, I), whereas it was higher ($p < 0.05$) in rearing water than in embryos (at 48 hpf) and larvae (at 3 dph) in the highest stocking density (Figures 5 H, J).

3.2.2 Beta diversity

For comparison of bacterial communities of European eel offspring and water between the lowest and highest stocking densities and between European eel offspring and water in highest and lowest stocking densities at each developmental stage, PERMANOVA tests were performed based on Bray–Curtis and Sørensen–Dice dissimilarities. For a complete overview, see Table 2.

When PCoA plots based on Bray–Curtis and Sørensen–Dice dissimilarities were used to evaluate β-diversity in each sample (Figure 6), it was shown that the bacterial communities of the unadulterated inflowing water differed from those of the initial egg samples (Figures 6 A, B). Overall, the bacterial communities of the rearing water were different from those of embryos or larvae regardless of stocking density (lowest and highest) and stage (48 hpf and 3 dph). At 48 hpf, the two stocking densities investigated yielded different bacterial communities in embryos and rearing water (Figures 6 C, D), and differences in bacterial communities between the lowest and highest stocking densities were still visible in the newly hatched larvae at 0 dph (Figures 6 E, F). At 3 dph, however, larval bacterial communities at the lowest and highest stocking densities no longer differed (Figures 6 G, H).

To compare the bacterial communities of European eel offspring or water across relevant developmental stages at each stocking density treatment, PERMANOVA tests were performed based on Bray–Curtis and Sørensen–Dice dissimilarities (Table 3). For both the lowest and highest stocking densities, these tests revealed that the bacterial community in the initial eggs significantly differed from that in all other developmental stages (Table 3). For the lowest stocking density, the bacterial community in the embryos at 48 hpf was different from that in the newly hatched larvae (at 0 dph) according to Bray–Curtis analysis, but not according to Sørensen–Dice indices. However, both indices showed that for the highest stocking density, bacterial communities in embryos at 48 hpf and newly hatched larvae (0 dph) were comparable. For both the highest and lowest stocking densities, both indices revealed differences between the bacterial communities of embryos at 48 hpf and larvae at 3 dph as well as between newly hatched larvae (0 dph) and 3 dph larvae. As for the water, bacterial communities of unadulterated inflowing water (at 0 hpf) and rearing water (inside the incubators) at 48 hpf were different at both the lowest and highest stocking densities. Moreover, both indices show that the bacterial communities in incubation rearing water at 48 hpf and in the Kreisel tank rearing water at 3 dph differed at both the lowest and highest stocking densities.

3.2.3 Relative abundances at order level

The bacterial community of the initial egg samples was unique for each parental cross (Figure 7), while communities of the unadulterated inflowing water, mainly consisting of the orders of Rhodobacterales, Alteromonadales and “Unassigned”, were relatively stable for each batch

(parental cross) of eggs. Moreover, Rhodobacterales and Alteromonadales were prominent in the bacterial communities of embryos and rearing water at 48 hpf and of larvae at hatch (0 dph), regardless of the stocking density. At 3 dph, the contribution of these two orders to the bacterial communities of the larvae decreased, whereas the contribution of other orders such as Vibrionales, Oceanospirillales, and Pseudomonadales increased. At the same time, the bacterial community of the rearing water at 3 dph was mainly dominated by the orders Vibrionales, Rhodobacterales, Alteromonadales, Oceanospirillales, Pseudomonadales, Rhizobiales and "Unassigned".

3.2.4 Differential abundance Analysis

Analysis of differences in the bacterial community composition of embryos at 48 hpf (Figure 8 A) revealed that more ASVs were significantly abundant in the lowest stocking density than in the highest stocking density. These ASVs belonged to several phyla, including Proteobacteria, Actinobacteria, Fusobacteria and Firmicutes. Moreover, at the highest stocking density, all the ASVs that were significantly more abundant than those in the lowest stocking density belonged to the phylum Proteobacteria. Interestingly, six ASVs belonging to the Vibrionales order were significantly abundant in the highest stocking density, whereas only one ASV belonging to this order was significantly abundant in the lowest stocking density.

DESeq2 analysis of the rearing water revealed that bacterial communities differed between the lowest and highest stocking density treatments, mainly due to differences in the abundance of ASVs belonging to the phylum Proteobacteria (Figure 8 B). Moreover, ASVs belonging to the phyla Actinobacteria, Bacteroidetes, and Planctomycetes were more abundant in the lowest stocking density than in the highest stocking density. Strikingly, several ASVs belonging to the Vibrionales order were more abundant in the rearing water with the highest stocking density, while no significantly abundant ASVs belonging to this order were detected in the water at the lowest stocking density. Differences in the bacterial communities in newly hatched larvae (at 0 dph) at the lowest and highest stocking densities were mainly due to the significantly different abundances of the ASVs belonging to the phylum of Proteobacteria (Figure 8 C).

Comparison of rearing water and embryos at 48 hpf revealed that more ASVs were significantly abundant in the bacterial community of rearing water, regardless of the stocking density (Figures 9 A, B). Moreover, the differences between rearing water and embryos were mainly due to the significantly different abundances of ASVs belonging to the phylum of Proteobacteria at both the lowest and highest stocking densities. ASVs of the phylum Actinobacteria (Order Propionibacteriales) were abundant in embryos, regardless of the stocking density. At 3 dph, more ASVs were significantly abundant in rearing water than in larvae, regardless of the stocking density (Figure 9 C, D). Again, these differences were mainly due to the differences in the abundances of the Proteobacteria ASVs. Additionally, Bacteroidetes, Firmicutes, and

Planctomycetes ASVs were more abundant in the bacterial community of rearing water than in larvae at both the highest and lowest stocking densities. Furthermore, Propionibacteriales ASVs were more abundant in the bacterial community of embryos than in the rearing water at both the highest and lowest stocking densities.

Differences observed between initial eggs (at 0 hpf) and embryos at 48 hpf were mainly due to differential abundances of Proteobacteria ASVs, regardless of the stocking density (Figure 10 A, B). At the highest stocking density, ASVs belonging to potentially harmful orders such as Vibrionales, Rhodobacterales, Oceanospirillales and Alteromonadales were more abundant in the bacterial community of embryos at 48 hpf than in the bacterial community of initial eggs (Figure 10 B). At the lowest stocking density, similar orders contribute to the differences between initial eggs (at 0 hpf) and embryos at 48 hpf, but with a lower number of ASVs (Figure 10 A).

Moreover, in connection with the major drop in survival during the embryonic phase, the bacterial communities of embryos (at 48 hpf) and larvae (at 3 dph) were compared at the lowest and highest stocking densities. At the lowest density, a higher number of ASVs belonging to different phyla (Proteobacteria, Actinobacteria, Bacteroidetes, Chloroflexi and Planctomycetes) were detected in 3 dph larvae (117 ASVs) than in 48 hpf embryos (38 ASVs; Figure 10 C). The few ASVs that were found to be more abundant in 48 hpf embryos mainly belonged to the phylum Proteobacteria (primarily Rhodobacteriales and Alteromonadales). At the highest stocking density, 125 ASVs were more abundant in 48 hpf embryos, and 124 of these ASVs belonged to the phylum Proteobacteria (mainly in the orders Vibrionales, Rhodobacteriales, Oceanospirillales, and Alteromonadales) (Figure 10 D). Significantly more abundant ASVs belonged to different phyla in 3 dph larvae than in 48 hpf embryos.

In the comparison of embryonic incubation water and larval rearing water, all the ASVs that were more abundant in the egg incubation water at highest stocking density belonged to the phylum Proteobacteria, including ASVs of opportunistic and potentially harmful orders such as Rhodobacterales, Alteromonadales, Oceanospirillales, and Vibrionales (Figure 10 F). Similarly, at lowest stocking density, most of the ASVs that were more abundant in the egg incubation water belonged to the Proteobacteria phylum, including members of the Rhodobacterales, Alteromonadales, and Oceanospirillales orders (Figure 10 E). While ASVs of those potentially harmful orders were detected in the bacterial communities of larval rearing water, several additional ASVs belonging to other taxa were also detected at higher abundances in the bacterial communities in larval rearing water, regardless of the stocking density.

3.3 Expression of immune and stress related genes

Expression patterns of immune and stress-related genes were not affected by stocking density or the stage × stocking density interaction. However, all genes were affected by stage, except for *lysc* and *tlr18*. An interesting pattern was observed for the two complement components investigated in this study, where *c1qc* was expressed nearly at basal levels until hatch (0 dph) and was then upregulated at 3 dph (Figure 11 A), while expression levels of *c3b1* were stable at high levels until hatch and dropped on reaching 3 dph (Figure 11 B). Moreover, while the expression of anti-inflammatory cytokine *il10* decreased (Figure 11 C), the expression of pro-inflammatory cytokine *il1b* increased throughout development (Figure 11 D). A similar expression pattern was observed in the cytokines *irf3* and *irf7*, where expression levels were low until hatch and then upregulated at 3 dph (Figures 11 E, F). For the two adaptive immune genes studied, the expression of *mhc2* at hatch was downregulated (Figure 11 G) and the expression of *igm* was upregulated (Figure 11 H). Furthermore, expression of *hsp90* was highest at 48 hpf and significantly decreased ($p < 0.001$) to stable low levels after hatch (Figure 11 I). In contrast, *hsp70* was expressed at stable low levels until hatch and was significantly ($p < 0.01$) upregulated at 3 dph (Figure 11 J). Two of the genes studied (*tlr18* and *lysc*) were expressed stably throughout development (Figures 11 K, L).

4 Discussion

In the current pioneering stage of the captive production of European eel offspring, efforts to successfully complete the entire lifecycle in captivity are facing the obstacle of high mortality rates during initial developmental stages. In this study, most early mortalities took place during the incubation period of the embryos rather than in the initial stages of larval rearing. Furthermore, we observed a stage-specific shift in the composition of bacterial communities, which may elucidate the role of bacterial interference in these mortalities. Additionally, stocking density had an impact on survival rates, likely due to unfavourable interactions between offspring and bacteria. Notably, the early immune system seems to have been incapable of effectively countering these bacteria, as suggested by the absence of a density-dependent effect on the molecular immune response. In the following sections, we delve into each of these key findings and provide practical insights for selecting an optimal egg incubation density in European eel hatchery production.

Stage-specific and density-related bacterial community structure dynamics

The lowest ASV richness and the highest evenness were found in the bacterial community of the initial eggs. Here, we observed a strong maternal effect on the initial egg bacterial communities, which were unique to each parental cross. It has been suggested that this initial bacterial

community of recently spawned eggs protects the eggs by acting as a barrier against colony formation by pathogens and opportunists (Olafsen, 2001). In this process, the surfaces of fish (egg chorion, skin, digestive tract etc.) can be initially colonised by bacteria both transmitted vertically from the parent or horizontally among individuals of the same generation in a shared environment (Sylvain and Derome, 2017). However, the vertical transmission of bacteria is often interrupted in farmed fish, especially when egg disinfection protocols are applied post-fertilisation (Assefa and Abunna, 2018). While the use of antibiotics and disinfection treatments to reduce microbial coverage and activity on fertilised European eel eggs improves the hatching success and longevity of the resulting larvae (Sørensen et al., 2014), egg surface disinfection could potentially be detrimental to bacterial community composition. Eliminating the natural bacterial communities that may play a protective role on the egg surface might encourage the proliferation of potentially harmful r-selected bacteria during recolonisation, especially in high-density incubation with abundant DOM availability (Vadstein et al., 2018). In the present experiment, as eggs were not disinfected after fertilisation, both vertical and horizontal transmission of bacteria may have played an important role in the bacterial community succession of the offspring. Even though the unfertilised eggs had a unique bacterial community, the selective forces during rearing diluted the initial maternal effects over time, eventually leading to an opportunist-dominated bacterial community. It is also important to mention that age-dependent variations in susceptibility to bacterial challenges have been demonstrated (Nakase et al., 2015) for the offspring of the closely related captive-bred Japanese eel (*Anguilla japonica*). The authors suggest that as larvae mature, their resistance to bacterial challenges increases, possibly due to enhanced defensive mechanisms. Therefore, controlled bacterial colonisation of water and offspring might be crucial at the egg stage to yield a community composition capable of restraining the adhesion of harmful bacteria. This would help avoid the uncontrolled bacteria-influenced mortalities characteristic of the embryonic phase and might potentially prove important in the first-feeding stage (not addressed in this study), where more drivers (diet-offspring-environment interactions) come into play. Whereas first studies exploring gut-priming and/or pre-feeding options (Politis et al., 2023) have now established the foreground for culturing eel larval strategies, further research is needed targeting alternative tools and methods or investigating natural larval intestinal microbiota and host-microbiota interactions.

Interestingly, density-related differences in bacterial composition were most prominent at 48 hpf, coinciding with the period of highest (~75%) mortality (Figure 2), but then diminished throughout development until communities no longer differed at 3 dph (Table 2). Moreover, at the lowest stocking density, a gradual increase in richness was observed throughout development, whereas at the highest stocking density, ASV richness remained stable during egg incubation and early larval rearing. Here, due to the high availability of DOM, the eggs/embryos incubated at the

highest stocking density might have been overgrown by fast-growing (r-strategic) bacteria occupying the area over the egg chorion shortly after the initiation of incubation. On the other hand, at the lowest stocking density, eggs/embryos might be sparsely colonised by bacteria, leaving space for further colonisation over time. This could be linked to the higher offspring survival at the lowest stocking density (500 eggs/L). The differential abundance analysis, comparing the bacterial communities of initial eggs and embryos (at 48 hpf), confirmed the presence of strong selective forces at the highest stocking density, favouring the growth of opportunistic and rapid-growing bacteria. Several ASVs belonging to potentially harmful orders such as Vibrionales, Rhodobacterales, Oceanospirillales and Alteromonadales were markedly more abundant in the bacterial community of highly stocked embryos at 48 hpf than in the unfertilised eggs. Several members of the Vibrionales order are pathogens in farmed fish (reviewed in Ina-Salwany et al. (2019); Kashulin et al. (2017)), and an increase in the Oceanospirillales and Alteromonadales orders has often been associated with stressed and diseased marine invertebrates (Bourne et al., 2008; Bourne and Munn, 2005; Meron et al., 2011; Sunagawa et al., 2009). It is likely that in our experiment, the higher availability of substrate (DOM) for bacteria at the highest stocking density increased the carrying capacity in the incubators and promoted fast-growing, opportunistic bacteria, leading to an r-selected bacterial community (Vadstein et al., 2018). Thus, detrimental host–microbe interactions may be the most likely explanation for the lower survival observed during the embryonic incubation phase and at the highest stocking density.

Interactions between bacteria associated with offspring and in the rearing environment

A clear difference was observed between bacterial communities in water and in offspring, where a more diverse bacterial community (due to higher ASV richness and evenness) was found in the unadulterated inflowing water than in the newly spawned unfertilised eggs (Figure 5). This trend continued throughout ontogeny, as water bacterial communities showed higher ASV richness than the corresponding offspring samples (embryos or larvae), corroborating previous findings on tilapia and Atlantic cod (Giatsis et al., 2015; Vestrum et al., 2021, 2018). Moreover, the present results showed that ASV richness in rearing water increased throughout embryonic development, while evenness decreased (Figure 3). This suggests that ASVs associated with eggs/embryos influenced the colonisation of the rearing water, while selective forces (e.g., DOM supply) favoured some ASVs, which then went on to dominate the water bacterial community during incubation. Moreover, the evenness in the bacterial communities of the offspring (embryos at 48 hpf and larvae at 3 dph) was comparable to that of the rearing water at the lowest stocking density, whereas at the highest stocking density, evenness was lower in the bacterial community of the offspring than in the rearing water. This seems to indicate that the higher the stocking density, the

stronger the selective forces in rearing water, which in turn act on bacterial communities of offspring, favouring the growth of opportunistic and rapid-growing bacteria and leading to reduced evenness in bacterial communities.

Gene expression - molecular ontogeny of immune and stress related mechanisms

We did not detect any significant effects of stocking density on the expression of immune and stress related genes during the embryonic and early larval phase of European eel. In Siberian sturgeon (*Acipenser baerii*) offspring, however, upregulation of stress-related *hsp90* has previously been noticed with increasing stocking density during later developmental stages (complete yolk-sac absorption phase), but not during earlier developmental stages (Aidos et al., 2020). It is possible that a similar (later) stage specificity of stress response to crowding may exist in European eel, even though eel eggs and embryos do not appear to be sensitive to crowding. Interestingly, we did detect the expression of the *lysc* gene in eggs, embryos, and early larval stages. Lysozyme participates in bacteriolytic mechanisms in various tissues and mucus secretions in higher vertebrates, including fish eggs, either alone or cooperatively with complement and specific antibodies (Hansen and Olafsen, 1999). The occurrence of high levels of lysozyme in the eggs of salmonids and its effect on potentially harmful bacteria have previously been documented (Yousif et al., 1994, 1991). In our study, however, we did not see an upregulation of this gene during the embryonic phase, where the embryos were exposed to potentially harmful bacteria. In their natural oligotrophic environment (Rowe et al., 2012), the early stages of European eel are probably exposed to fewer or different bacteria than they encounter in hatchery incubators, where native bacteria are absent, or where nutrients are abundant, and thus bacterial loads can be high. Therefore, the early developmental stages of European eel may not yet be equipped with fully matured/functional underlying mechanisms to induce the defensive responses needed to counter the opportunistic bacteria they encounter in hatcheries, which may be related to the mortalities observed in our study during the embryonic incubation phase.

In this connection, we studied two genes (*mhc2* and *igm*) related to adaptive immunity. MHC molecules engage in presenting pathogen-associated peptides and molecules (i.e., antigens) to T cells, which initiate an immune response against pathogens. MHC class I molecules are expressed by nearly all host cells, whilst MHC class II molecules are only expressed by professional antigen-presenting cells-APCs (Zhu et al., 2013). In the present study, the expression of *mhc2* in embryos (at 48 hpf) suggests that professional APCs are already present at the embryonic stage. This is similar to zebrafish (*Danio rerio*), where the first production of APCs (e.g., macrophages) has been detected during primitive hematopoiesis at the 12–24 hpf period (Rombout et al., 2005). However, further experimentation is needed to confirm this. Moreover, in our study, *igm*

expression was low in embryos (at 48 hpf), peaked at hatch and decreased to basal levels at 3 dph, confirming previous results for European eel (Miest et al., 2019). As such, *igm* expression at such early developmental stages (e.g., embryos and newly hatched larvae), suggests that *igm* might be transferred maternally to the offspring, which has also been reported in various other fish species (Castillo et al., 1993; Swain and Nayak, 2009).

In our results, the complement component *c3b1* was already expressed in the embryos (at 48 hpf), while its expression levels had decreased by 3dph, suggesting the maternal origin of this gene. Maternal transfer of *c3b1* is in line with previous studies of European eel (Miest et al., 2019) and other fish species (reviewed in Zhu et al. (2013)). In contrast, expression of the complement component *c1qc* was at basal levels until hatch and peaked in larvae at 3 dph, suggesting the involvement of this gene in the development of its own larval immunity. This pattern of expression could be associated with a regulatory mechanism designed to avoid excessive complement activation, as this can result in anaphylaxis and cellular damage (Walport, 2001). Similarly, expression levels of *il1b*, which codes for a pro-inflammatory cytokine, were at basal levels at the embryonic stage and then increased with development. Expression of *il1b* is known to induce a cascade of immune reactions leading to inflammation, and it is mediated through the up-regulation or down-regulation of other cytokines (Dinarello, 1998). It follows that the observed up-regulation of *il1b* might be a response to the downregulation of the anti-inflammatory cytokine, *il10*. On the other hand, both genes related to interferon-regulating receptors (*irf3* and *irf7*) were increasingly expressed throughout development, which is in line with previous observations in European eel (Miest et al., 2019), where the potential for mounting anti-viral response at a very early developmental stage has been suggested.

In sum, the early developmental stages of European eel possess components of both innate and adaptive arms of the immune system, either of maternal origin or of own production. However, the lack of response in the studied immune and stress related genes suggests that the immune system of early developmental stages of European eel offspring is not evolutionarily prepared to handle hostile bacterial communities. This may help explain the mortalities observed in this study during embryonic incubation and at high stocking densities.

Egg incubation density: Practical considerations for hatchery production

Although the incubation of eggs at low density (500 eggs/L) appears to be a valid strategy to attain higher offspring survival, it compromises the efficient use of hatchery infrastructure, increases handling demand and complicates husbandry procedures (Broach et al., 2017). European eels are generally characterised by high fecundity, ranging from 1,440,000 to 7,455,000 oocytes per female (Reismann and Frankowski, 2022). Thus, from a cost-benefit point of view,

the minor decrease in survival (~5%) observed in the highest stocking density (4000 eggs/L) might be an acceptable compromise allowing a more efficient utilisation of infrastructure and labour. Further support for this is that we found no evidence of stocking density-related effects on hatching success. Studies of other marine species such as North American burbot (Jensen et al., 2008) and striped bass (Harper et al., 2010) have shown that egg stocking densities higher than 10,000 eggs/L did not affect hatching success when incubating eggs were exposed to high water exchange rates that could flush the toxic metabolites and replenish the dissolved oxygen. Interestingly, the hydraulic retention time in the incubators for the latter study (33.7 min) was roughly similar to the hydraulic retention time in our experiment (27.7 min), which might thus be close to an optimum water exchange rate. This may partly explain why we did not detect density-dependent effects on hatching success. Consequently, egg incubation at high stocking densities (up to 4000 eggs/L) can be recommended from a practical point of view, given that the tools for bacterial community management are incorporated to avoid r-selection during the embryonic incubation phase.

Our findings clearly demonstrate the contribution of adverse interactions between offspring and bacteria to significant early mortalities, as well as the limited capacity of the early immune system to manage such interactions. However, these factors alone do not fully account for the relatively low survival and hatching rates observed in the present experiment. This suggests that suboptimal rearing conditions may be at play and that additional factors beyond the ones tested in our experiment may have exerted an even stronger influence. A variety of other factors may influence the developmental competence of early life stages. Factors which should be taken into consideration for future improvements in hatchery techniques include the conditions under which broodstock are reared and fed, the implementation of hormonal therapies for assisted reproduction, an exploration of genetic compatibility dynamics among gametes and the physical rearing environment and hydrodynamics affecting early life stages. Moreover, the current study has neither provided qualitative assessments of inorganic toxic metabolites nor examined factors that influence oxygen demand, such as bacterial quantities and the provision of dissolved organic matter (DOM). These topics should be addressed in future research.

5 Conclusions

In this study of European eel, we found a stocking density-dependent effect on offspring survival and on the bacterial communities of water and offspring. Most of the mortality was recorded during embryonic development, but not during the early larval phase. Interestingly, we noticed a shift in the bacterial community of offspring from a unique and inherent bacterial community in unadulterated eggs to a community dominated by rapid-growing (r-selected) opportunistic bacteria on embryos. This was most evident at the highest stocking density. Moreover, a more

diverse bacterial community was observed in the larval rearing water than in the embryonic incubation water, where opportunistic and potentially harmful bacteria were more abundant. Thus, detrimental host–microbe interactions might be a key driver for the increased mortality observed not only during the embryonic phase but also at the highest stocking density, where selective forces (e.g., availability of DOM) might have favoured potentially harmful bacteria and thus negatively affected the commensal relationship between indigenous bacteria and opportunistic pathogens. Stress-related and immune-related genes were not affected by density, indicating that the molecular immune system is not fully matured and is thus incapable of handling such a challenging microbial environment in captivity during the early life stages. This could be the result of an evolutionary adaptation to the early natural oligotrophic oceanic environment of the European eel, but it aggravates the challenges faced during hatchery production. Despite the minor decrease in survival (~5%), we conclude that densities as high as 4000 eggs/L constitute an acceptable stocking density, allowing efficient utilisation of infrastructure and labour and compensated for by the high fecundity of the European eel broodstock. However, bacterial community management tools should be applied to avoid r-selection in the bacterial communities of offspring and rearing water, and, to counter bacteria-associated mortalities, especially during the embryonic incubation phase. Moreover, a useful strategy to enhance survival during the early hatchery phase may be to shift the ontogeny of the immune system earlier towards embryonic development and hatching, possibly by enhancing maternal immunity or by applying immune-stimulating products during offspring rearing.

CRediT author statement

All authors contributed to this manuscript.

Kasun A. Bandara: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. **Sebastian Nikitas Politis**: Conceptualization, Methodology, Investigation, Data curation, Validation, Visualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Daniela Eliana Sganga**: Investigation, Data curation, Formal analysis, Writing – review & editing. **Sune Riis Sørensen**: Resources, Conceptualization, Visualization, Writing – review & editing, Funding acquisition. **Paraskevas Koumpiadis**: Methodology, Investigation, Writing – review & editing. **Jonna Tomkiewicz**: Conceptualization, Methodology, Resources, Data curation, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Olav Vadstein**: Conceptualization, Methodology, Resources, Data curation, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Table 1. Primers used for molecular analysis of immune and stress related gene expression (FW: Forward, RV: Reverse)

Function	Gene name	Abbr.	Primer sequence		Accession
Reference	40S ribosomal S18	*rps18*	FW	AGAGCAGGGGAACTGACTGA	XM_035428274.1
			RV	ACCTGGCTGTATTTGCCATC	
	Elongation Factor 1a	*ef1*	FW	CTGAAGCCTGGTATGGTGGT	XM_035428800.1
			RV	CATGGTGCATTTCCACAGAC	
Complement system	Complement component C3, tandem duplicate 1	*c3b1*	FW	AATATGTGCTCCCAGCCTTC	XM_035404253.1
			RV	GATAACTTGCCGTGATGTCG	
	Complement component 1, Q subcomponent, C chain	*c1qc*	FW	ACAATGTCGACACAGGCAAG	XM_035380402.1
			RV	ACTTGGTTGAGGTTGGAGGTC	
Pro-inflammatory Cytokines	Interleukin 1β	*il1β*	FW	ATTGGCTGGACTTGTGTTCC	XM_035380403.1
			RV	CATGTGCATTAAAGCTGACCTG	
Anti-inflammatory cytokine	Interleukin 10	*il10*	FW	CCTGCAAGAAACCCTTTGAG	XM_035382126.1
			RV	TGAACCAGGTGTCAATGCTC	
Induce type I Interferon (ant-viral)	Interferon regulatory factor 7	*irf7*	FW	TTCCTTGGAAGCACAACTCC	XM_035396683.1
			RV	TGTCGTTCGGATTCTCTCTG	
	Interferon regulatory factor 3	*Irf3*	FW	GAAGAGGTGGCAGCAAAATC	XM_035405522.1
			RV	GGAAAAAGAGGGGGATTCAC	
Pathogen recognition	Toll like receptor 18	*tlr18*	FW	TGGTTCTGGCTGTAATGGTG	XM_035421803.1
			RV	CGAAATGAAGGCATGGTAGG	
Antibacterial response	Lysozyme Type C	*lysc*	FW	ACGGCATCTTCCAGATCAAC	XM_035389795.1
			RV	TGGAGCACGGGATATTACAG	
Antigen presentation to immune cells	Major histocompatibility complex, Class II	*mhc2*	FW	TCAAATTGACCTGGCTGAGAG	XM_035425009.1
			RV	TTTCCATTAGCCAGCTCCTC	
Antibody	Immunoglobulin M	*igm*	FW	CCAAGGACCATTCTTTCGTC	EF062515.1
			RV	ACTGGCTTTCAGGAAGATGC	
Stress/repair	Heat shock protein 70	*hsp70*	FW	TCAACCCAGATGAAGCAGTG	XM_035380750.1
			RV	GCAGCAGATCCTGAACATTG	
	Heat shock protein 90	*hsp90*	FW	ACCATTGCCAAGTCAGGAAC	XM_035392491.1
			RV	ACTGCTCATCGTCATTGTGC	

Table 2. PERMANOVA p values based on Bray–Curtis and Sørensen–Dice dissimilarities for comparisons of bacterial communities of European eel (*Anguilla anguilla*) offspring or water between the highest and lowest stocking densities and for comparisons of bacterial communities between European eel (*Anguilla anguilla*) offspring and water at the lowest and highest stocking densities at each developmental stage. The significance level was set at <0.05.

Stage	Comparison	p value	
		Bray–Curtis	Sørensen–Dice
0 hpf	eggs *vs*. unadulterated inflowing water	**0.001**	**0.001**
48 hpf	Lowest density embryos *vs*. highest density embryos	**0.001**	**0.001**
	Lowest density water *vs*. highest density water	**0.001**	**0.001**
	Lowest density embryos *vs*. rearing water	**0.001**	**0.001**
	Highest density embryos *vs*. rearing water	**0.019**	**0.001**
0 dph	Lowest density larvae *vs*. highest density larvae	**0.019**	**0.034**
3 dph	Lowest density larvae *vs*. highest density larvae	0.070	0.080
	Lowest density water *vs*. highest density water	0.190	0.390
	Lowest density larvae *vs*. rearing water	**0.001**	**0.001**
	Highest density larvae *vs*. rearing water	**0.009**	**0.001**

Table 3. PERMANOVA p values based on Bray–Curtis and Sørensen–Dice dissimilarities for comparison of bacterial communities in samples of European eel (*Anguilla anguilla*) offspring and water across relevant developmental stages at each stocking density treatment. The significance level was set at <0.05.

Sample type	Stocking density	Comparison	p value	
			Bray–Curtis	Sørensen–Dice
Offspring	Lowest	0 hpf eggs *vs*. 48 hpf embryos	**0.001**	**0.001**
		0 hpf eggs *vs*. 0 dph larvae	**0.001**	**0.001**
		0 hpf eggs *vs*. 3 dph larvae	**0.001**	**0.001**
		48 hpf embryos *vs*. 0 dph larvae	**0.014**	0.244
		48 hpf embryos *vs*. 3 dph larvae	**0.001**	**0.001**
		0 dph larvae *vs*. 3 dph larvae	**0.001**	**0.001**
	Highest	0 hpf eggs *vs*. 48 hpf embryos	**0.001**	**0.001**
		0 hpf eggs *vs*. 0 dph larvae	**0.001**	**0.001**
		0 hpf eggs *vs*. 3 dph larvae	**0.001**	**0.001**
		48 hpf embryos *vs*. 0 dph larvae	0.064	0.186
		48 hpf embryos *vs*. 3 dph larvae	**0.001**	**0.001**
		0 dph larvae *vs*. 3 dph larvae	**0.001**	**0.001**
Water	Lowest	Inflowing *vs*. rearing water at 48 hpf	**0.001**	**0.001**
		Rearing water at 48 hpf *vs*. 3 dph	**0.001**	**0.001**
	Highest	Inflowing *vs*. rearing water at 48 hpf	**0.001**	**0.001**
		Rearing water at 48 hpf *vs*. 3 dph	**0.001**	**0.001**

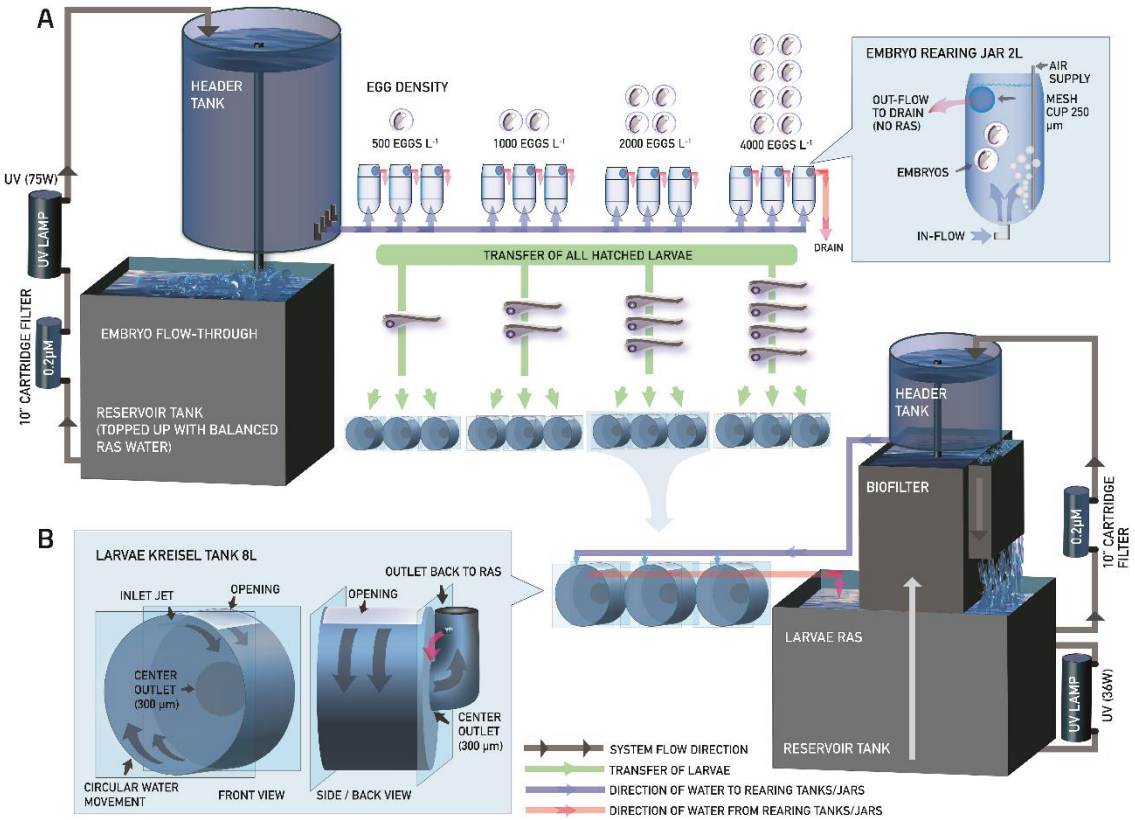

Figure 1. Schematic representation of the experimental setup for embryonic incubation (A) and larval rearing (B) of European eel (*Anguilla anguilla*). Eggs/embryos of each parental cross batch (n = 5) were incubated in replicated 2 L flow-through incubation jars (n = 3) at each of four different densities (500, 1000, 2000 and 4000 eggs/L) from fertilisation until hatch, resulting in 60 experimental units. Upon hatching, larvae were transferred to acrylic 8 L Kreisel tanks connected to a recirculating aquaculture system (RAS) and were reared until 3 days post-hatch.

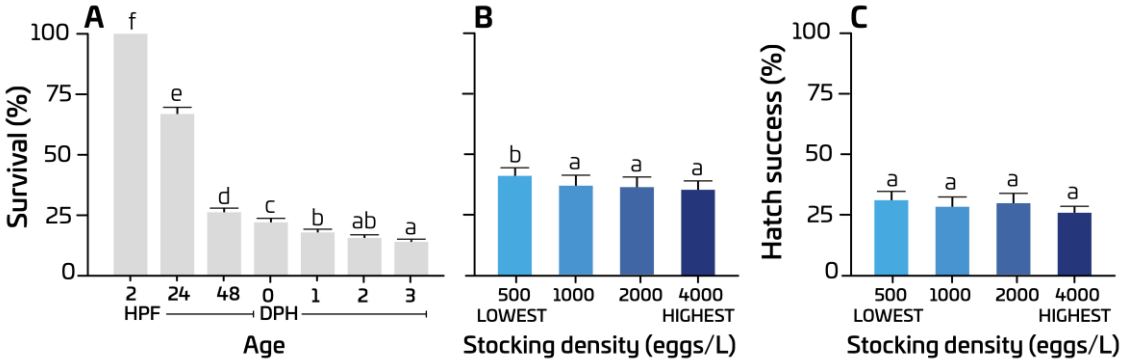

Figure 2. Effect of age (A) and stocking density (B) on offspring survival and effect of stocking density on hatching success (C) in European eel (*Anguilla anguilla*). Age is shown in hours post fertilisation (HPF) and days post-hatch (DPH). Stocking densities were 500, 1000, 2000 and 4000 eggs/L. Values represent means (± SEM), while different lower-case letters represent significant differences ($p < 0.05$).

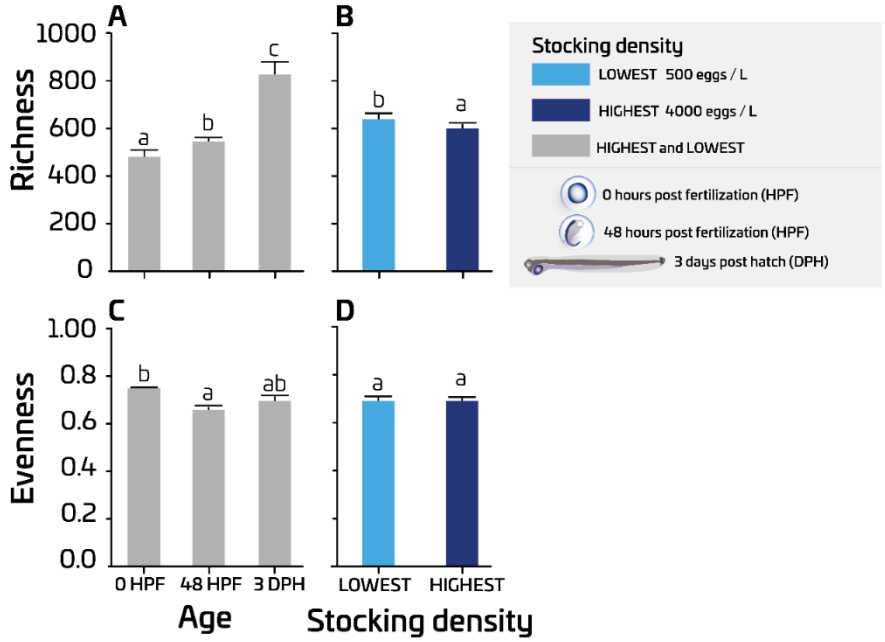

Figure 3. Effect of stage (A, C) and stocking density (B, D) on ASV richness and evenness in water samples. As no significant stage × stocking density interaction was detected, the main effects of stage and stocking density are displayed. Unadulterated water was sampled at 0 hpf, and rearing water at 48 hpf and 3 days post-hatch. Values represent means (± SEM), while different lower-case letters represent significant differences ($p < 0.05$).

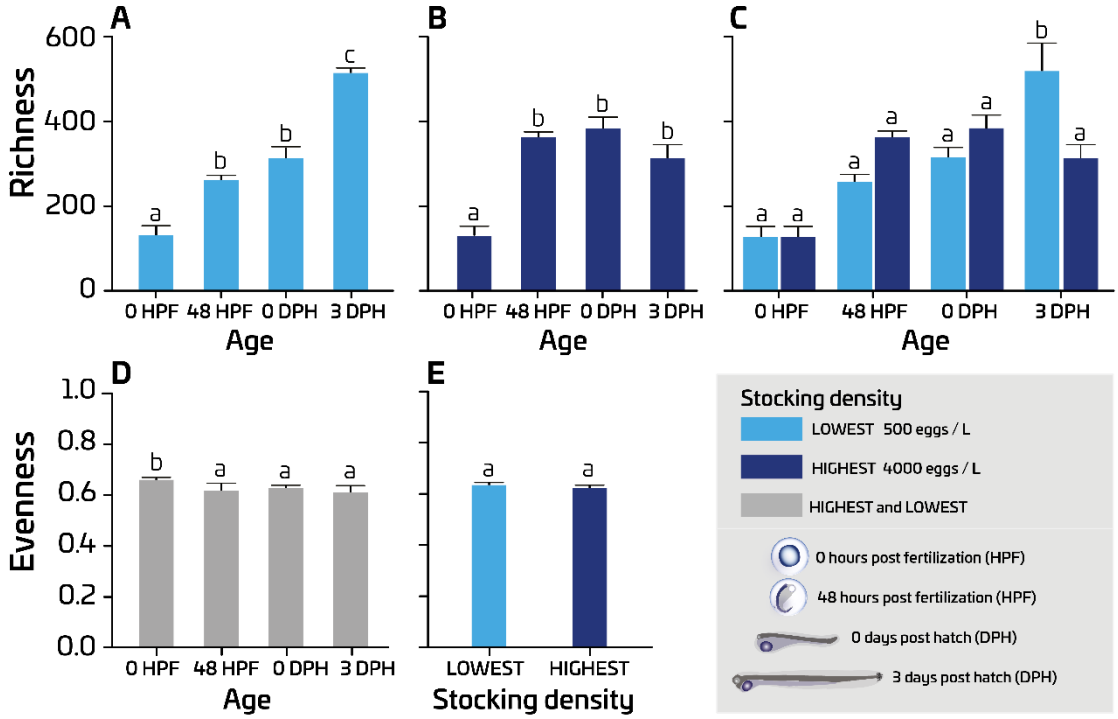

Figure 4. Effect of stage (A, B, D) and stocking density (C, E) on ASV richness and evenness in offspring of European eel (*Anguilla anguilla*). As a significant stage × stocking density interaction was detected for ASV richness, the model was decomposed into a series of reduced ANOVA models to determine the effect of the stage at the lowest (A) and highest (B) stocking densities, as well as the effect of stocking density at each stage (C). For evenness, no significant stage × stocking density interaction was detected, and thus, the main effects of stage (D) and stocking density (E) are displayed. Values represent means (± SEM), while different lower-case letters represent significant differences ($p < 0.05$).

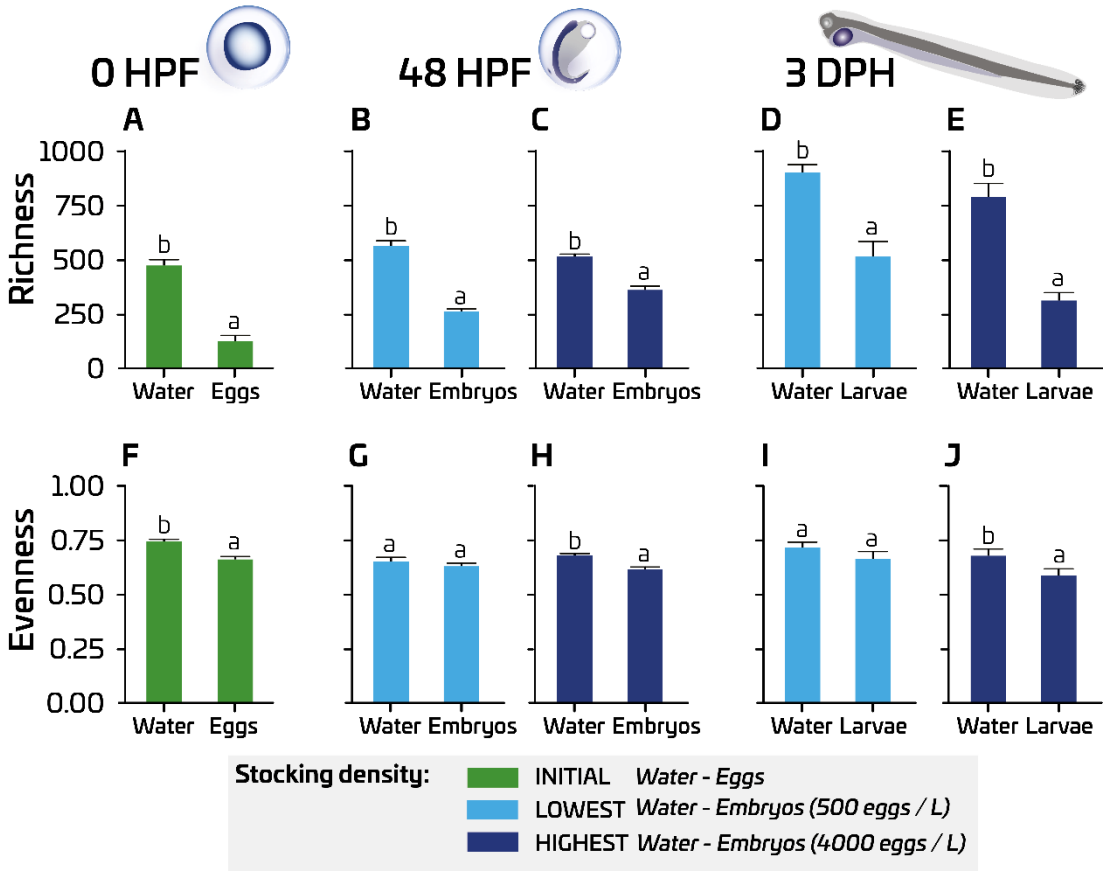

Figure 5. Comparison of alpha diversity indices between water and European eel (*Anguilla anguilla*) offspring samples. ASV richness (A) and evenness (F) in unadulterated inflowing water *vs.* initial eggs; ASV richness in rearing water *vs.* embryos at 48 hpf for lowest (500 eggs/L) (B) and highest (4000 eggs/L) (C) stocking densities; ASV richness in rearing water *vs.* larvae at 3 dph for lowest (D) and highest (E) stocking densities; ASV evenness in rearing water *vs.* embryos at 48 hpf for lowest (G) and highest (H) stocking densities; and ASV evenness in rearing water *vs.* larvae at 3 dph for lowest (I) and highest (J) stocking densities. Values represent means (± SEM), while different lower-case letters represent significant differences ($p < 0.05$).

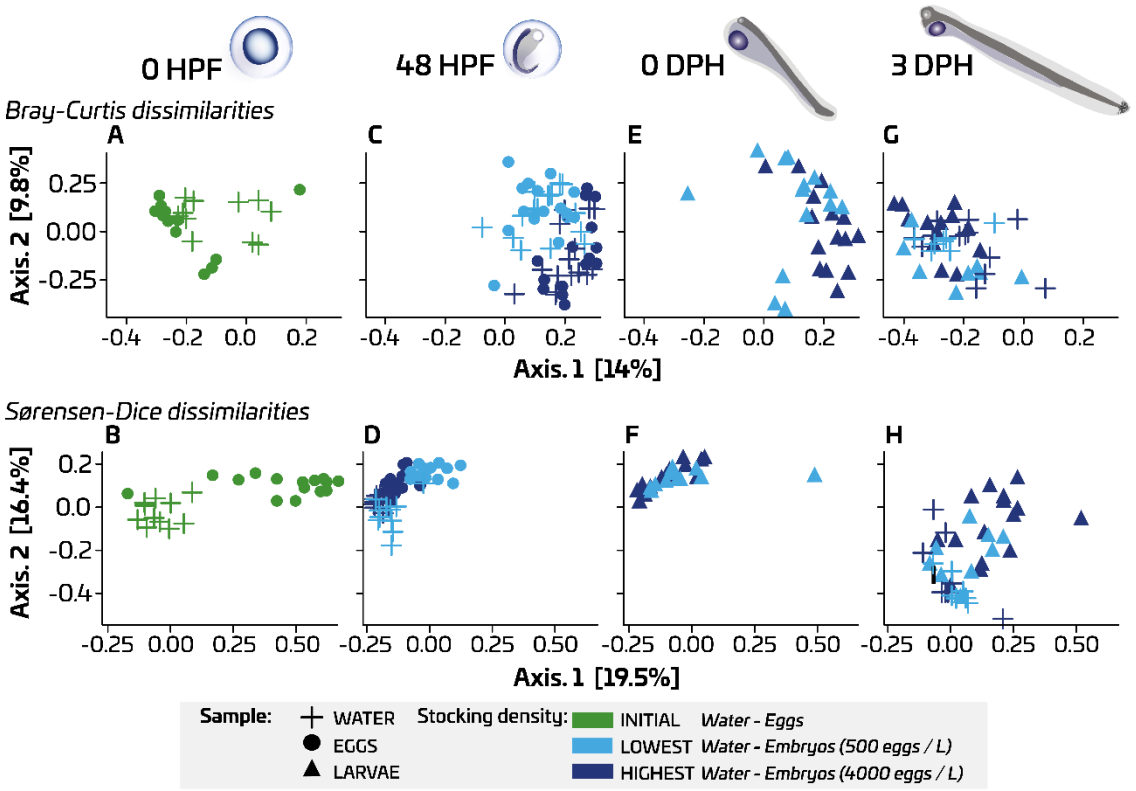

Figure 6. PCoA ordination plots based on Bray–Curtis and Sørensen–Dice dissimilarities for comparison of the bacterial communities of European eel (*Anguilla anguilla*) offspring and water in highest and lowest stocking densities at different developmental stages. The same ordination was plotted independently for each stage. Shapes indicate sample type, and colours denote stocking densities (Initial = before stocking, Lowest = 500 eggs/L, Highest = 4000 eggs/L).

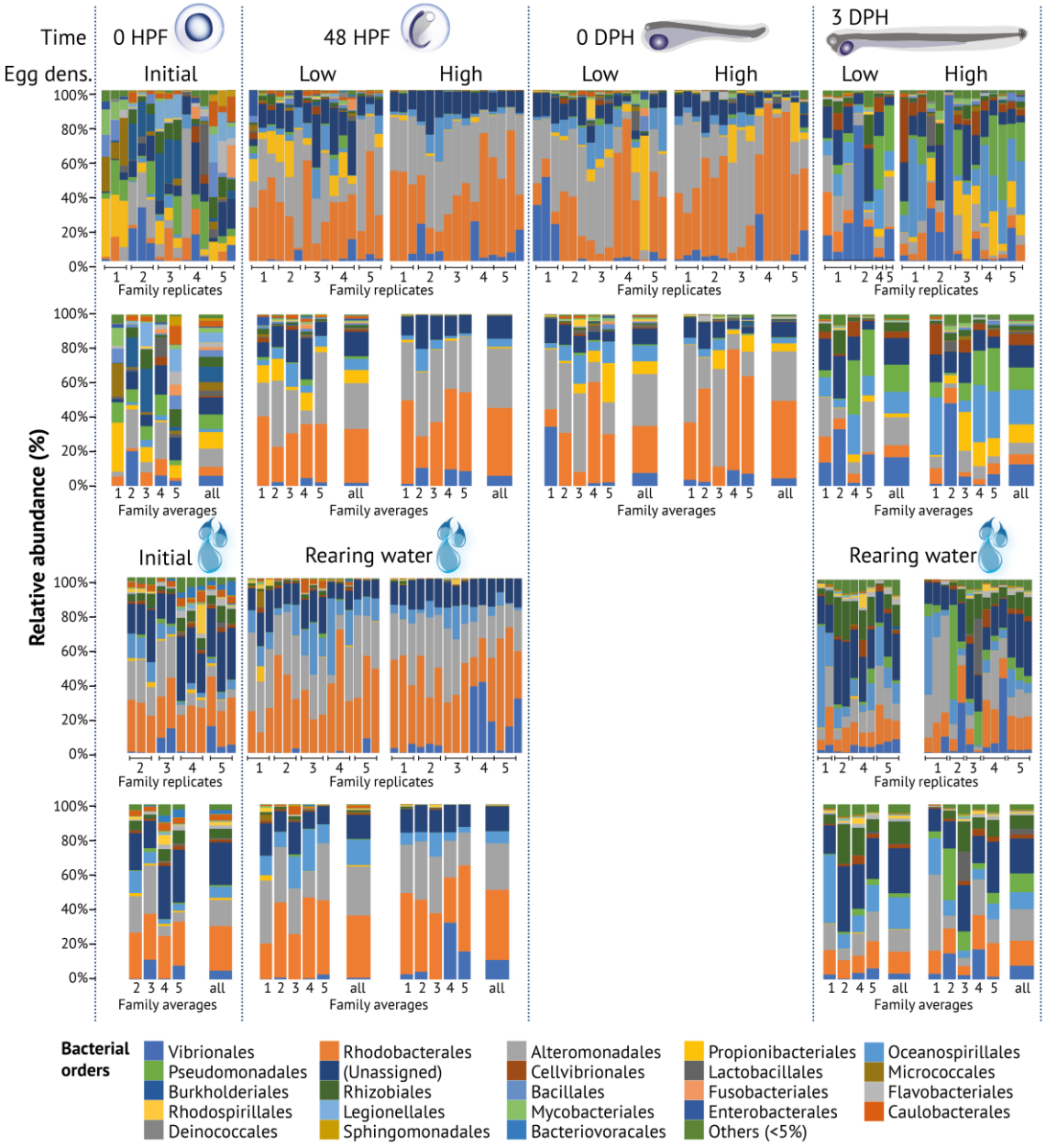

Figure 7. Relative abundances of bacterial orders detected in initial European eel (*Anguilla anguilla*) eggs and unadulterated water (flowing into the incubators) as well as in embryos, larvae, and rearing water of the two extreme stocking densities (Low = 500 eggs/L *vs.* High = 4000 eggs/L) at 48 hours post fertilisation (HPF), hatch [0 days post hatch (DPH)], and 3 DPH. Stacked bars represent the relative abundance of bacterial orders detected in each replicated sample (top rows), the average relative abundance of bacterial orders for each parental cross, or the average relative abundance of bacterial orders for two extreme stocking densities (Low = 500 eggs/L and High = 4000 eggs/L; bottom rows).

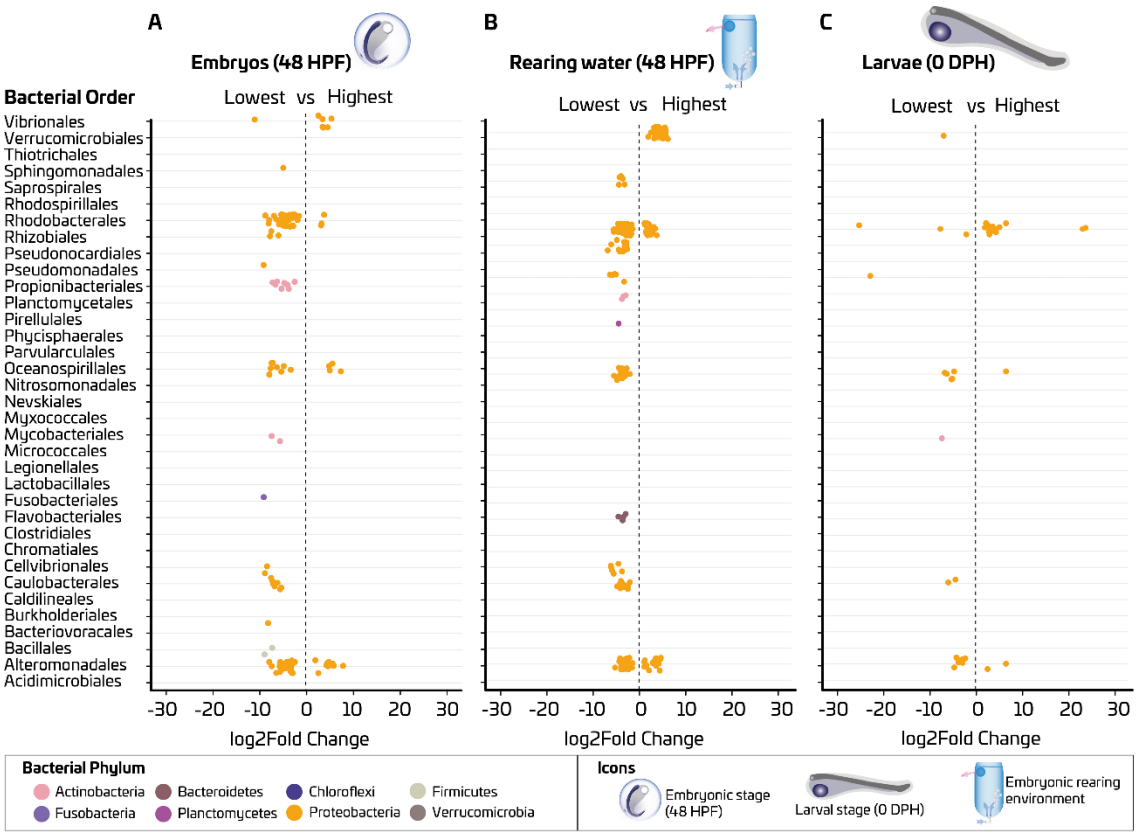

Figure 8. Results of DESeq2 analysis to determine significantly different abundances of taxa in the lowest (reference sample) and highest stocking density treatments in European eel (*Anguilla anguilla*) embryos (A) and rearing water (B) at 48 hours post fertilisation (HPF) and in European eel larvae at hatch [0 days post hatch (DPH)] (C). Each dot represents an ASV, and the $\log_2$-fold difference in the abundance of each ASV compared to the reference sample is shown. In DESeq2 analysis, only ASVs that are statistically significant ($p < 0.05$) are reported.

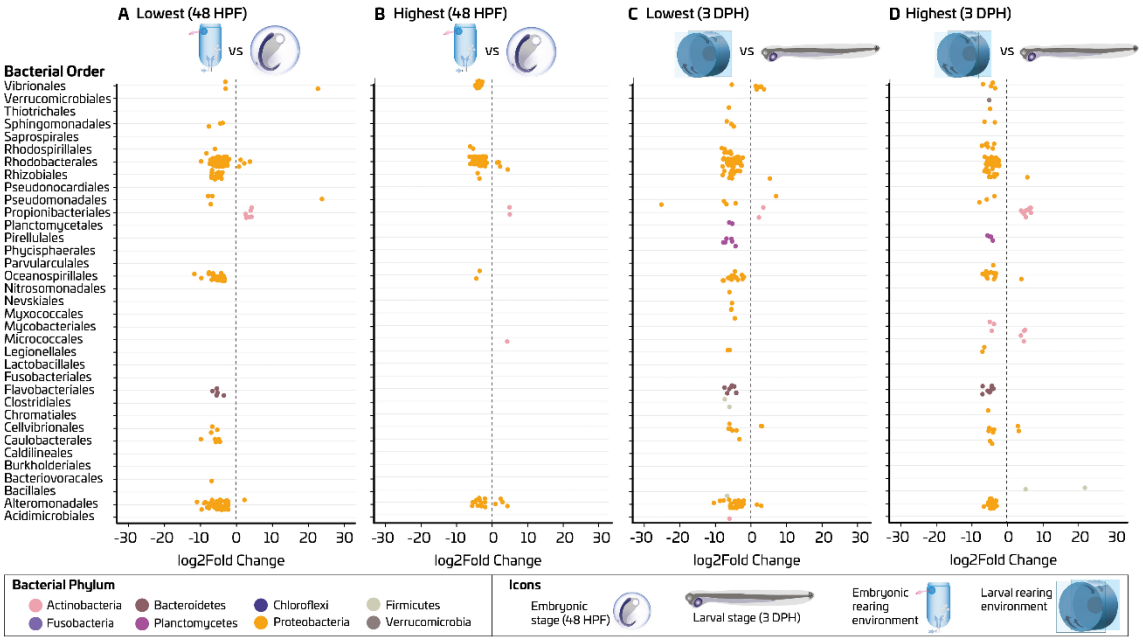

Figure 9. Results for DESeq2 analysis to determine significantly different abundances of taxa between rearing water (reference sample) and European eel (*Anguilla anguilla*) embryos of the lowest (A) and highest stocking density (B) at 48 hours post fertilisation (HPF) and between rearing water (reference sample) and European eel (*A. anguilla*) larvae at the lowest (C) and highest stocking density (D) at 3 days post hatch (DPH). Each dot represents an ASV, and the $\log_2$-fold difference in the abundance of each ASV compared to the reference sample is shown. In DESeq2 analysis, only ASVs that are statistically significant ($p < 0.05$) are reported.

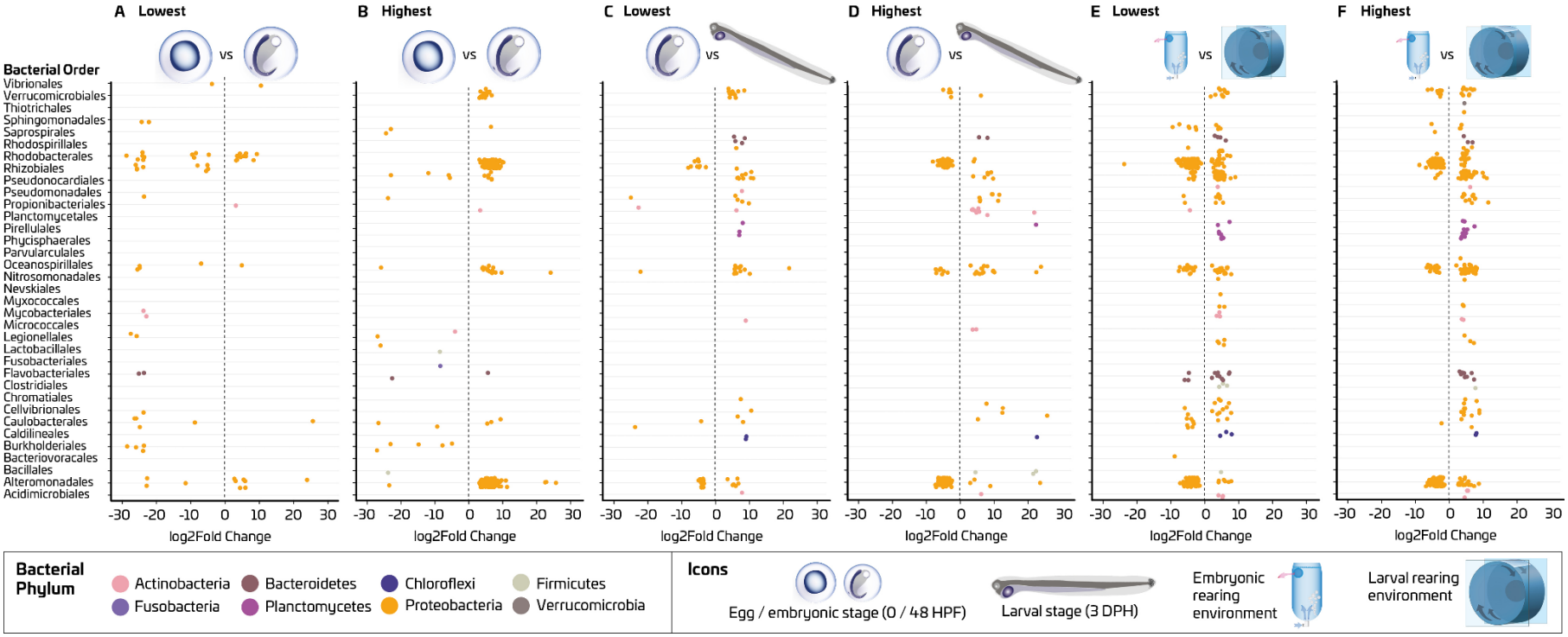

Figure 10. Results for DESeq2 analysis to determine significantly different abundant taxa between European eel (*Anguilla anguilla*) eggs prior to fertilisation [at 0 hours post fertilisation (HPF)] (reference sample) and embryos at 48 HPF at the lowest (A) and the highest (B) stocking densities, between embryos at 48 HPF (reference sample) and larvae at 3 days post hatch (DPH) at the lowest (C) and the highest (D) stocking densities and between embryonic incubation water at 48 HPF (reference sample) and larval rearing water at 3 DPH at the lowest (E) and highest (F) stocking densities. Each dot represents an ASV and $\log_2$-fold difference in the abundance of each ASV is compared to the reference sample. In DESeq2 analysis, only ASVs that are statistically significant ($p < 0.05$) are reported.

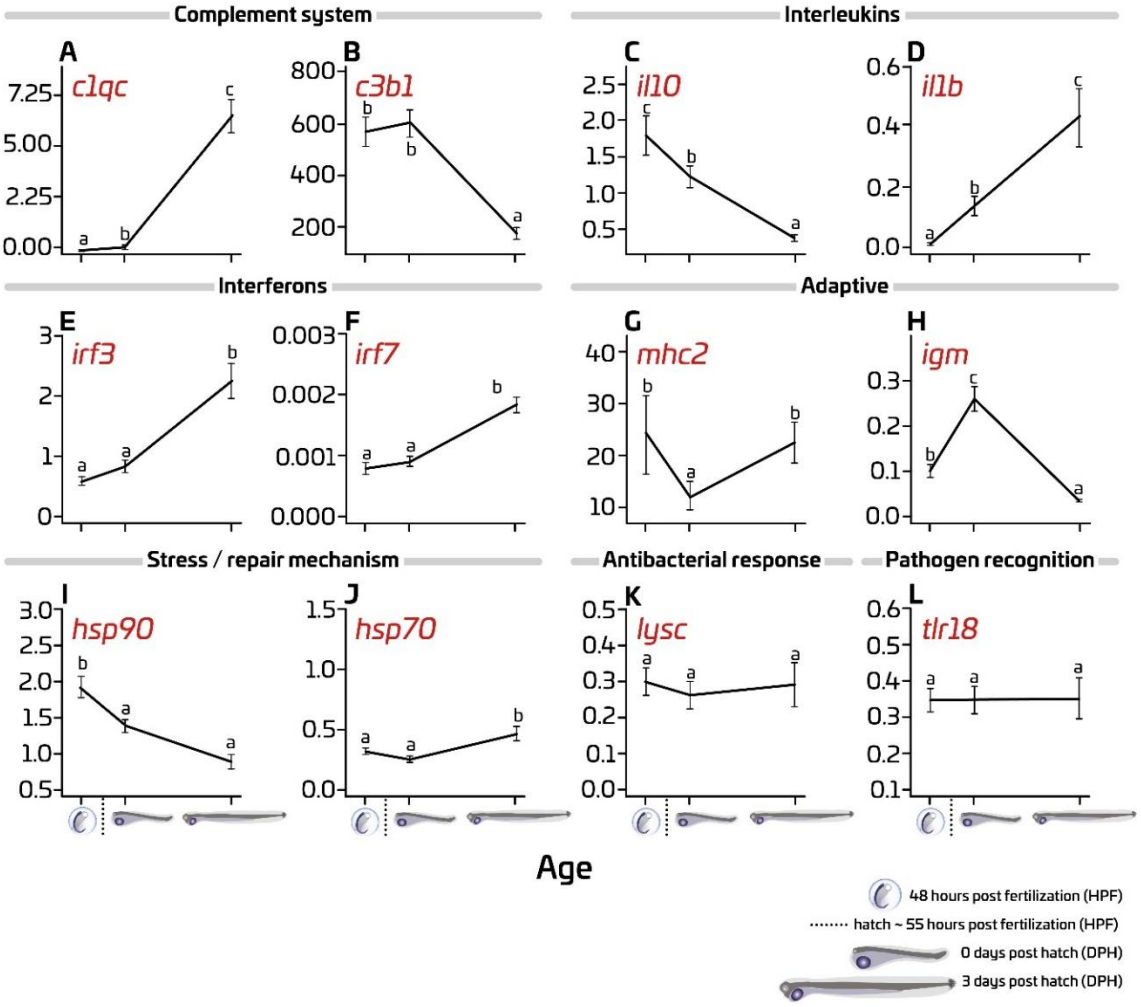

Figure 11. Effect of developmental stage [48 hours post fertilisation (HPF), hatch- 0 days post hatch (DPH) and 3 DPH] on the relative expression of immune and stress related genes in European eel (*Anguilla anguilla*). Values represent means (± SEM) among five crosses at each stage. Different lower-case letters represent significant differences ($p < 0.05$).

Study 2:

Exploring bacterial community composition and immune gene expression of European eel larvae (*Anguilla anguilla*) in relation to first feeding diets

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RESEARCH ARTICLE

Exploring bacterial community composition and immune gene expression of European eel larvae (*Anguilla anguilla*) in relation to first-feeding diets

Kasun A. Bandara[1,2]*, Elisa Benini[1], Sebastian N. Politis[1], Luis E. C. Conceição[3], André Santos[3], Sune Riis Sørensen[1], Jonna Tomkiewicz[1], Olav Vadstein[2]

1 Technical University of Denmark, National Institute of Aquatic Resources, Lyngby, Denmark, **2** Department of Biotechnology and Food Science, NTNU—Norwegian University of Science and Technology, Trondheim, Norway, **3** SPAROS Lda, Área Empresarial de Marim, Lote C, Olhão, Portugal

* amuka@aqua.dtu.dk

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Abstract

European eel (*Anguilla anguilla*) is a commercially important species for fisheries and aquaculture in Europe and the attempt to close the lifecycle in captivity is still at pioneering stage. The first feeding stage of this species is characterized by a critical period between 20 to 24 days post hatch (dph), which is associated with mortalities, indicating the point of no return. We hypothesized that this critical period might also be associated with larvae-bacterial interactions and the larval immune status. To test this, bacterial community composition and expression of immune and stress-related genes of hatchery-produced larvae were explored from the end of endogenous feeding (9 dph) until 28 dph, in response to three experimental first-feeding diets (Diet 1, Diet 2 and Diet 3). Changes in the water bacterial community composition were also followed. Results revealed that the larval stress/repair mechanism was activated during this critical period, marked by an upregulated expression of the *hsp90* gene, independent of the diet fed. At the same time, a shift towards a potentially detrimental larval bacterial community was observed in all dietary groups. Here, a significant reduction in evenness of the larval bacterial community was observed, and several amplicon sequence variants belonging to potentially harmful bacterial genera were more abundant. This indicates that detrimental larvae-bacteria interactions were likely involved in the mortality observed. Beyond the critical period, the highest survival was registered for larvae fed Diet 3. Interestingly, genes encoding for pathogen recognition receptor TLR18 and complement component C1QC were upregulated in this group, potentially indicating a higher immunocompetency that facilitated a more successful handling of the harmful bacteria that dominated the bacterial community of larvae on 22 dph, ultimately leading to better survival, compared to the other two groups.

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Competing interests: The authors have declared that no competing interests exist.

1. Introduction

Global consumption of aquatic food has increased and is expected to rise continuously in coming years [1]. The sum of fisheries and aquaculture production was 178 million tonnes in 2020, and aquaculture production alone was 87.5 million tonnes. As one of the fastest growing animal production sectors in the world, aquaculture plays an important role in fulfilling the nutritional requirements of the growing human population globally [1]. Marine fishes in particular are a rich source of omega-3 fatty acids, besides protein and minerals [2, 3]. Aquaculture also helps to release the pressure on the natural marine fishery resources [4]. In this regard, it is the closing of the life cycle of fish in captivity that is the basis for development of successful aquaculture, by ensuring reliable, high-quality, and year-round hatchery production of offspring to sustain the commercial farming [5]. Still, unreliable availability of hatchery produced offspring is a constraint for successful marine fish culture, among other, as a sustainable alternative to wild-caught juveniles [6].

European eel (*Anguilla anguilla*) is a marine finfish species of high market value with substantial fisheries and aquaculture potential [7]. However, aquaculture of eels for human consumption and stock enhancement relies entirely on wild juveniles called "glass eels", which are caught in targeted fisheries [8]. Moreover, the stock has declined sharply, and the European eel is ranked as critically endangered on the IUCN red list [9]. It is therefore imperative to develop hatchery technology for sustainable aquaculture, stock management and conservation plans. Currently, efforts in applied research to establish hatchery techniques and technology have led to a stable production of European eel larvae entering the feeding stage (around 9 dph) [10]. This progress involves assisted reproduction technology and methodology resulting in viable fertilised eggs, their incubation until hatch (~2 days post fertilisation) and development into the feeding stage (10–12 days post hatch (dph)). Present challenges, aiming at establishing protocols for rearing of feeding larvae, include identification of specific nutritional requirements, optimisation of rearing techniques and microbial management in recirculating aquaculture systems (RAS).

For Anguillid eels in general, a major constraint during first feeding of larvae is the establishment of effective diets and adequate feeding regimes. This is mainly due to the limited knowledge about the natural diet of eel larvae during the first-feeding phase and long-lasting migratory phase, from oceanic spawning areas (i.e., the leptocephalus stage) to continental waters, where they metamorphose into glass eels. One enigma of the larval feeding ecology has been that they, in contrast to most other fish larvae, do not appear to feed on zooplankton [11]. Recent studies of leptocephali gut contents suggest that their natural diet consists of amorphous material of different origin, such as larvacean houses, faecal pellets, gelatinous zooplankton, and materials associated with marine snow (bacteria, protists, fungi, and other microorganisms) [11, 12]. This is in sharp contrast to diets that have proven successful for hatchery-reared eel larvae. For first feeding of larval of Japanese eel (*Anguilla japonica*), a species closely related to European eel, a diet based on egg yolk of spiny dogfish (*Squalus acanthias*) was successful [13]. This slurry-type diet sustained larval survival until 26 dph. A gradual modifications and improvements of the diet, together with improved rearing techniques, has enabled closure of the life cycle of Japanese eel and thus production of further generations of captive propagated offspring [14].

For European eel, establishing first feeding larval culture protocol is at a pioneering stage, where successful production of viable larvae and enhanced larval culture technology has increased survival of larvae entering to feeding stage and recently enabled feeding experiments [15, 16]. A recent study showed upregulated expression of genes involved in growth and digestion when pre-fed a slurry type diet based on pasteurized egg yolk from thornback ray (*Raja*

clavata), suggesting a potential benefit of early feeding for improved transition from endogenous to exogenous feeding [17]. Moreover, a further study [18] testing three different slurry-type diets based on spiny dog fish eggs throughout the first feeding window, and documented improved larval growth, survival and digestion, and growth-related gene expression at the end of the experiment (28 dph). Notably, this study represents a landmark as the first documentation of European eel larvae overcoming the bottleneck during the first feeding window and surviving beyond the point of no return. Interestingly, the study revealed two periods of high mortalities: shortly after introduction of feed (10–12 dph) and during the period 20–24 dph. This indicates that besides nutritional (in)appropriateness, other factors such as detrimental larvae-bacteria interactions might be in play.

In marine fish larval culture, negative larvae-bacteria interactions are known to cause low and unpredictable growth and survival [19, 20]. The importance of microbial management during egg incubation and larval culture of European eel has previously been demonstrated, where hatching success and larval longevity were negatively impacted by microbial activity [21]. During feeding with a slurry-type diet, a rapid deterioration of water quality in the rearing tanks is observe [17, 22]. This is likely due to leakage of nutrients to the water and stimulation of microbial growth. In this regard, eel larvae, which in nature thrive in an oligotrophic environment with low bacterial densities [23], might in culture be challenged by detrimental larvae-bacteria interactions caused by the introduction of these easily degradable feeds into the rearing tanks. This challenge may be amplified by a not fully developed larval immune system [24, 25].

In general, newly hatched fish larvae are highly sensitive to detrimental bacteria as their immune system is not fully developed and the intensive rearing causes stress [26]. In marine fish larvae, it can take up to three months until their immune response is fully functional, a long period during which larvae depend largely on the innate part of the immune system and are vulnerable to detrimental interactions and dysbiosis in the microbiota [27, 28]. Since innate immunity plays an important role in protecting fish larvae against microbial interference, larval survival might closely be linked to their immune ontogeny [26]. In fact, a previous study investigating immune gene expression during early life history of European eel, indicated a sensitive phase during which larvae are potentially immuno-compromised [25].

We hypothesized that the 2nd period with increased mortality, described during the first feeding stage of European eels, might be linked to changes in microbial communities and the molecular ontogeny of the immune system. The present study complements the study of [18], by elucidating the impact of the three slurry type diets on the succession of bacterial communities throughout and beyond the first feeding period. Furthermore, the study attempts to disclose the interplay between larvae-bacteria interactions, immune and stress/repair related gene expression and larval performance (survival).

2. Materials and methods

2.1 Ethics statement

All fish were handled according to the European Union regulations concerning the protection of experimental animals (Dir 2010/63/EU). Experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2020-15-0201-00768). Broodstock was anaesthetised individually before tagging, biopsy, and stripping of gametes, and euthanised after stripping (females) or at the end of the experiment (males) by submergence in an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg/L, Sigma Aldrich, Germany) [29]. Larvae were anaesthetised and euthanised

using tricaine methanesulfonate (MS-222, Sigma Aldrich, Germany) at a concentration of 7.5 and 15 mg/L, respectively [29].

2.2 Broodstock husbandry, offspring production and rearing

The experiment was conducted at EEL-HATCH, Hirtshals, Denmark (57.5858410˚N, 9.9853423˚E), an experimental hatchery of DTU Aqua. Female European eel broodstock originated from Saltbæk Vig, Zealand, Denmark, while farm-raised males were obtained from a Danish commercial fish farm (Royal Danish Fish A/S, 57.1226075˚N, 8.6243243˚E). The broodstock was acclimatised to a salinity of 36 PSU and a temperature of 18–20˚C. Gametes were obtained through assisted reproduction as described earlier [30]. After fertilisation of eggs using standardised procedures [31], embryos were incubated in 60 L conical bottom incubators supplied with filtered and UV-treated North Sea water adjusted to a salinity of ~36 PSU with Sea Salt (Aquaforest, Brzesko, Poland) [32] and a temperature of ~18˚C [33]. At ~52 hours post-fertilisation (hpf), aeration was stopped, and embryos hatched at ~56 hpf. Within 6 h after hatching, larvae were transferred to a 77 L tank connected to a 1.7 m^3 RAS and reared until 9 dph (Fig 1A). During this pre-feeding period, the temperature was maintained at 18–20˚C and salinity at ~36 PSU [17]. Water flow was set to 600 mL/min, and rearing was in constant darkness [34].

2.3 Experimental design and implementation

The present study is part of a feeding experiment in which various aspects of larval development and culture conditions were investigated and related to larval survival. The present study focuses on the succession of bacterial communities and larval immune gene expression, whereas another study targeted the effect of diets on larval appetite, feeding success and growth as well as related gene [18].

For the experiment, a batch of larvae was selected on 9 dph. Larvae were stocked in replicated (n = 9) 8 L Kreisel tanks at a density of ~60 larvae/L. These nine Kreisel tanks were randomly assigned in triplicates to three different experimental groups based on the type of diet (i.e., Diet 1, Diet 2, and Diet 3; three tanks per diet) and connected to three separate RAS units (one RAS per diet) (Fig 1B and 1C). In total we used: 3 diets × 3 reps × ~60 larvae/L × 8 L = ~4320 larvae. The RAS units, maintained at a salinity of 18 PSU [35] and temperature of 20˚C [33], were identical in terms of design. Each was composed of an upper sump reservoir of 370 L, which housed an 80 L wet/dry trickling filter, filled with RK bio-elements (240 m^2 surface area or 0.12 m^2 per L), a lower sump reservoir (260 L), a protein skimmer (Aquamedic 5000 single 6.0, Bissendorf, Germany) and UV treatment (ProCristal UV-C 11W, JBL GmbH & Co. Neuhofen, Germany). Each system was connected to an extra reservoir of 160 L to create head pressure before reaching the rearing tanks. Flow rates into the tanks were kept at ~ 420 mL/min, i.e., a hydraulic retention time of 0.32 h, except during feeding. Light (~500 lux) was turned on only during feeding [15].

Throughout the feeding period, water quality parameters were measured regularly and maintained within the optimum range. Temperature and salinity were 20 ± 0.5˚C and 18 ± 0.4 PSU, respectively. The range of dissolved oxygen (DO) during the experiment was within acceptable levels. DO levels at the end of feeding (after flow has been stopped for 30 mins to allow larvae to settle to bottom and eat) was 6.5 ± 0.2 mg O_2/L. When the water flow was on, DO level was 7.8 ± 0.1 mg O_2 /L. Water pH was 8 ± 0.2. The toxic nitrogen components in water (NH_4^+/NH3 and NO_2^-), which were measured with standard test kits were below the detection limits.

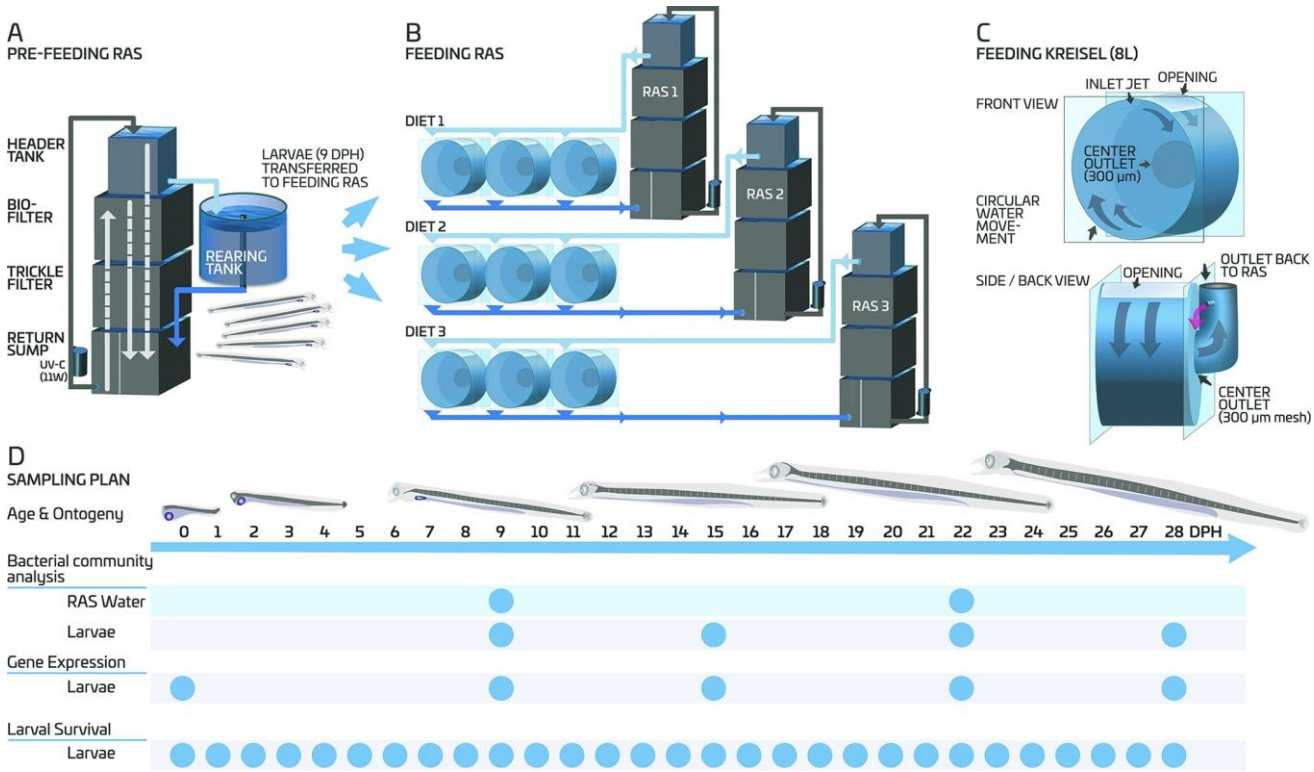

Fig 1. European eel (*A. anguilla*) larvae were reared in a common larval rearing tank during the pre-feeding (endogenous feeding) period (from 0 to 9 days post hatch (DPH)) (A) and in 8L acrylic Kreisel tanks (n = 3 for each diet) throughout and beyond the first-feeding window (B and C). Water from the RAS was sampled before (on 9 DPH) after (on 15 DPH) the initiation of the feeding for bacterial community composition analysis and larvae were sampled at different ages for bacterial community composition analysis and molecular analysis of immune and stress related genes, while larval survival was calculated through enumeration of dead larvae daily (D).

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The proximal compositions of the three diets applied during the experiment are given in Table 1 and explained in detail previously [18]. Diet 1, which consisted of pasteurised spiny dogfish (*S. acanthias*) egg yolk (Sterna Seafood AS, Snarøya, Norway), krill extract, soybean peptides and water, contained less protein and more lipids compared to the other diets. Diets 2 and 3 represented modifications of Diet 1, where spiny dogfish (*S. acanthias*) egg yolk was partially substituted with hydrolysed proteins. Diet 2 contained fish hydrolysate (CPSP90, Sopropeche, France) encapsulated in whey (Volacactive UltraWhey 80 Instant, Volac International Ltd, Hertfordshire, UK), whereas Diet 3 contained only whey. The diets were comparable in energy content.

The different experimental groups were fed with their respective diet five times per day at 2 h intervals. Before feeding, lights in the larval rearing room were turned on and water flow to

Table 1. Composition of the three diets used during the experiment (see also [18]).

	Diet 1	Diet 2	Diet 3
Dry matter (%)	27.1 ± 0.12	29.8 ± 0.09	36.01 ± 0.10
Protein (%)	50.89 ± 0.35	61.12 ± 0.17	59.09 ± 0.81
Lipid (%)	37.52 ± 0.25	27.49 ± 0.10	27.51 ± 0.28
Ash (%)	3.33 ± 0.04	3.21 ± 0.12	2.91 ± 0.03
Energy (kJ/g)	29.30 ± 0.18	27.93 ± 0.17	28.46 ± 0.18

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the tanks was stopped. The lights were programmed to start with a low intensity and gradually increase the intensity to minimise the stress caused by exposure to light. Then, diets were pipetted on the bottom of the tank at a concentration of 0.5 mL/L of water. After allowing the larvae to feed for 30 min, lights were turned off and the water flow was started. The remaining food on the tank bottom was flushed with a gentle jet of water. Water in the rearing tanks flowed through for 30 min (by disconnecting the tanks from the rest of the recirculating unit) before the tanks were reconnected to each corresponding RAS. To compensate for the loss of water, each RAS unit was refilled by adding water pre-adjusted to 20˚C and 18 PSU. The larvae were moved into clean tanks each day [17].

2.4 Sampling and data collection

2.4.1 Larval survival. Larval survival was monitored daily during the exogenous feeding period through counting and removing dead larvae. Additionally, all larvae at the end of the experiment (28 dph) as well as those sampled from each experimental unit were enumerated. Larval survival was then calculated as a percentage from 9 until 28 dph (Fig 1D).

2.4.2 Characterisation of bacterial community composition by amplicon sequencing. For characterisation of the bacterial community associated with larvae, pools (n = 4) of ~10 larvae were sampled from the common larval rearing tank before the onset of exogenous feeding (9 dph) (Fig 1D). Also, at two intermediate time points during the first feeding period (on 15 and 22 dph), pools (n = 2) of ~10 larvae from each replicate (n = 3) of the experimental groups (n = 3) were collected for microbiome analysis. At the end of the experiment (28 dph), larval samples were collected for microbiome analysis only from the groups fed with Diets 2 and 3. Sampled larvae were immediately euthanised, rinsed and stored at -20˚C for later analysis. To investigate whether the three different experimental groups were exposed to a similar initial water microbiome, water samples (n = 4) from each RAS (n = 3) were collected (from the inlet tubes that supplied water to the rearing tanks). To check whether the feeding caused a shift in the water microbiome, water samples (n = 4) from each RAS (n = 3) were collected on day 22 post larval hatch. Here, 250 mL of water from each sample was vacuum filtered through 0.22 μm white gridded filters (diameter = 47 mm; Merck KGaA, Darmstadt, Germany) using a Büchner funnel and the filters were stored in sterile cryotubes stored at −20˚C until processing [36].

DNA from larvae (pools of 10 whole larvae) and water were isolated using the MagAttract PowerSoil Pro DNA Kit (Qiagen, Germany) following the protocol developed by the supplier for automated high-throughput isolation of DNA with the Thermo Scientific⅂ KingFisher⅂ Flex platform. Briefly, samples (pools of ~10 larvae, or filter papers) were homogenised in bead-beating tubes containing ~0.55 g of 0.1 mm glass beads (Bertin Technologies, France) and 800 μL of lysis buffer, using a Precellys 24 tissue homogeniser (Bertin Technologies, France) at 5500 rpm for two times 30 s with a 15 s break in between. The tubes containing the lysates were centrifuged at 15000 × g for 1 min and the supernatants transferred into 1.5 mL Eppendorf tubes. Then, 300 μL of CD2 solution was added to each Eppendorf tube, vortexed to mix and centrifuged at 15000 × g for 1 min. Prepared lysates i.e., supernatants from the previous step, were transferred to the KingFisher Flex platform (Thermo Fisher Scientific), where total genomic DNA was captured on specialised magnetic beads in the presence of buffers, washed on the beads and then eluted.

The V3 and V4 regions of the bacterial 16S rRNA gene were amplified from the DNA isolates using the forward primer, Ill-341F_K1 (5^0– NNNNCCTAC GGGNGGCWGCAG –3^0) and the reverse primer, Ill805R (5^0– NNNNGACTACNVGGGTATCTAAKCC–3^0) [37]. Each PCR reaction contained 0.02 U/μL Phusion Hot Start II DNA polymerase (Thermo Scientific), 0.2 mM of each dNTP (VWR), 0.3 μM of each primer (SIGMA), 1x Phusion HF buffer

(containing 7.5 mM MgCl2) (Thermo Scientific) and PCR grade water (VWR) up to a total reaction volume of 25 μL, and 1 μL of DNA extract as a template. The PCR reactions were run with 35 cycles (T100TM Thermal Cycler, Bio-Rad) [38]. The PCR amplicons were purified and normalized using SequalPrep Normalization Plate (96) kit (Invitrogen, USA), following the protocol provided by the supplier. Using the Nextera1 XT DNA Sample Preparation Kit (Illumina), a unique pair of index sequences that represented the PCR amplicons, originating from each sample, was added by an additional PCR step with 10 cycles. The indexed PCR products were purified and normalized using the SequalPrep Normalization Plate (96) kit (Invitrogen, USA). Finally, the samples were pooled and concentrated with AmiconUltra 5.0 Centrifugal Filter (Merck Millipore, Ireland) following the manufacturer's protocol. The amplicon library was sequenced in a MiSeq run (Illumina, San Diego, CA) with V4 reagents (Illumina) at the Norwegian Sequencing Centre (NSC), University of Oslo.

The Illumina sequencing data were processed using USEARCH (version 11) (https://www.drive5.com/usearch/). Merging the paired reads, trimming off primer sequences and filtering out reads shorter than 380 base pairs were carried out using the command Fastq_mergepairs. The Fastq_filter command (with an expected error threshold of 1) was used for further processing, which included the steps, demultiplexing, removal of singleton reads, and quality trimming. Unoise3 command was used for chimaera removal and generation of amplicon sequence variants (ASVs) (https://drive5.com/usearch/manual/cmd_unoise3.html). Taxonomy was assigned by applying the SINTAX script [39] with a confidence value threshold of 0.8 against the RDP reference data set (version 18). Before analysing the data, ASVs representing eukaryotic amplicons (e.g., algae, fish DNA), Archaea and Cyanobacteria/Chloroplast were removed from the ASV table. Moreover, the ASVs that were highly abundant in the DNA extraction kit blank and reported as common contaminants were removed. ASVs of special interest were further investigated with the SeqMatch tool to find the matches of those DNA sequences at the RDP website (https://academic.oup.com/nar/article/42/D1/D633/1063201).

2.4.3 Analysis of expression of immune and stress-related genes. For analysis of immune- and stress-related gene expression, larvae were collected during the endogenous (at hatch and 9 dph) and exogenous feeding periods (15, 22, and 28 dph) (Fig 1D). During the endogenous feeding period, three samples, each containing ~10 larvae were collected from the common larval rearing tank, where the larvae were housed before moving into the different experimental groups. On 15 and 22 dph, pooled samples of ~10 larvae were randomly collected from each replicate (n = 3) of the three experimental groups. On 28 dph, sampling for molecular analysis was possible only for Diet 3, as not enough larvae were available for sampling for the other two diets. Sampled larvae were immediately euthanised, preserved in RNAlater (Sigma-Aldrich St Louis, USA), and stored at -20˚C until analysis [33].

Total RNA from samples was extracted using the NucleoSpin (Mini) RNA isolation kit, following the protocol provided by the supplier (Macherey-Nagel GmbH & Co. KG, Düren, Germany). RNA concentration (110 ± 43 ng/mL) and purity (260/280 = 2.09 ± 0.03, 230/260 = 2.02 ± 0.12) were determined through spectrophotometry using Nanodrop ND-1000 (Peqlab, Germany) and normalized to 100 ng/mL with HPLC water. From the resulting total RNA, 450 ng was reverse transcribed using the qScriptTM cDNA Synthesis Kit (Quantabio, Germany) according to the manufacturer's instructions, including an additional gDNA wipeout step before transcription [PerfeCtaR DNase I Kit (Quantabio, Germany)].

Expression levels of 4 target and 3 reference genes were determined by quantitative real-time PCR (qRT-PCR). Primers were designed using primer 3 software v 0.4.01 based on sequences available in Genbank databases (Table 2). All primers were designed for an amplification size ranging from 75 to 200 nucleotides. Expression of genes in each larval sample from each tank (n = 3), diet (n = 3), and larval age (n = 5) were analysed in technical replicates

Table 2. Oligos used for molecular analysis of immune- and stress-related gene expression.

Function	Gene name	Abbreviation	Primer sequence (FW: Forward, RV: Reverse)		Accession no.
Reference	40S ribosomal S18	*rsp18*	FW	AGAGCAGGGGAACTGACTGA	XM_035428800.1
			RV	ACCTGGCTGTATTTGCCATC	
	Tubulin β	*tubb*	FW	TGATGAGCACGGTATTGACC	XM_035419873.1
			RV	TGGCACATACTTTCCACCAG	
	Elongation Factor 1a	*ef1a*	FW	CTGAAGCCTGGTATGGTGGT	XM_035428274.1
			RV	CATGGTGCATTTCCACAGAC	
Complement system	Complement component 1, Q subcomponent, C Chain	*c1qc*	FW	TCTGCTGTCATGTTCACCCA	XM_035433127.1
			RV	CTTCTCGCCATCCCTTCCAT	
Pro-inflammatory Cytokines	Interleukin 1β	*il1b*	FW	ATTGGCTGGACTTGTGTTCC	XM_035380403.1
			RV	CATGTGCATTAAAGCTGACCTG	
Pathogen recognition	Toll like receptor 18	*tlr18*	FW	TGGTTCTGGCTGTAATGGTG	XM_035421803.1
			RV	CGAAATGAAGGCATGGTAGG	
Stress/ repair	Heat shock protein 90	*hsp90*	FW	ACCATTGCCAAGTCAGGAAC	XM_035392491.1
			RV	ACTGCTCATCGTCATTGTGC	

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(n = 3) of each gene using the qPCR Biomark™ HD system (Fluidigm) based on 96.96 dynamic arrays (GE chips). In brief, a pre-amplification step was performed with a 500 nM primer pool of all primers in TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.3 mL cDNA per sample for 10 min at 95˚C; 14 cycles: 15 s at 95˚C and 4 min at 60˚C. Obtained PCR products were diluted at 1:10 with low EDTA-TE buffer. The pre-amplified products were 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 mM. The chip was run according to the Fluidigm 96.96 PCR protocol with a Tm of 60˚C.

The genes 40S ribosomal S18 (*rps18)*, tubulin β (*tubb*) and elongation factor 1a (*ef1a*) were chosen as reference genes, as they have been suggested to be the most stable in fish larvae and thus, the most reliable reference genes [40]. Their stability was statistically confirmed, as their expression was not significantly different across treatments. The relative quantity of target gene transcripts (ΔCT) was normalized to the geometric mean of the three reference genes chosen above. The coefficient of variation (CV) of technical replicates was calculated and checked. Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method [41] to calculate the expression of targeted genes relative to the levels at hatch.

2.5 Statistical analysis

2.5.1 Measures of microbial diversity. Different packages developed for R statistical software (version 4.2.0) were used to calculate diversity and perform statistical analyses. Alpha-diversity measures including estimated ASV richness (Chao-1) [42], observed ASV richness and evenness, were calculated using the vegan community ecology package (version 2.6.2). Observed ASV richness and evenness were analysed using a series of mixed model ANOVAs. Residuals were evaluated for normality and homoscedasticity (plot of residuals vs. predicted values) to ensure that they met model assumptions. Data were transformed appropriately to meet these assumptions when necessary. Beta-diversity analyses were performed on the ASV table that had been filtered to remove any ASVs that had less than 2 counts in at least two samples and rarefied by sub-sampling ten times at 11503 reads per sample (the threshold was chosen based on the sample with the lowest number of reads). Ordination by principal coordinate

analysis (PCoA, 999 permutations) based on Bray–Curtis [43] and Sørensen–Dice dissimilarity was used to visualise differences in microbial community composition between different samples using the function plot_ordination within the phyloseq package (version 1.40.0). Permutational multivariate analysis of variance (PERMANOVA) [44] based on the Bray-Curtis and Sørensen–Dice dissimilarities were used to test for differences in community composition (beta diversity) as a function of diet and age. Pairwise differences were tested using the function pairwise.adonis2 in vegan package (version 2.6.2). The package DESeq2 (version 1.36.0) was used on the unrarefied ASV table to assess the differential abundance of ASVs between the samples that were found to be significantly different by PERMANOVA. DESeq2 includes a model based on the negative binomial distribution and Wald's post hoc test for significance testing. The P-values adjustment method used was the Benjamin and Hochberg method [45], which accounts for multiple comparisons.

2.5.2 Expression of immune and stress-related genes and larval survival. R studio statistical analysis software (version 4.2.0) was used to perform all the statistical analyses. Residuals were evaluated for normality and homoscedasticity (plot of residuals vs. predicted values) to ensure that they met model assumptions. Data were transformed appropriately to meet these assumptions when necessary. Alpha was set at 0.05 for testing the main effects and interactions. Larval survival and gene expression data were analysed using a series of mixed model ANOVAs, where the main model variables were treatment (Diet 1, Diet 2, and Diet 3) and age, whereas replicated tanks were considered random. The initial model tested included an interaction effect between treatment and age. The model was reduced, when possible, validated through analyses of the residuals, and treatment means were contrasted using Tukey's honestly significant difference test (Tukey's HSD).

3. Results

3.1 Effect of type of diet on larval survival

Independent of the diet, survival significantly decreased over time. An initial sharp drop was observed during the first 3 days after initiation of feeding, followed by a period with relatively stable survival from 13 to 19 dph (Fig 2A–2C). Thereafter, a second sharp drop in survival occurred between 20 and 24 dph. A significant age × diet interaction was detected for the survival. Thus, the model was decomposed into a series of reduced one-way ANOVAs to analyse the survival as a function of age for each diet and to determine the effect of diet at each day-post-hatch; see also [18]. Diets 1 and 3 equally outperformed Diet 2 until 16 dph, while from 17 to 19 dph, highest survival was observed in the larvae fed Diet 1 (Fig 2D). However, beyond 22 dph, highest survival was observed for larvae fed Diet 3 with a 4% survival at the end of the experiment.

3.2 Bacterial community composition analysis

3.2.1 Alpha diversity. Both ASV richness and evenness in inflowing water were significantly higher than in larvae on both, 9 and 22 dph, regardless of the diet used. There was no significant age × diet interaction for the alpha diversity indices, neither for larval nor for inflowing water samples. For the larval samples, both richness and evenness were affected significantly by age, but not by diet. Richness was 68% higher ($p = 0.034$) on 22 dph compared to 15 dph (Fig 3A). On the other hand, evenness was 14% lower ($p = 0.042$) on 22 dph compared to 15 dph. The richness in the inflowing water of the RAS allocated to Diet 1 was 11% higher ($p = 0.001$) than in the inflowing water of the other two RAS units (Fig 3B). There was no effect of age on the richness of ASVs in inflowing water. Whereas diet had not significantly affected

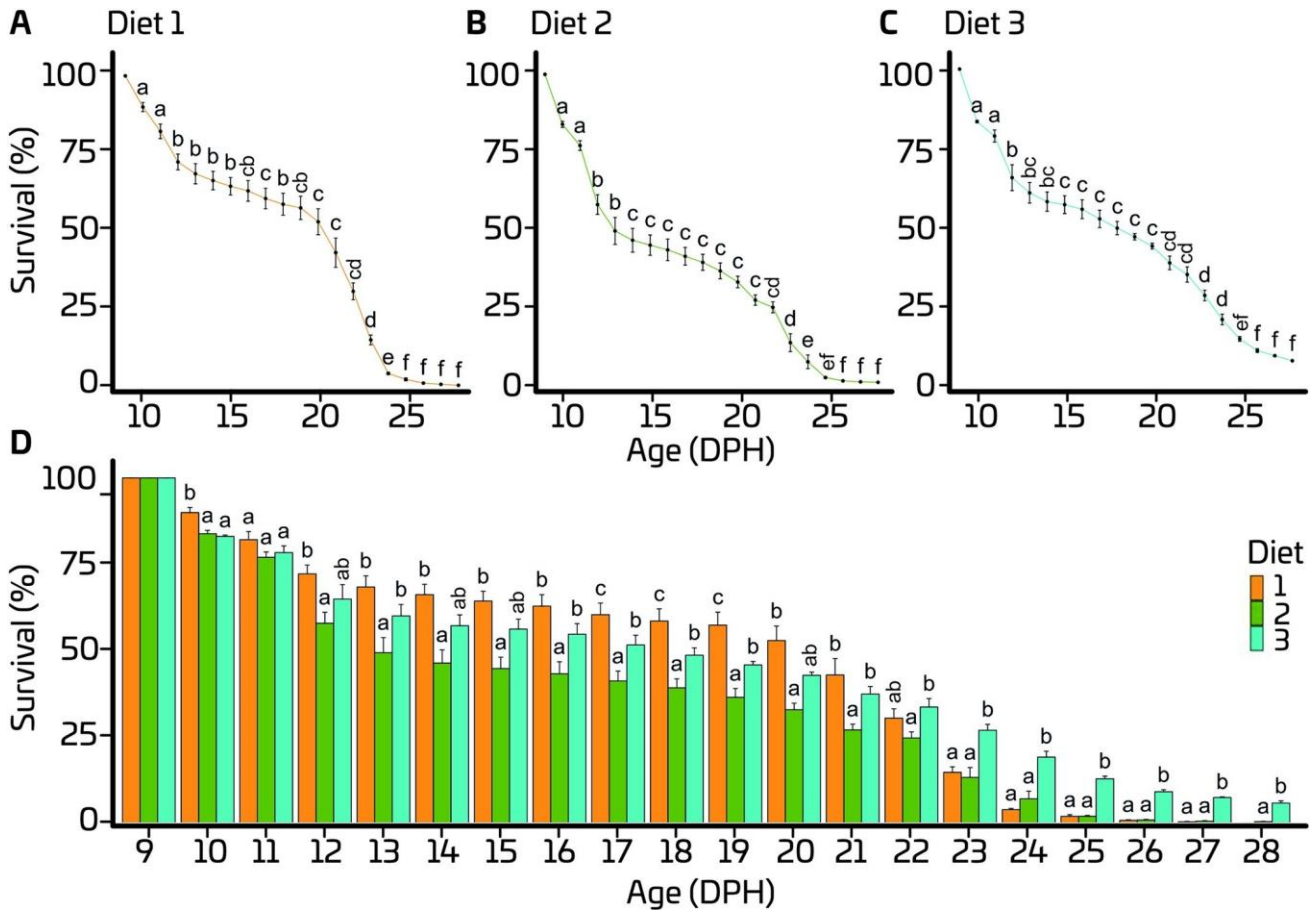

Fig 2. Effect of age for each diet (A-C) and effect of diet at each day-post-hatch on survival of European eel (*A. anguilla*) larvae. Values represent means (± SEM), while different lower-case letters represent significant differences ($p < 0.05$).

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evenness in inflowing water, a significant effect of age was detected, with 3% higher evenness on 22 dph than on 9 dph (Fig 3B).

On 9 dph, samples from inflowing water in all three RAS units were dominated by the two orders "unassigned" (>33.4%) and Alteromonadales (>11.9%) (Fig 4). Orders Rhodobacterales, Rhizobiales, Mycobacteriales, Oceanospirillales, Cellvibrionales, and Flavobacteriales were also detected in inflowing water on 9 dph, but at low abundances (<4.6%). Notably, in the inflowing water of the RAS allocated to Diet 3, bacteria belonging to the Vibrionales order, showed a higher relative abundance (20.9%) than in inflowing water of the other two RAS units. On 22 dph, the contribution of "unassigned" (>44.4%), Flavobacteriales (>3.4%) and Oceanospirillales (>3%) orders to the bacterial community in the rearing water was increased, and the share of Alteromonadales (<10.5%) was reduced compared to 9 dph. The abundance of Vibrionales was also decreased in inflowing water of the RAS allocated to Diet 3 on 22 dph compared to 9 dph. On the other hand, the larval bacterial community on 9 dph was dominated mainly by the Rhodobacterales (26.6%), "unassigned" (24%), Pseudomonadales (18.7%) and Vibrionales (14.3%) orders. A shift in the larval bacterial community composition was observed from 15 dph to 22 dph, independent of the diet fed. On 15 dph, a more even larval bacteria community, where "unassigned" (>13.1%), Alteromonadales (>9.6%), Flavobacteriales (>8%), Oceanospirillales (>6.4%), Mycobacteriales (>4.5%) and Rhodobacterales

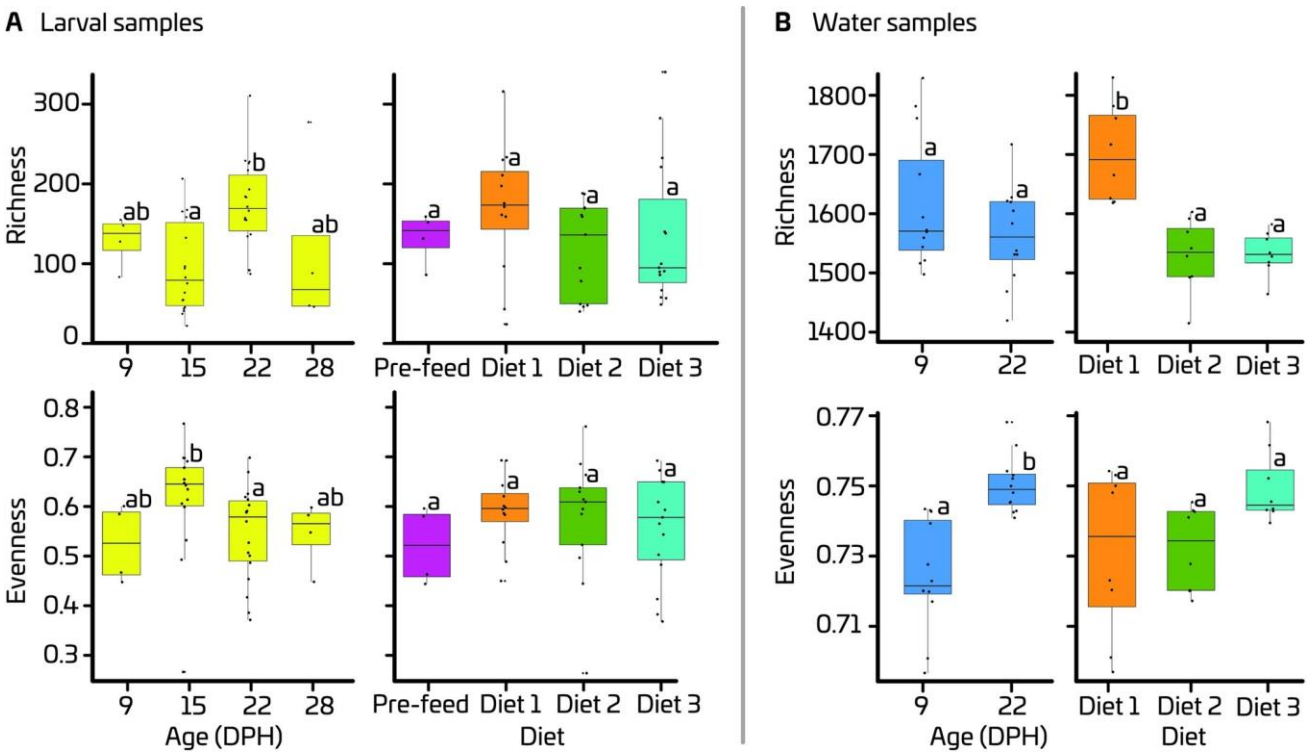

Fig 3. Effects of diets and age (Days Post Hatch (DPH)) on richness and evenness of ASVs in European eel (*A. anguilla*) larvae (A) and inflowing water (B). Yellow and blue colours indicate the larval and water samples, respectively. In each box plot, the solid black line indicates mean alpha diversity measure, and the surrounding box indicates the third quantile.

https://doi.org/10.1371/journal.pone.0288734.g003

(>0.4%) orders were abundant in all dietary groups. On 22 dph, the abundances of the orders Flavobacteriales (>22.2%), Alteromonadales (17.2%) and Oceanospirillales (14.2%) have increased comprising more than 60% of larval bacterial community composition independent of the diet. On 28 dph, larval samples were not available for Diet 1. For Diet 2, one larval sample was available, and the bacterial community of this sample was dominated by the orders, Flavobacteriales (59.2%) and Rhodobacterales (22.4%). For Diet 3, again a more even bacterial community was detected on 28 dph, where contribution of Rhodobacterales (25.7%), Lactobacillales (19.0%) and Mycobacteriales (7.6%) was increased, while contribution of Flavobacteriales (11.7%), Oceanospirillales (11.4%), and Alteromonadales (2.5%) was decreased compared to 22 dph.

3.2.2 Beta diversity. We assessed the microbial diversity between different diets, as well as over the age by performing PCoA using the Bray-Curtis and Sørensen–Dice distance metrices. The three axes of the PCoA plot based on the Sørensen–Dice distances captured a higher amount of variation (49.9%), compared to the plot based on the Bray Curtis distances (35.1%) (Fig 5). Overall, water samples were clustered tightly, whereas the larval samples showed a wider spread. Discrete grouping of the inflowing water and larval samples can be seenmainly along the axis 1 in both PCoA plots. For the inflowing water, samples were clustered based on the age, mainly along the axis 2 in both PCoA plots. Moreover, on 9 dph, inflowing water samples from the RAS allocated for Diet 3 clustered separately from other two diets in the PCoA plot based on the Bray-Curtis distances, whereas no clear separation was observed in the plot based on Sørensen–Dice distances. However, on 22 dph, no clear separation of the inflowing water samples was observed depending on the diets in PCoA plots based on both indices.

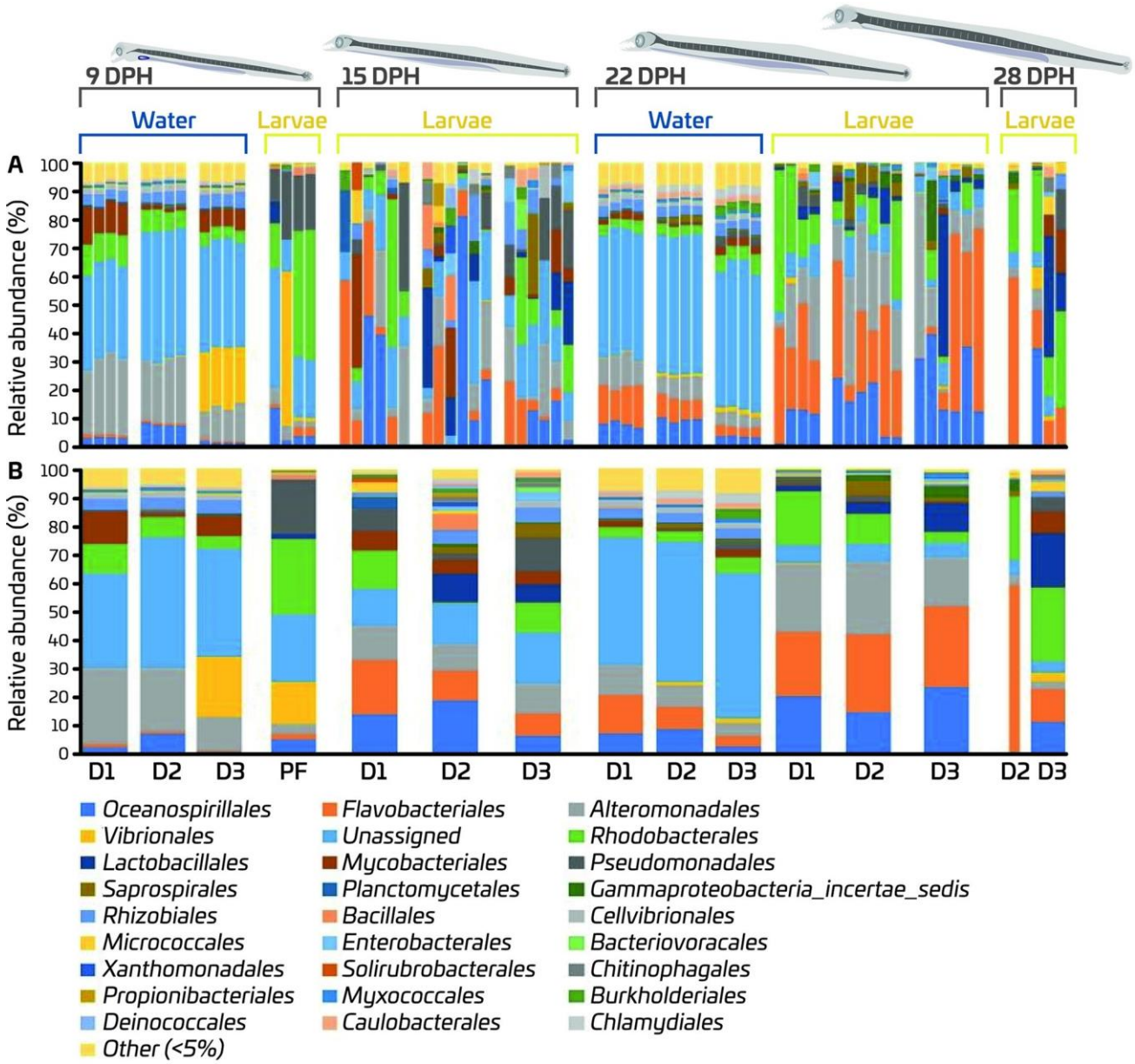

Fig 4. Relative abundances of the bacterial orders detected in European eel (*A. anguilla*) larvae as a function of feed and age, and in inflowing water from corresponding RAS units. Each stacked bar represents the relative abundances of bacterial orders detected in each replicate sample (A) and the average of relative abundances in replicate samples (B). Unassigned is ASVs that could not be classified reliably at the order level. (D1 = Diet 1, D2 = Diet 2, D3 = Diet 3, PF = Pre-feeding).

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Interestingly, samples pre-feeding larvae (at 9 dph) were distinctly clustered from the samples of feeding larvae mainly along the axis 3 especially in both PCoA plots. No clear clustering of larval samples was observed depending on the diets fed both on 15 and 22 dph. On 28 dph, samples were not enough to see the differences in larvae based on the diet they were fed.

PERMANOVA indicated that RASs allocated for three diets yielded different bacterial communities in inflowing water both on 9 and 22 dph (Table 3). Consistently, the larval bacterial community was significantly different from the bacterial community of the inflowing water to

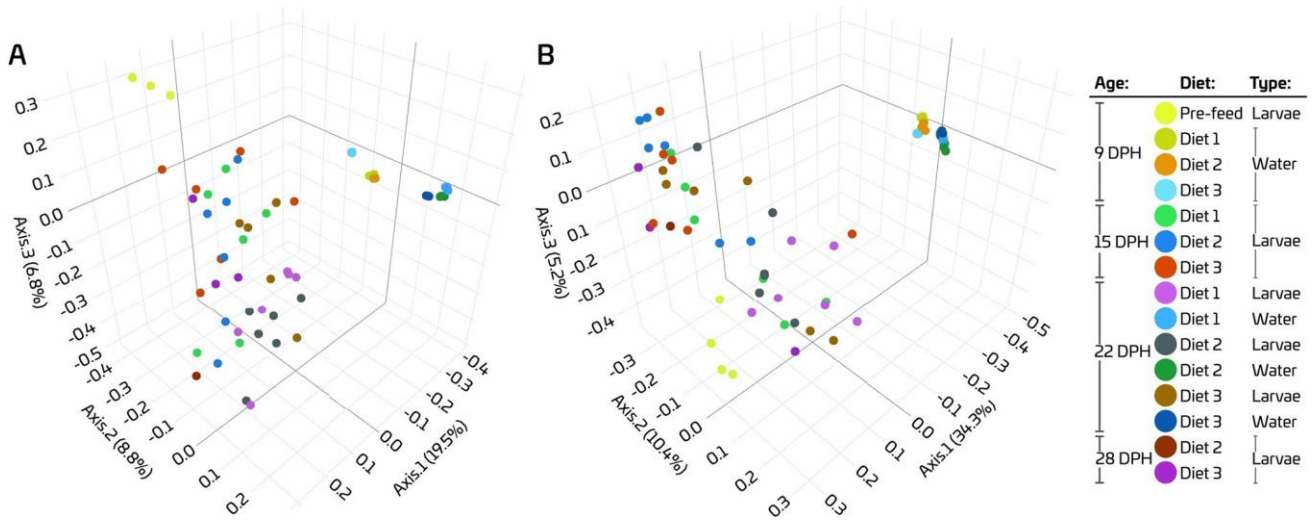

Fig 5. PCoA ordination plots based on Bray–Curtis (A) and Sørensen–Dice (B) dissimilarities for comparison of the bacterial communities of inflowing water and of European eel (*A. anguilla*) larvae feeding on different diets. Different colours indicate different samples. Bray-Curtis and Sørensen-Dice are both percent dissimilarities based on relative abundance and presence/absence, respectively.

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the rearing tank in all dietary treatments. Bacterial communities of the larvae were not significantly different between experimental groups, indicating that the diet did not influence the bacterial community composition of the larvae. Regardless of the diet, larval bacterial communities on 9 dph were significantly different from bacterial communities on 15 and 22 dph

Table 3. PERMANOVA R^2 and p-values based on Bray–Curtis and Sørensen–Dice indices for comparisons of bacterial communities between RAS water allocated for different diets, between European eel (*A. anguilla*) larvae and RAS water, and between larvae fed different diets at different developmental stages. Significance level was set at <0.05 and significant p values were bolded.

Age	Sample type	Pairwise comparison	Bray-Curtis		Sørensen-Dice	
			R^2 value	P value	R^2 value	P value
9 dph	System water	Diet 1 vs Diet 2	0.866	**0.027**	0.537	**0.034**
		Diet 1 vs Diet 3	0.891	**0.025**	0.564	**0.029**
		Diet 2 vs Diet 3	0.889	**0.031**	0.550	**0.019**
	System water vs larvae	Diet 1	0.719	**0.033**	0.735	**0.022**
		Diet 2	0.722	**0.028**	0.721	**0.023**
		Diet 3	0.694	**0.03**	0.718	**0.025**
15 dph	Larvae	Diet 1 vs Diet 2	0.094	0.378	0.129	0.079
		Diet 1 vs Diet 3	0.103	0.289	0.096	0.357
		Diet 2 vs Diet 3	0.096	0.314	0.105	0.186
22 dph	System water	Diet 1 vs Diet 2	0.822	**0.027**	0.552	**0.019**
		Diet 1 vs Diet 3	0.831	**0.027**	0.559	**0.029**
		Diet 2 vs Diet 3	0.856	**0.035**	0.578	**0.022**
	Larvae	Diet 1 vs Diet 2	0.143	0.078	0.137	0.071
		Diet 1 vs Diet 3	0.152	0.074	0.146	0.116
		Diet 2 vs Diet 3	0.178	0.054	0.125	0.153
	System water vs larvae	Diet 1	0.516	**0.003**	0.649	**0.004**
		Diet 2	0.576	**0.006**	0.577	**0.006**
		Diet 3	0.399	**0.007**	0.530	**0.006**
28dph	Larvae	Diet 2 vs Diet 3	0.352	0.500	0.385	0.250

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Table 4. PERMANOVA R^2 and p-values based on Bray–Curtis and Sørensen–Dice indices for comparisons of bacterial communities in European eel (*A. anguilla*) larvae and RAS water allocated for different diets between different ages. Significance level was set at <0.05 and significant p values were bolded.

Diet	Sample type	Pairwise comparison	Bray-Curtis		Sørensen-Dice	
			R^2 value	P value	R^2 value	P value
Diet 1	Larvae	9 DPH vs 15 DPH	0.262	**0.009**	0.242	**0.002**
		9 DPH vs 22 DPH	0.443	**0.009**	0.446	**0.007**
		15 DPH vs 22 DPH	0.165	**0.024**	0.171	**0.014**
Diet 2	Larvae	9 DPH vs 15 DPH	0.277	**0.005**	0.280	**0.005**
		9 DPH vs 22 DPH	0.468	**0.007**	0.362	**0.005**
		9 DPH vs 28 DPH	0.538	0.200	0.505	0.200
		15 DPH vs 22 DPH	0.185	**0.008**	0.169	**0.013**
		15 DPH vs 28 DPH	0.145	0.848	0.181	0.293
		22 DPH vs 28 DPH	0.319	0.156	0.337	0.144
Diet 3	Larvae	9 DPH vs 15 DPH	0.263	**0.008**	0.258	**0.004**
		9 DPH vs 22 DPH	0.321	**0.007**	0.316	**0.006**
		9 DPH vs 28 DPH	0.406	**0.036**	0.353	**0.028**
		15 DPH vs 22 DPH	0.145	**0.008**	0.117	0.14
		15 DPH vs 28 DPH	0.166	0.063	0.149	0.197
		22 DPH vs 28 DPH	0.180	0.151	0.192	0.151
Diet 1	Water	9 DPH vs 22 DPH	0.885	**0.029**	0.498	**0.019**
Diet 2	Water	9 DPH vs 22 DPH	0.894	**0.028**	0.471	**0.027**
Diet 3	Water	9 DPH vs 22 DPH	0.878	**0.029**	0.551	**0.029**

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(Table 4). For the groups fed Diets 1 and 2, bacterial communities on 15 and 22 dph were significantly different based on both dissimilarity measures. However, for the larvae fed Diet 3, bacterial communities on 15 and 22 dph were different based only on Bray-Curtis index. Moreover, for the larvae fed Diet 3, bacterial community on 28 dph was significantly different compared to 9 dph, while being comparable to bacterial communities on 15 and 22 dph. Overall, different bacterial communities in the pre-feeding and feeding larvae were detected indicating that the initiation of feeding influenced the bacterial community composition in the larvae.

3.2.3 Differential abundance testing. Differential abundance analysis revealed that ASVs of potentially harmful genera were significantly more abundant in the bacterial community of larvae on 22 dph than on 15 dph (Table 5). Moreover, we used the RDP SeqMatch tool for more precise taxonomy based on the DNA sequences of those ASVs. Interestingly, we found that the sequences of ASVs that had significantly higher abundance on 22 dph had high similarity to sequences of potentially harmful bacterial strains (Table 5) regardless of the diet fed. However, in the bacterial communities of the larvae fed Diet 1 and 3, we found significantly higher abundances of ASVs belonging to the potentially beneficial genus *Bacteriovorax* on 15 dph compared to 22 dph. However, AVSs belonging to this genus were not differentially abundant on 15 dph compared to 22 dph, in the group fed Diet 2. Differential abundance analysis revealed that potentially detrimental bacteria were more abundant on 22 dph than on 15 dph, and the abundances of ASVs belonging to the potentially beneficial genus *Bacteriovorax* decreased on 22 dph compared to 15 dph.

3.3 Expression of immune and stress-related genes

Mixed model ANOVAs were used to analyse the relative gene expression data, where the main model variables were age (9, 15, 22 and 28 dph) and treatment (Diet 1, Diet 2, and Diet 3). The

Table 5. ASVs of potentially harmful genera that were found by DESeq2 analysis to be significantly more abundant ($p < 0.05$) in larval bacterial communities on 22 dph compared to 15 dph in different experimental groups and nearest matches of the DNA sequences of those ASVs as found by the RDP SeqMatch tool.

ASV ID	Diet fed	Genus	log_2 increase in abundance	RDP match	S_ab score
ASV 94	Diet 1	*Vibrio*	>21	*Vibrio harveyi*	1.00
ASVs: 133 and 296	Diet 1	*Vibrio*	> 21	*Vibrio alginolyticus*	> 0.92
ASV 154	Diet 1 and 3	*Vibrio*	>21	*Vibrio campbellii*	1.00
ASVs: 15 and 1921	Diet 1 and 3	*Enterococcus*	>7	*Enterococcus faecalis*	> 0.90
ASV 3808	Diet 1	*Enterococcus*	>7	*Enterococcus faecalis*	> 0.90
ASV 96	Diet 1	*Pseudomonas*	>10	*Pseudomonas stutzeri*	1.00
ASV 2691	Diet 1	*Shewanella*	>10	*Shewanella sp.*	1.00
ASV 4	Diet 2	*Lactococcus*	>21	*Lactococcus lactis*	1.00
ASV 127	Diet 2	*Pseudomonas*	>21	*Pseudomonas psychrophile*	1.00
ASV 156	Diet 2 and 3	*Flavobacterium*	>21	*Flavobacterium columnare*	1.00
ASV 1425	Diet 2	*Stenotrophomonas*	>21	*Stenotrophomonas maltophilia*	0.95
ASV 236	Diet 2	*Vibrio*	>7	*Vibrio fluvialis*	0.96
ASV 48	Diet 3	*Micrococcus*	>25	*Micrococcus luteus*	1.00
ASV 55	Diet 3	*Mycobacterium*	>25	*Mycobacterium frederiksbergense*	1.00

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initial model tested included an interaction effect between age and treatment. Expression of all selected immune and stress related genes were affected by the age × treatment interaction ($p < 0.01$). Thus, the model was decomposed into a series of one-way ANOVAs to determine the effect of age for each diet and the effect of diets for each age. In larvae fed Diet 1, relative expression level of pro-inflammatory cytokine, *il1b* was 50% and 62% lower ($p < 0.0001$) on 9 dph than on 15 and 22 dph, respectively (Fig 6A i). In the group fed Diet 2, *il1b* increased ($p < 0.0001$) throughout larval development and peaked on 22 dph (Fig 6A ii). In larvae fed Diet 3, this gene was upregulated ($p < 0.0001$) until 22 dph and then remained relatively stable at high levels (Fig 6A iii). On 15 dph, no effect of diet was found on expression of *il1b*, whereas higher ($p < 0.0001$) expression of this gene was found in the larvae fed Diet 3 than in the larvae fed Diet 1 on 22 dph (Fig 6A iv).

A pathogen recognition molecule, *tlr18* was expressed at a stable level throughout development in larvae fed Diet 1 (Fig 6B i). In larvae fed Diet 2, expression of *tlr18* was downregulated ($p < 0.01$) on 15 and 22 dph compared to 9 dph (Fig 6B ii). The expression level of this gene peaked ($p < 0.0001$) on 22 dph compared to other ages in larvae fed Diet 3 (Fig 6B iii). On 15 dph, a higher (p ⧀ 0.0001) expression level of *tlr18* was observed in larvae fed Diet 1 than in larvae fed Diet 2 and 3. On 22 dph, the highest ($p < 0.0001$) expression level of this gene was detected in larvae fed Diet 3, whereas the lowest ($p < 0.001$) expression levels were detected in larvae fed Diet 2 (Fig 6B iv).

Expression of the complement component, *c1qc*, was downregulated ($p < 0.0001$) throughout development in larvae fed Diet 1 (Fig6C i). In larvae fed both Diet 2 and 3, a peak ($p < 0.0001$) in expression of *c1qc* was observed on 9 dph, after which the expression levels were stable at relatively low levels (Fig 6C ii and iii). On 15 dph, higher ($p < 0.0001$) expression of this gene was observed in larvae fed Diet 1 compared to larvae fed Diet 2. On 22 dph, *c1qc* was expressed at higher ($p < 0.0001$) levels in the larvae fed Diet 3 compared to larvae fed Diet 1 and 2 (Fig 6C iv).

The expression of the gene *hsp90*, which is related to cellular stress response and repair mechanism, peaked ($p < 0.05$) on 22 dph in all larval groups, regardless of the diet fed (Fig 6D i, ii and iii). No differences in expression levels of this gene were detected on 15 dph among the larval groups fed different diets. However, on 22 dph, *hsp90* expression was higher

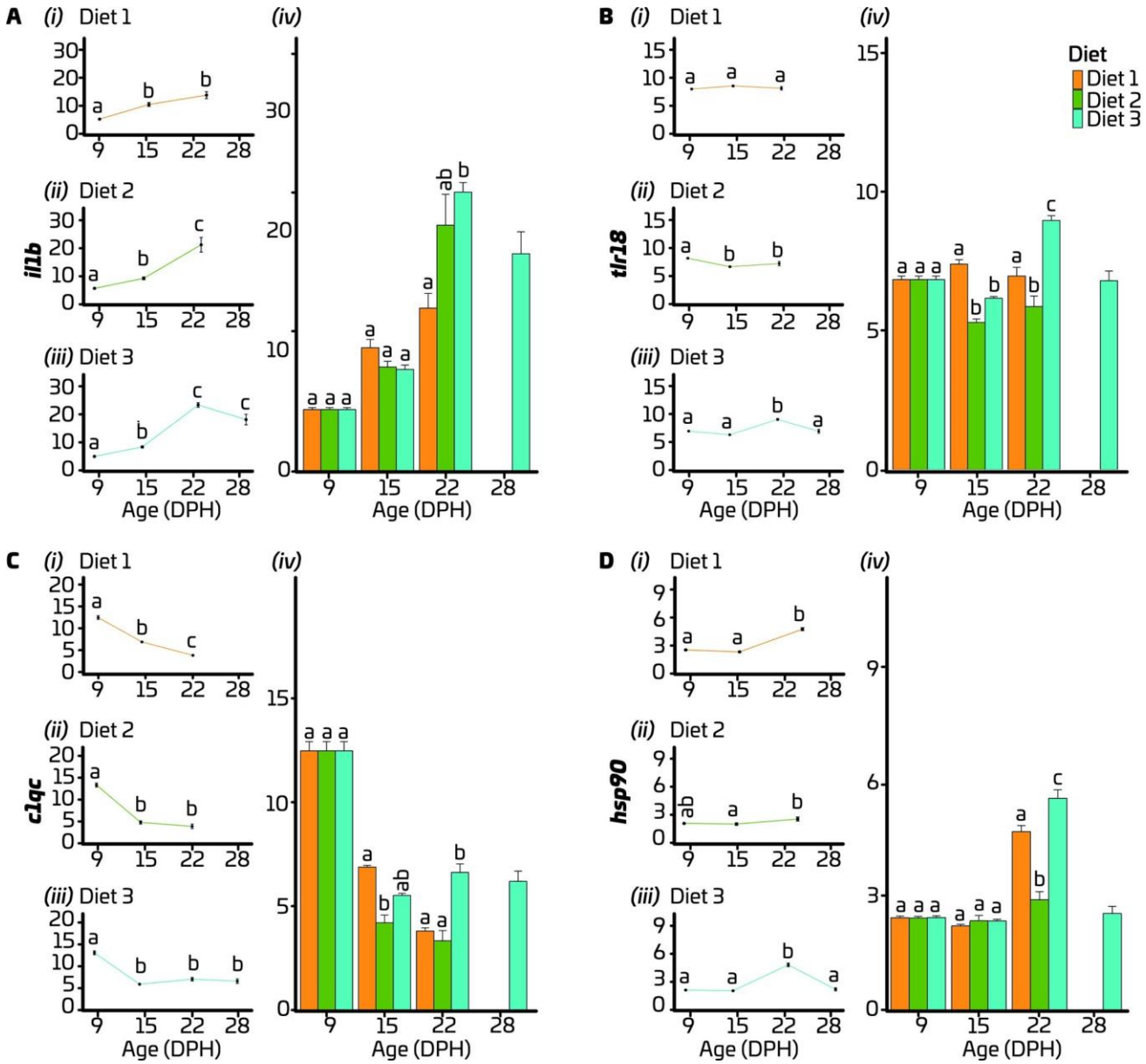

Fig 6. Expression of immune-related genes, *il1b* (A), *tlr18* (B), *c1qc* (C) and stress-related gene, *hsp90* (D) in European eel (*A. anguilla*) larvae fed three different diets. All y-axes display gene expression relative to the expression at hatch (0 dph). In each facet, the graphs, i, ii and iii show the effect of age on expression of the relevant gene for each diet, whereas the graph iv shows the effect of diets on gene expression at each age. Values represent means (± SEM), and different lower-case letters represent significant differences ($P < 0.05$).

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($p < 0.05$) in larvae fed Diet 1 compared to larvae fed Diet 2 (Fig 6D iv) and highest ($p < 0.0001$) in larvae fed Diet 3.

Overall, a gradual increase in the expression levels of *il1b* was detected with the age independent of the diet fed. The highest expression levels of *c1qc* were observed on 9 dph in all experimental groups. Moreover, a significant peak in the expression of *hsp90* was observed on 22 dph independent of the diet fed. The group fed Diet 3 showed significantly higher expression levels of *tlr18* and *c1qc* on 22 dph compared to other two dietary groups. The expression

levels of *il1b* in this group was higher than that in the group fed Diet 1, while being comparable to the levels in the group fed Diet 2.

4. Discussion

Improvements in assisted reproduction, embryonic incubation and larval rearing have led to a stable production of European eel larvae that are ready to enter the feeding stage. A recent study [18] tested three different experimental slurry-type diets and reported the first successful rearing of European eel beyond the first feeding window. However, two periods of high mortalities were observed, whereas initial mortality occurred shortly after feeding initiation and then again during the period from 20 to 24 dph, indicating the point of no return. In this regard, initiation of feeding, especially with a formulated and slurry-type diet, might lead to deterioration of water quality, due to effusion of nutrients. Consequently, during first-feeding the larvae might be challenged to live in a microbially hostile environment. Here, the innate immune system of larvae might play an important role in protecting the larvae against microbial interference. As such, in the present study, we explored the succession of bacterial communities in larvae and RAS water, but also followed expression patterns of immune and stress-related genes in response to first-feeding diets in European eel. Furthermore, we attempted to disclose the association among bacterial community composition, immune gene expression and larval performance.

Interestingly, several ASVs showing higher similarities to *Bacteriovorax* sp. were found in higher abundances ($> \log_2 22$) on 15 compared to 22 dph in the bacterial communities of larvae fed with Diets 1 and 3. *Bacteriovorax* species are well known for their ability to predate on Gram-negative bacteria, by attaching and penetrating through the cell wall to form a bdelloplast and multiply in the periplasmic space of the host bacterium [46, 47]. They play an important role by reducing the bacterial density, while altering microbial community composition through predation and are known to predate on potentially harmful members of Gammaproteobacteria in aquaculture (e.g., bacteria belonging to genera *Vibrio* and *Aeromonas*) [48, 49]. As such, ASVs of the *Bacteriovorax* genus might have played an important role in maintaining a healthy larval bacterial community on 15 compared to 22 dph. We have no data suggesting reasons for the decline in relative abundance of *Bacteriovorax*.

Regarding the characteristic mortality patterns during the first feeding period (as described in [18]), in addition to that the larvae reached the point of no return, the second sharp drop of larval survival could be associated with a shift towards a detrimental larval bacterial community, dominated by potentially harmful bacteria. Interestingly, the analysis of bacterial community composition, conducted in the present study revealed that evenness in the larval bacterial community on 22 dph was lower than on 15 dph, which was noticeable also at the order level of taxonomy. On 22 dph, bacteria of the orders Oceanospirillales, Flavobacteriales, and Alteromonadales have dominated the bacterial community in larvae, regardless of the diet fed. In some cases, these orders have previously been reported to have detrimental effects on marine organisms. For instance, an increase in abundances of bacteria of orders Oceanospirillales and Alteromonadales, which belong to the class of Gammaproteobacteria, have often been associated with stressed and diseased marine invertebrates [50–53]. As such, dominancy of these potentially harmful bacterial orders in the community of 22 dph larvae supports our hypothesis that the second sharp drop in larval survival is linked to a shift towards a deleterious larval bacterial community.

Independent of the diet fed, several ASVs that were more abundant in the larval bacterial communities on 22 dph have been reported as pathogens or opportunistic pathogens. For instance, ASV 94 is similar to *Vibrio harveyi*, which is pathogenic to aquatic organisms [54].

Similarly, DNA sequences of ASVs 133 and 296 matched the sequence of *Vibrio alginolyticus*, frequently reported as the putative agent for outbreaks of vibriosis in cultured fish, such as Gilt-head sea bream (*Sparus aurata*) [55] and grouper (*Epinephelus malabaricus*) [56], and is also associated with abdominal swelling in larvae of several fish species [57, 58]. Moreover, ASV 154 matched *Vibrio campbellii*, which is a pathogen in shrimp hatcheries [59], while ASVs 15, 1921, and 3808 had highest similarity to *Enterococcus faecalis*, an opportunistic fish pathogen, which is one of the causative agents of haemorrhagic septicaemic disease in fish and associated with high mortalities [60–62]. Furthermore, ASV 96 was similar to *Pseudomonas stutzeri*, which is an immerging pathogen in Red Sea seabream (*Diplodus noct*) [63], while ASV 2691 matches *Shewanella* sp., which is also an opportunistic pathogen in fish [64]. Therefore, our hypothesis that the larval bacterial community was dominated by potentially detrimental bacteria on 22 dph and that this could be the reason for parts of the mortality, was supported also at the ASV level.

Considering the inflowing RAS water, a more even bacterial community structure was observed on 22 compared to 9 dph, where the fraction of Vibrionales, which appeared in the RAS allocated to Diet 3, clearly decreased by 22 dph. In aquaculture, several members of the order Vibrionales are known pathogens or opportunistic pathogens for reared finfish, shellfish, and shrimp [65]. In this regard, the ability of RAS to lower the fraction of opportunistic bacteria by developing and maintaining a more even and stable microbial community composition with higher species diversity has previously been described [19]. Moreover, in the present study, we observed that the bacterial communities of the larvae were dissimilar to the bacterial communities of the inflowing water. This might be an indication that even though the inflowing water probably sourced the bacteria for the larval bacterial community, selection inside the rearing tanks played an important role for community assembly [66]. Here, especially due to the atypical feeding behaviour of European eel larvae in captivity, where they dive into a puddle of slurry-type diet, it is important to mention that food-related bacteria might also be substantially driving the larval bacterial community structure. However, further experimentation is required to confirm this.

At the same time, on 22 dph, in all groups of larvae, regardless of the diet, expression of *hsp90* peaked on 22 dph. Expression patterns of heat shock proteins are affected in a wide variety of fish cells and tissues in response to both biotic stressors (such as infectious pathogens) as well as abiotic stressors (such as temperature). Generally, heat shock proteins are constitutively expressed in cells to maintain a number of critical cellular processes relating to protein folding, fidelity and translocation with the task to ensure survival by protecting vital cellular functions [67]. As such, this family of proteins is also commonly referred to as “stress/repair proteins” and an upregulation of *hsp90* has been previously described in several fish species, including the responses of silver sea bream (*Sparus sarba*) to *Vibrio alginolyticus* [68], miiuy croaker (*Miichthys miiuy*) to *Vibrio anguillarum* [69], and marbled eel (*Anguilla marmorata*) to *Aeromonas hydrophila* [70]. Thus, in the present study, the upregulation of *hsp90* observed on 22 dph, might be a response to the presence of potentially harmful bacteria in the larval bacterial community, triggering the activation of the stress/repair mechanism, which might be of key essence, especially considering that the larval immunocompetence is not yet fully developed.

Regarding immunocompetence, generally, the innate immune system recognizes conserved pathogen-associated molecular patterns (PAMPs) through their interaction with specific pattern recognition receptors (PRRs) and can facilitate a direct successful removal of pathogens, e.g. by phagocytosis, or may trigger additional protective responses through induction of adaptive immune responses [71]. In this regard, toll-like receptors (TLRs) are evolutionarily conserved PRRs that play a crucial role in innate immune responses by activation of immune cells that combat invading pathogens [72]. For instance, TRL18 is a member of the fish-specific

TLR1 subfamily, reported to directly interact with adaptor proteins and signal downstream to modulate the production of pro-inflammatory cytokines and numerous other immune-related proteins [73]. Cytokines, are small proteins interacting with cells, ligands, and receptors to activate cell-mediated immune responses that aid both, innate and acquired immune system [74]. Here, expression of IL-1β, a pro-inflammatory cytokine, leads to activation of lymphocytes and synthesis of acute phase proteins and thus, activation of the complement system [75]. On the other hand, C1QC is a member of the complement system, aiding several immune functions, including pathogen opsonization, phagocytosis, and inflammatory reactions [76]. In the present study, highest expression levels of the PRR *tlr18* and complement component *c1qc* were detected on 22 dph in the larvae fed with Diet 3. Similarly, expression of the pro-inflammatory cytokine *il-1b* was higher in the larvae fed Diet 3 compared to Diet 1. In other fish species, upregulation of these immune genes has been linked to challenges with pathogenic bacteria or PAMPs [73, 77–81]. As such, the immune components related to pathogen recognition, cellular signalling pathway and complement proteins, important for opsonization, phagocytosis and eventual lysis of harmful microorganisms, which were expressed at higher levels on 22 dph in the larvae fed with Diet 3, potentially indicate a higher immunocompetency, probably assisted in handling the potentially harmful bacteria that dominated the larval bacterial community at this stage, leading to better performance in terms of survival.

Overall, Diet 3 has sustained physiological mechanisms vital for life compared to the other two diets tested, allowing larvae to survive throughout and beyond the first-feeding window [18]. As such, a factor contributing to the higher survival observed in larvae fed Diet 3 might be a higher immunocompetency, which could potentially be supported by the immunomodulatory properties of the included dietary whey proteins, allowing the larvae to better cope with their hostile microbial environment. Whey protein concentrate is considered to be an immune stimulating agent, because it contains bioactive compounds such as α-lactalbumin, β-lactoglobulin, all casein fractions, and lactoferrin (reviewed in [82]). Inclusion of whey protein to replace up to 27.7% of fish meal in Nile tilapia (*Oreochromis niloticus*) fingerling diets has shown to improve performance in terms of growth and survival as well as immune status of fish challenged with *A*. *hydrophila* [83]. Similarly, improved lysosomal and phagocytic activity has been reported in response to inclusion of whey in Barramundi (*Lates calcarifer*) diets [84]. In our study, it is important to mention that an elevated immune response and higher survival were only observed in the larvae fed Diet 3 and not Diet 2, which also included whey proteins, but in much lower levels, indicating that this inclusion level might be insufficient to facilitate the same immunomodulatory effect. Thus, dietary inclusion of whey proteins might be of value, but inclusion levels need to be considered in future.

5. Conclusion

To conclude, first-feeding culture of European eel larvae was characterized by a critical period close to the end of the first-feeding window (around 22 dph), which was marked by a sharp drop in survival and an increased activity of the stress/repair mechanism as indicated by elevated expression of *hsp90*. At the same time, the larvae were exposed to a hostile microbial environment, where potentially harmful bacteria dominated the larval bacterial community, indicating that the steep drop in survival observed during the critical period was probably linked partly to detrimental larvae-bacteria interactions. Moreover, the larval group fed Diet 3 that contained whey protein (10%) showed a better immunocompetency on 22 dph, which presumably aided this larval group in handling the potentially detrimental bacteria better than the other two groups, and thus perform (i.e., survival) better after the critical period. The inclusion of whey seems to benefit the European eel larvae; however, inclusion levels need to be

optimised. For the first time, the present study reveals the bacterial interference during rearing of the feeding European eel larvae and the significance of larval immunocompetency for sustaining larval survival in a microbially hostile environment.

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Author Contributions

Conceptualization: Kasun A. Bandara, Sebastian N. Politis, Luis E. C. Conceição, Jonna Tomkiewicz, Olav Vadstein.

Data curation: Kasun A. Bandara, Elisa Benini, Sebastian N. Politis, Luis E. C. Conceição, Jonna Tomkiewicz, Olav Vadstein.

Formal analysis: Kasun A. Bandara.

Funding acquisition: Sebastian N. Politis, Jonna Tomkiewicz, Olav Vadstein.

Investigation: Kasun A. Bandara, Elisa Benini, Sebastian N. Politis.

Methodology: Kasun A. Bandara, Elisa Benini, Sebastian N. Politis, Luis E. C. Conceição, André Santos, Jonna Tomkiewicz, Olav Vadstein.

Project administration: Jonna Tomkiewicz, Olav Vadstein.

Resources: Luis E. C. Conceição, Sune Riis Sørensen, Jonna Tomkiewicz, Olav Vadstein.

Supervision: Sebastian N. Politis, Jonna Tomkiewicz, Olav Vadstein.

Validation: Kasun A. Bandara, Sebastian N. Politis, André Santos, Jonna Tomkiewicz, Olav Vadstein.

Visualization: Kasun A. Bandara, Sune Riis Sørensen.

Writing – original draft: Kasun A. Bandara.

Writing – review & editing: Kasun A. Bandara, Elisa Benini, Sebastian N. Politis, Luis E. C. Conceição, André Santos, Sune Riis Sørensen, Jonna Tomkiewicz, Olav Vadstein.

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Study 3:

Effect of food amounts on larval performance, bacteriome and molecular immunologic development during first-feeding culture of European eel

Kasun A. Bandara, Sebastian N. Politis, Sune Riis Sørensen, Elisa Benini, Jonna Tomkiewicz, Olav Vadstein

Manuscript

Effect of food amounts on larval performance, bacteriome and molecular immunologic development during first-feeding culture of European eel

Authors:

Kasun A. Bandara[1,2*], Sebastian N. Politis[1], Sune Riis Sørensen[1], Elisa Benini[1], Jonna Tomkiewicz[1], Olav Vadstein[2]

Affiliation:

[1]Technical University of Denmark, National Institute of Aquatic Resources, 2800 Kgs. Lyngby, Denmark

[2]Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, 7491 Trondheim, Norway

Abstract

Closure of the life cycle in captivity is essential to ensure the sustainability of commercial aquaculture and assist stock management attempts of the European eel (*Anguilla anguilla*), which is categorised as “Critically Endangered” on the IUCN red list. While prior research has successfully produced larvae entering the feeding stage, challenges remain in cultivating feeding larvae. Initial studies on first-feeding diets showed that European eel larvae can survive beyond the point of no return on slurry-type food, but refining feeding regimes is necessary. The present study focused on increasing food amounts without compromising healthy larval-bacterial interactions and well-being to potentially enhance feeding success and growth. Accordingly, first-feeding European eel larvae (at 9 days post-hatch (dph)) were fed two different slurry-type food amounts (Low = 0.5 mL food / L water *vs.* High = 1.5 mL food / L water) until 30 dph to assess the effects on feeding success, growth, and survival. Also, the expression of genes related to immunity, stress, and food ingestion was examined. Additionally, the bacteriomes of water, larvae, and food, along with changes in the larval bacteriome throughout ontogeny were studied. Our findings indicate that approximately 75% of larvae ingested the provided diet in both food amount treatments. Interestingly, larvae in the High-food group had 45.2% more ($p < 0.001$) food in their guts compared to the Low-food group. Moreover, the gene encoding ghrelin (*ghrl*), known as the "hunger hormone" was upregulated in the Low-food group, suggesting starvation. On the other hand, none of the studied genes related to stress and immunity were affected by food amounts. However, the upregulation of the stress/repair-related gene encoding heat shock protein 90 (*hsp90*) on 25 and 30 dph possibly indicates nutritional inadequacy. Notably, while survival was initially lower in the High-food group, both treatments had comparable survival by the end of the experiment. Furthermore, the effect of food amount on the larval bacteriome became evident by 15 dph, however, only in the High-food group, with significant differences observed between experimental groups by 30 dph. Here, the High-food group exhibited a healthier bacteriome with a higher abundance of potentially beneficial orders (e.g., Lactobacillales and Bacillales), whereas the Low-food group showed more potentially harmful orders (e.g., Vibrionales, Rhodobacterales and Alteromonadales). This shift towards a healthier bacteriome in the High-food group was likely influenced by the food-associated bacteriome, primarily composed of the Lactobacillales order (90%). In conclusion, feeding European eel larvae with High food amounts resulted in several advantages, including increased gut fullness together with the molecular evidence of reduced *ghrl* expression (no starvation), faster growth, absence of induced cellular stress responses, and the presence of healthier bacteriome at 30 dph.

Keywords: *Anguilla anguilla*, aquaculture, bacterial interference, molecular immune response

1 Introduction

European eel (*Anguilla anguilla*) is a commercially important finfish species for aquaculture and fisheries in Europe, but commercial aquaculture and stock enhancement rely entirely on wild-caught juveniles called glass eels (Nielsen and Prouzet, 2008). However, the European eel is ranked as critically endangered on the IUCN red list as the stock has diminished (Pike et al., 2020). Therefore, the establishment of hatchery techniques and technology for this species is indispensable to ensure the sustainability of aquaculture, but also assisting stock management and conservation plans. Ongoing research regarding biology and technology for assisted reproduction, embryo incubation, and larval rearing has made it possible to consistently produce European eel larvae that reach the feeding stage within 10-12 days post-hatching (dph) (Tomkiewicz et al., 2019). At present, challenges in raising feeding larvae revolve around optimising their diets and feeding schedules (including food quantities, timing, and duration), refining rearing methods, and managing microbial aspects.

For Anguillid eels in general, it is a curtailment to establish effective diets and feeding regimes for rearing first-feeding larvae, due to limited knowledge of their natural diets during that stage. The gut content of wild leptocephalus larvae consists of amorphous material of different origins, such as appendicularian houses, faecal pellets of zooplankton, gelatinous zooplankton, and other materials associated with marine snow (Ayala et al., 2018; Miller et al., 2019). In sharp contrast to their natural diets, the diets that were proven successful for captive-reared Japanese eel (*Anguilla japonica*) (Tanaka et al., 2001) and European eel (Benini et al., 2023) larvae are slurry-type diets, based on egg yolk of spiny dogfish (*Squalus acanthias*). With this type of diet, the lifecycle of the Japanese eel has been closed in captivity, leading to the production of further generations of captive propagated offspring (Tanaka, 2015). However, establishing a first feeding larval culture protocol is at a pioneering stage for the European eel. Specifically, knowledge regarding feeding behaviour, stage-specific diets and feeding regimes is still lacking.

At present, European eel larvae are reared in low salinity of ~18 PSU (Politis et al., 2018), while water circulation is turned off to enable feeding, which due to negative buoyancy, occurs at the bottom of the tank. Here, the slurry-type diet is poured in creating a "food puddle" for larvae to dive into. Thus, the addition of sufficient food amount, which is evenly distributed on the tank bottom, providing enough food and space for all larvae to feed, is crucial. Currently, European eel larvae are fed 0.5 mL of food / L of rearing water (Benini et al., 2023), which is three times less than the amount (1.5 mL food / L of rearing water) fed to the closely related Japanese eel (Okamura et al., 2019). Thus, we hypothesised that increasing the amount of food given to European eel larvae might increase the feeding success in terms of feeding incidence (i.e., the fraction of larvae that ingest food) and gut fullness (i.e., the gut fraction with food present). On

the other hand, we considered that the addition of higher food amounts might be selecting for detrimental and opportunistic bacteria due to the higher availability of substrate (i.e., dissolved organic matter – DOM) for bacterial growth.

When fish larvae experience a microbially hostile environment, the larval immune system plays an important role in handling the response towards potentially harmful bacteria. However, the immune system of newly hatched fish larvae is generally not fully developed and thus, highly sensitive to detrimental bacteria, especially under intensive rearing conditions that might cause stress (Vadstein et al., 2013). In European eel, a sensitive phase was reported during early life, where recently hatched larvae are potentially immuno-compromised (Miest et al., 2019), while nutritional aspects (such as dietary composition or specific ingredients) can influence immune gene expression in feeding eel larval culture, potentially linking to improved survival even though the larvae were subjected to potentially harmful opportunistic bacteria during the earlier first-feeding stage (Bandara et al., 2023).

In this regard, the bacterial community structure of a fish larval rearing system depends on the supply of bacteria and DOM as well as the selective forces inside the rearing tank and the water source. The supply of DOM is mainly the limiting factor for the growth of heterotrophic bacteria, the group interacting directly with fish larvae and defining their carrying capacity (CC; the number of bacteria that the system can sustain over time) (Attramadal et al., 2012; Vadstein et al., 1993). In the case of rearing eel larvae, the addition of food, which is composed of perishable ingredients (e.g., shark egg yolk), quickly results in elevated and oscillating DOM loading, and thus, higher CC in the rearing tank. The resulting perturbed environment and sudden increase in DOM concentration, creates niches for colonisation by r-selected opportunists (Hess-Erga et al., 2010). Therefore, we hypothesised that increasing the amount of food added to the rearing tank might increase the magnitude of selective forces favouring r-selection and thus, the growth of opportunistic bacteria in the rearing tank. Here, r-selected and opportunistic bacteria are known to negatively interact with marine fish larvae, causing low and unpredictable growth and survival during rearing (Attramadal et al., 2012; Vadstein et al., 2004). For the European eel, bacterial interference during egg incubation and early larval rearing has been demonstrated, where hatching success and longevity of non-feeding larvae were negatively affected (Sørensen et al., 2014). Moreover, a drop in survival has been associated with the selection for opportunistic bacteria that dominated the larval bacterial community during the rearing of European eel feeding larvae (Bandara et al., 2023).

As such, the present experiment was designed to identify the appropriate food amount that should be used to feed European eel larvae with the aim to gain potential benefits, such as improved feeding success and growth, but without compromising healthy larvae-bacterial interactions and

larval wellbeing. For this, two slurry-type food amounts were tested: Low - 0.5 mL food / L water (as in Benini et al., 2023) and High - 1.5 mL food / L water (as in Tanaka et al., 2001) to evaluate the effects on feeding success, growth, and survival of European eel larvae. Moreover, the bacterial community composition of water, larvae, and food, as well as the changes in the larval bacterial community over time were studied. Finally, the expression of genes related to immunity, stress, and food ingestion was investigated in response to both food amounts.

2 Materials & methods

2.1 Ethics statement

All fish were handled according to the European Union regulations concerning the protection of experimental animals (Dir 2010/63/EU). Experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2020-15-0201-00768). Broodstock was anaesthetised individually before tagging, biopsy, and stripping of gametes, while euthanised after stripping (females) or at the end of the experiment (males) by submergence in an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg/L, Sigma Aldrich, Germany). Larvae were anaesthetised and euthanised using tricaine methanesulfonate (MS-222, Sigma Aldrich, Germany) at a concentration of 7.5 and 15 mg/L, respectively.

2.2 Experimental animals

The experiment was conducted at EEL-HATCH, Hirtshals, Denmark (57.5858410°N, 9.9853423°E), an experimental hatchery that belongs to DTU Aqua. European eel larvae that were used in this experiment were obtained from a batch originating from a farm-reared broodstock, sourced from Lyksvad Fish farm K/S, Vamdrup, Denmark (55.4311115°N, 9.4007440°E) and Royal Danish Fish A/S, Hanstholm, Denmark (57.1226075°N, 8.6243243°E), respectively. Gametes were obtained through assisted reproduction as previously described (Kottmann et al., 2020). After fertilisation, using standardised procedures (Butts et al., 2014), fertilised eggs were incubated in 60 L conical bottom incubators supplied with filtered and UV-treated North Sea water. Here, salinity was adjusted to ~36 PSU using Sea Salt (Aquaforest, Brzesko, Poland) (Sørensen et al., 2016) and temperature kept at ~18°C (Politis et al., 2017). At ~52 hours post-fertilisation (hpf), aeration was stopped, while embryos hatched at ~56 hpf. Newly hatched larvae were transferred to a 77 L rearing tanks connected to a recirculating aquaculture system (RAS). The RAS was composed of a biofilter (RK elements, 750 m^2 per 1 m^3, RK BioElements, Skive, Denmark), a protein skimmer (Turboflotor 5000 single 6.0, Aqua Medic GmbH, Bissendorf, Germany) and a UV light (11 W, JBL ProCristal, Neuhofen, Germany). Larvae were reared during this pre-feeding period (from hatch to 9 dph), under constant darkness

(Politis et al., 2014), while temperature and salinity were maintained at ~20°C and ~36 PSU (Benini et al., 2022), respectively.

2.3 *Experimental design*

On 9 dph, the salinity of the rearing water was reduced by connecting the rearing tank to a similar RAS (described in 2.2) supplying water at ~18 PSU. At the end of day 9 (post-hatch), the larvae were transferred to replicated 8 L Kreisel tanks (n = 6) at a density of ~180 larvae/L and randomly connected to one of two separate but identical RAS units (three Kreisel tanks per RAS), which were allocated to one of the two experimental treatments (Low and High). The Low treatment received 0.5 mL food / L of water as described for the European eel earlier by Benini et al. (2023), while the High treatment received 1.5 mL food / L of water as described by Okamura et al. (2019) for the Japanese eel. Each RAS was composed of an upper sump reservoir of 370 L which housed an 80 L wet/dry trickle filter filled with RK bio-elements (240 m^2 surface area or 0.12 m^2 per L), a lower sump reservoir (260 L) and a protein skimmer (Aquamedic 5000 single 6.0, Bissendorf, Germany). An extra water reservoir of 160 L created head pressure, while the water was UV-treated (ProCristal UV-C 11W, JBL GmbH & Co. Neuhofen, Germany) before reaching the rearing tanks. During the exogenous feeding period of the experiment, (i.e., from 10 to 30 dph) temperature and salinity of the rearing water were maintained at ~20°C (Politis et al., 2017) and ~18 PSU (Politis et al., 2021), respectively. Flow rates of water into the tanks were kept at ~ 420 mL/min, except during feeding, where water flow was shut off. Light (~500 lux) was turned on only during feeding (Butts et al., 2016)

The slurry-type diet was based on the egg yolk of spiny dogfish (*S. acanthias*) as previously described (Benini et al., 2023). The different experimental groups were fed with their respective amount of food (i.e., Low or High) five times per day, at 2 h intervals. Before feeding, lights in the larval rearing room were turned on and the water flow to the rearing tanks was stopped, to allow the larvae to settle on the tank bottom. Then, the treatment-specific amount of slurry-type diet was pipetted on the bottom of the tank. After allowing the larvae to feed for 30 min, lights were turned off and the water flow was started. The remaining food on the tank bottom was flushed away with a jet of water. To prevent overloading the biofilter of each RAS, the water in the rearing tanks was flowed through for 30 min (i.e., by disconnecting the tanks from the rest of the recirculating unit) before the tanks were reconnected to the RAS. Larvae were moved into clean tanks daily after the last feeding of each day (Benini et al., 2022).

2.4 Sampling and data collection

2.4.1 Sampling

For digital imaging, ~30 larvae were randomly sampled from the initial 77 L common larval rearing tank, before the larvae were allocated into different experimental groups (i.e., Low and High food) on 9 dph. On 15, 20, 25, and 30 dph, ~20 larvae were randomly sampled from each replicate (n = 3) and each treatment (n = 2; Low or High). Sampled larvae were anaesthetised and digitally imaged using a digital camera (Digital Sight DS-Fi2, Nikon Corporation, Japan) mounted to a stereomicroscope (SMZ 1270, Nikon Corporation, China), with the aid of NIS-Elements D software (Version 5.20.00).

For molecular analysis, three samples, each containing ~10 larvae were collected on 9 dph from the 77 L common larval rearing tank before the larvae were randomly grouped into the two experimental groups. On 15, 20, 25 and 30 dph, samples of ~10 larvae were collected from each replicate (n=3) and each treatment (n = 2). Sampled larvae were immediately euthanised, rinsed, and preserved in RNAlater (Sigma-Aldrich St Louis, USA) and subsequently, stored at -20°C for later analysis (Politis et al., 2017).

For investigating bacterial community composition, samples were analysed only for two replicated tanks from each experimental group. From the common larval rearing tank before the onset of exogenous feeding (9 dph) pools (n = 4) of ~10 larvae were sampled. At 15, 25 and 30 dph, pools (n = 2) of ~10 larvae from each replicate (n = 2) of the experimental groups (n = 2) were collected. Sampled larvae were immediately euthanised, rinsed and stored at -20°C for later analysis. To investigate the influence of feeding initiation on the RAS water bacterial community, RAS water samples (n = 4) were collected from the inlet tubes that supplied water to the rearing tanks on 9 and 15 dph. Additionally, water was sampled on 15 dph, to evaluate changes in the tank water bacterial communities in response to food addition. Moreover, sampling included out-flowing water (n = 4) from the tank outlet from each replicate (n = 2) and treatment (n = 2) that were collected ~30 min after feeding, when tanks were about to be re-connected to the corresponding RAS. Here, 250 mL of water from each sample was vacuum filtered through 0.22 µm white gridded filters (diameter = 47 mm; Merck KGaA, Darmstadt, Germany) using a Büchner funnel, while the filters were collected in sterile cryotubes and stored at −20°C until processing (Bakke et al., 2013). To investigate the bacteria associated with the food, samples of food (n = 4) were also collected in sterile cryotubes and stored at −20°C until processing. Before the isolation of DNA, 1 mL of food was centrifuged and the pellet was used for the extraction of bacterial DNA.

2.4.2 Image analysis for determining larval body area, feeding incidence and gut fullness

Larval images (see section 2.4.1) were later analysed for body area using NIS-Elements Analysis D software (Version 5.20.00). Based on images of larvae, the presence of food in the gut was visually assessed and "feeding incidence" was calculated as the percentage of larvae with food in the gut compared to the total number of larvae. Moreover, the total gut area of the larvae and the area of the gut containing food were measured to calculate "gut fullness" as the percentage of area with food relative to the total gut area (Butts et al., 2016).

2.4.3 Larval survival

Larval survival was monitored daily during the exogenous feeding period through assessment of mortality, i.e., counting and removing dead larvae from all experimental units. Additionally, larvae sampled from each experimental unit and all larvae at the end of the experiment (30 dph) were recorded. Then, "larval survival" was calculated as a percentage based on the initial total number of larvae stocked at 9 dph.

2.4.4 Gene expression analysis

Total RNA from samples was extracted using the NucleoSpin (Mini) RNA isolation kit, following the protocol provided by the supplier (Macherey-Nagel GmbH & Co. KG, Düren, Germany). RNA concentration (161.1 ± 43 ng/mL) and purity (260/280 = 2.14 ± 0.02, 230/260 = 1.96 ± 0.38) were determined by spectrophotometry using Nanodrop ND-1000 (Peqlab, Germany). The concentration was normalised to 100 ng/mL with HPLC water. From the resulting total RNA, 450 ng was reverse transcribed using the qScript™ Ultra SuperMix cDNA Synthesis Kit (Quantabio, Germany) according to the manufacturer's instructions, including an additional gDNA wipe-out step before transcription [PerfeCtaR DNase I Kit (Quantabio, Germany)].

Expression levels of 6 target and 2 reference genes were determined by quantitative real-time PCR (qRT-PCR). Primers were designed using primer 3 software v 0.4.01 based on sequences available in Genbank databases (Table 1). All primers were designed for an amplification size ranging from 75 to 200 nucleotides. Expression of genes in each larval sample from each replicate tank (n = 3) and food amount (n = 2) were analysed in technical replicates (n = 3) using the QuantStudio5 (Applied Biosystems, ThermoFisher Scientific, USA) qPCR system.

The qPCR assays were performed in a final volume of 10 µL reaction mixtures, containing 4 µL of cDNA template, 0.5 µL of UltraPure™ DNase/RNase-free distilled water (Thermo Fisher Scientific, USA), 6 µL of PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific, USA) and 0.5 µL of each primer (Table 1). The mixture was vortexed and distributed in low-profile 0.2 ml optical 8-tube strips (BIO-RAD, USA), covered with flat optical 8-cap strips (BIO-

RAD, USA), and kept on ice until placed in the real-time PCR thermal cyclers. Here, the following PCR thermal profile was used: initial denaturation at 95°C for 2 min, followed by 40 amplification cycles (at 95°C for 15 sec, 60°C for 1 min and 90°C for 15 sec) and a final step at 60°C for 1 min and 90°C for 15 sec (melting curve). Ct values and quality of the run were then visualised with Design and Analysis Software version 2.5.1 (Thermo Fisher Scientific, USA). Ribosomal protein S18 (*rps18)* and elongation factor 1a (*ef1a*) were chosen as reference genes, as they have been previously suggested to be the most stable in fish larvae and are thus, the most reliable reference genes (McCurley and Callard, 2008). Their stability was statistically confirmed, as their expression was not significantly different across treatments. The relative quantity of target gene transcripts (ΔCT) was normalised to the geometric mean of the two reference genes. The coefficient of variation (CV) of technical replicates was calculated and checked. Further analysis of gene expression was carried out according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), to calculate the expression of targeted genes relative to the levels at hatching.

2.4.5 Characterisation of bacterial community composition by amplicon sequencing

DNA from larvae, water and food were isolated using the MagAttract PowerSoil Pro DNA Kit (Qiagen, Germany) following the protocol developed by the supplier for automated high-throughput isolation of DNA with the Thermo Scientific® KingFisher® Flex platform. Briefly, samples (pools of ~10 larvae, filter papers or food) were homogenised in bead-beating tubes containing ~0.55 g of 0.1 mm glass beads (Bertin Technologies, France) and 800 µL of lysis buffer, using a Precellys 24 tissue homogeniser (Bertin Technologies, France) at 5500 rpm for two times 30 s with a 15 s break in between. The tubes containing the lysates were centrifuged at 15000 g for 1 min, and the supernatants were transferred into 1.5 mL Eppendorf tubes. Then, 300 µL of CD2 solution was added to each Eppendorf tube, vortexed to mix and centrifuged at 15000 g for 1 min. Prepared lysates i.e., supernatants from the previous step, were transferred to the KingFisher Flex platform (Thermo Fisher Scientific), where total genomic DNA was captured on specialised magnetic beads in the presence of buffers, washed on the beads and then eluted.

The V3 and V4 regions of the bacterial 16S rRNA gene were amplified from the DNA isolates using the forward primer, Ill-341F_K1: 5′- NNNNCCTAC GGGNGGCWGCAG -3′ and the reverse primer, Ill805R: 5′- NNNNGACTACNVGGGTATCTAAKCC-3′ (Klindworth et al., 2013). Each PCR reaction contained 0.02 U/µL Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific), 0.2 mM of each dNTP (VWR), 0.3 µM of each primer (SIGMA), 1x Phusion HF buffer (containing 7.5 mM $MgCl_2$) (Thermo Fisher Scientific) and PCR grade water (VWR) up to a total reaction volume of 25 µL, as well as 1 µL of DNA extract as a template. The PCR reactions were run with 35 cycles (T100TM Thermal Cycler, Bio-Rad) (Bugten et al., 2022). The PCR amplicons were purified and normalised using the SequalPrep Normalisation Plate (96) kit

(Invitrogen, USA), following the protocol provided by the supplier. Using the Nextera®XT DNA Sample Preparation Kit (Illumina), a unique pair of index sequences that represented the PCR amplicons, originating from each sample, was added by an additional PCR step with 10 cycles. The indexed PCR products were purified and normalised using the SequalPrep Normalisation Plate (96) kit (Invitrogen, USA). Finally, the samples were pooled and concentrated with an AmiconUltra 5.0 Centrifugal Filter (Merck Millipore, Ireland), following the manufacturer's protocol. The amplicon library was sequenced in a MiSeq run (Illumina, San Diego, CA) with V4 reagents (Illumina) at the Norwegian Sequencing Centre (NSC), University of Oslo.

The Illumina sequencing data were processed using USEARCH utility (version 11) (https://www.drive5. com/usearch/). Merging the paired reads was carried out using the command Fastq_mergepairs. The Fastq_filter command (with an expected error threshold of 1), was used for further processing, including demultiplexing, removal of singleton reads, and quality trimming (trimming off primer sequences and filtering out reads shorter than 380 base pairs). Unoise3 command was used for chimaera removal and generation of amplicon sequence variants (ASVs) (https://drive5.com/usearch/manual/ cmd_unoise3.html). Taxonomy was assigned by applying the SINTAX script (Edgar, 2016), with a confidence value threshold of 0.8 against the RDP reference data set (version 18). Before analysing the data, ASVs representing eukaryotic amplicons (e.g., algae, fish DNA), Archaea and Cyanobacteria/Chloroplast were removed from the ASV table. Moreover, ASVs that were highly abundant in the DNA extraction kit blank and reported as common contaminants were removed. ASVs of interest were further investigated with the SeqMatch tool to find the “nearest neighbours” of those DNA sequences at the RDP website (https://academic.oup.com/nar/ article/42/D1/D633/ 1063201).

2.5 Statistical analysis

2.5.1 Larval survival, body area, feeding success and expression of immune and stress-related genes

R studio statistical analysis software (version 4.2.0) was used for all statistical analyses. Residuals were evaluated for normality and homoscedasticity (plot of residuals vs. predicted values) to ensure that they met model assumptions. Data were transformed appropriately to meet these assumptions when necessary. Alpha was set at 0.05 for testing main effects and interactions. Larval survival, body area, feeding incidence, gut fullness and gene expression data were analysed using a series of mixed model ANOVAs, where the main model variables were treatment (Low vs. High) and age, whereas replicated tanks were considered random. The initial model tested included an interaction effect between treatment and age. The model was reduced, when possible, and means were contrasted using Tukey's honestly significant difference test (Tukey's HSD).

2.5.2 Measures of microbial diversity

Different packages developed for R statistical software (version 4.2.0) were used to calculate diversity indices and perform statistical analyses. Beta-diversity analyses were performed on the ASV table that had been filtered to remove any ASVs that had less than 2 counts in at least two samples and rarefied by sub-sampling ten times at 17973 reads per sample (the threshold was chosen based on the sample with the lowest number of reads). Ordination by principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities (Bray & Curtis, 1957) (999 permutations) and Sørensen-Dice coefficient was used to visualise differences in microbial community composition between different groups of samples using the function plot_ordination within the phyloseq package (version 1.40.0). Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) based on the Bray-Curtis and Sørensen–Dice dissimilarities was used to test for differences in community composition (beta diversity) as a function of food amount and age. Pairwise differences were tested using the function pairwise.adonis2 in the vegan package (version 2.6.2). Alpha-diversity measures include diversity numbers of order 0 (ASV richness), 1(exponential Shannon – exp. Shannon) and evenness (Hill, 1973). These indices were calculated using the vegan community ecology package (version 2.6.2) and analysed using a series of mixed model ANOVAs. Residuals were evaluated to ensure that they met model assumptions. Data were transformed appropriately to meet these assumptions when necessary. The package DESeq2 (version 1.36.0) was used on the unrarefied ASV table to assess the differential abundance of ASVs between the samples that were found to be significantly different by PERMANOVA. DESeq2 includes a model based on the negative binomial distribution and Wald's post hoc test for significance testing. The P-values adjustment method used was the Benjamin and Hochberg method (Benjamini and Hochberg, 1995), which accounts for multiple comparisons.

3 Results

3.1 Feeding incidence, gut fullness, body area and larval survival

No significant age × treatment (i.e., food amount) interaction was detected for feeding incidence, gut fullness, and body area. Feeding incidence remained unchanged throughout larval age (Fig. 1A) and was not affected by the amount of food fed (Fig. 1B). Throughout the experiment, ~75% of the larvae were detected with food in their guts in both treatments. Gut fullness was significantly affected by both, age ($p = 0.023$) and treatment ($p < 0.001$). Gut fullness was 38.1% higher ($p = 0.012$) on 25 dph than on 15 dph (Fig. 1C). Interestingly, the larvae fed High amount of food contained 45.2% more ($p < 0.001$) food in their guts compared to the Low food treatment (Fig. 1D). Larval body area was significantly affected by both, age ($p < 0.001$) and treatment (p

= 0.024). Here, larvae increased in body area between 9 and 15 dph, followed by a sharp decrease on day 20 and successive increase again beyond 25 dph. The biggest (3.72 mm^2) and smallest (2.91 mm^2) larvae in terms of body area were observed on 15 and 20 dph, respectively, whereas the larvae had similar sizes on 9, 25 and 30 dph (Fig. 1E). Larvae fed High amount of food were 3.68% bigger than the larvae fed Low amount of food (Fig. 1F). A significant ($p = 0.023$) age × treatment interaction was detected for larval survival. A steep drop in survival was observed during the transition period from endogenous to exogenous feeding, independent on the amount of food fed (Fig. 1 G and H). In the group fed Low amount of food, the high mortality period spanned from 9 to 11 dph reaching 51% survival, whereas in the group fed High amount of food, high mortality occurred from 9 to 12 dph, reaching 36% survival. Despite the initial lower survival observed for larvae fed High amount of food (from 12 to 23 dph), survival reached similar levels in both treatments from 24 to 30 dph (Fig. 1I), reaching ~10% survival at the end of the experiment (30 dph).

3.2 Molecular analysis

The expression of the gene encoding ghrelin (*ghrl*), which is related to appetite and food intake, was significantly ($p = 0.033$) affected by the age × treatment interaction. In the larvae fed Low amount of food, expression of *ghrl* was relatively stable throughout larval age, except for a significant peak on 20 dph compared to 15 and 25 dph (Fig. 2A). In the larvae fed High amount of food, the expression of this gene remained stable until 25 dph and was downregulated significantly ($p = 0.012$) on 30 dph compared to 20 dph (Fig. 2B). Expression of *ghrl* was not significantly affected by the amount of food fed until 25 dph, whereas the expression of this gene was 256% higher ($p = 0.012$) in the larvae fed Low amount of food compared to the larvae fed High amount of food on 30 dph.

The age × treatment interaction was not significant for the expression of *hsp90*, which is related to the cellular stress response and repair mechanism. Expression of this gene was affected significantly ($p < 0.001$) by age (Fig. 2D), whereas an effect of food amount was not observed (Fig. 2E). Expression of *hsp90* remained relatively stable until 20 dph and was significantly upregulated on 25 and 30 dph compared to the expression levels on 9 and 20 dph.

For none of the immune-related genes studied an age × treatment interaction was detected. Expression of genes encoding actors in pathogen recognition (*tlr18*) and inflammatory response (*il10*), were neither affected by age nor the amount of food (Figs. 2F-I). However, the expression of the gene *tnfa*, which has a function in inflammatory response, was significantly affected by age ($p < 0.001$) (Fig. 2J), but not by the amount of food fed (Fig. 2K). Expression of this gene was significantly upregulated on 15 and 20 dph compared to 9 dph. Expression of the complement

system related gene (*c1qc*) was neither affected by age (Fig. 2L) nor the amount of food fed (Fig. 2M).

3.3 Bacterial community composition analysis

3.3.1 Alpha diversity - Diversity within samples

None of the alpha diversity indices (i.e., ASV richness, evenness, and exp. Shannon) for larval samples were affected by the age × treatment interaction. In the pre-feeding larvae (on 9 dph), ASV richness was lower compared to the feeding larvae (i.e., from 15 to 30 dph), where throughout the feeding period ASV richness remained unchanged (Fig.3A). Moreover, richness was not affected by the amount of food fed (Fig. 3B). Evenness of the larval bacterial community was not affected by age (Fig. 2C) nor the amount of food fed (Fig. 2D). Exp. Shannon in the bacterial community of pre-feeding larvae (on 9 dph) was lower than that in the feeding larvae on 15 and 30 dph (Fig. 3E). Throughout the feeding period (15 to 30 dph), exp. Shannon of the larval bacterial community remained relatively stable and was not affected by the amount of food fed (Fig. 3F).

Alpha diversity indices of the RAS water flowing into the rearing tanks were not different between 9 and 15 dph (Figs. 3G - I). On 15 dph, ASV richness in the bacterial community of inflowing water was similar to that of the outflowing water in the Low food treatment, whereas 13.4% higher ($p = 0.013$) compared to the High food treatment (Fig. 3J). However, ASV richness was not significantly different in outflowing water from Low and High food treatments. At the same time, the evenness of bacterial communities of the outflowing water from Low and High food treatments were not significantly different to each other, nor from the inflowing water (Fig. 3K). On 15 dph, exp. Shannon in the bacterial community of outflowing water from the Low food treatment was not different from that of the inflowing water (Fig. 3L). Contrastingly, a significant reduction in the exp. Shannon was noticed in the bacterial community of the outflowing water of the High food treatment, compared to the inflowing water.

Both ASV richness and exp. Shannon were higher ($p < 0.001$) in the bacterial community of rearing water compared to larvae, regardless of the amount of food added (Figs. 3M, O, P and R). Interestingly, the evenness in the bacterial community was similar between rearing water and larvae in the Low food treatment, but lower ($p = 0.004$) in the bacterial community of larvae than in the rearing water of High food treatment (Figs. 3N and Q).

3.3.2 Relative abundances at the order level

The bacterial community of pre-feeding larvae (on 9 dph) was mainly composed of bacteria belonging to the orders Alteromonadales (28.9%), “Unassigned” (10.7%), Vibrionales (8.8%),

Cellvibrionales (8.6%), Rhodobacterales (8.4%) and Rhizobiales (8.2%), summing up to 73.6% (Fig. 4). The contribution of bacteria belonging to the Lactobacillales order, which constituted 90% of the food bacterial community, represented only 3.36% of the bacterial community of pre-feeding larvae. The bacterial community of feeding larvae on 15 dph was mainly composed of bacteria belonging to the orders Flavobacteriales (22.3 and 13.4%), Oceanospirillales (15.4 and 30.0%), "Unassigned" (19.0 and 4.2%), Micrococcales (8.7 and 6.1%) and Vibrionales (0.4 and 5.4%), summing up to 65.4 and 59.1% for Low and High food treatments, respectively. Moreover, the order Lactobacillales represented 4.2 and 14.3% of the bacterial community of 15 dph larvae of Low and High food treatments, respectively. Notably, the contribution of this order increased on 25 dph, representing ~50% of the bacteria in the larval bacteria communities of both, Low and High food treatments. On 25 dph, bacteria belonging to the orders Rhodobacterales (18.6 and 10.9%), Alteromonadales (5.4 and 14.8%) and "Unassigned" (7.3 and 4.7%) constituted the larval bacterial communities, summing up to 31.3 and 30.4% of the bacterial community of Low and High food treatment larvae, respectively). At the same time (25 dph), bacteria belonging to the Vibrionales order represented 7.3% of the larval bacteria community of the High food treatment, whereas their contribution was negligible (< 0.01%) for Low food larvae. On 30 dph, 70.5% of the larval bacterial community of the Low food treatment was composed of the bacteria of the Rhodobacterales order, whose contribution to the larval bacterial community of the High food treatment was only 6.0%. Additionally, for Low food larvae, bacteria of the orders Flavobacteriales (11.4%), Pseudomonadales (3.9%), "Unassigned" (3.2%) and Vibrionales (2.6%) contributed to the bacterial community. For High food larvae, the major contributor (44.2%) to the bacterial community was the Lactobacillales order, which constituted only 3.06% to the bacterial community of Low food larvae. Moreover, on 30 dph bacteria of the orders Enterobacterales (20.8%), Bacillales (6.8%), Rhizobiales (6.2%) and Mycobacteriales (4.2%) also contributed considerably to the bacterial community of High food larvae. Overall, a clear increase in relative abundance of the Lactobacillales order, which was the major contributor (~90%) for the food bacterial community was noticed in the larval bacterial community of High food treatment on 30 dph compared to the Low food group.

The bacterial communities of the RAS water flowing into the rearing tanks consisted mainly of bacteria belonging to the orders "Unassigned" and Oceanospirillales, comprising more than 50% of the bacteria in the communities on both, 9 and 15 dph (Fig. 4). Moreover, presence of the Lactobacillales order, the main constituent of the food bacterial community was negligible (< 0.05%) in the bacterial community of inflowing water. Interestingly, bacteria of this order (Lactobacillales) constituted 19.3 and 44.2% of the bacterial communities of outflowing water of Low and High food treatments, respectively, on 15 dph.

3.3.3 Beta diversity – Comparison of samples

Principal component analyses (PCoA) based on Sørensen–Dice and Bray–Curtis dissimilarities were performed to evaluate β-diversity. Moreover, PERMANOVA tests based on Bray–Curtis and Sørensen–Dice indices were performed to extract significant differences. The two axes of the PCoA plot based on the Sørensen–Dice dissimilarities captured a higher amount of variation in the data (46.9%), compared to the plot based on the Bray-Curtis dissimilarities (33.9%) (Fig. 5). This reveals that the variability of the bacterial communities between the samples was mainly due to the presence or absence of the ASVs rather than their abundances. Larval, water and food samples were clustered apart from each other, revealing differences in their bacterial community composition - especially in the PCoA plot based on Sørensen–Dice dissimilarities (Fig. 5A). Interestingly, larval bacterial community composition became more similar to the food bacterial community with age.

On 9 dph, larval samples and inflowing water samples clustered separately (Fig. 5) and showed significantly different bacterial communities (Table 2). On 15 dph, inflowing and outflowing water clustered discretely, except for two of the outflowing water samples from the Low food treatment. These differences were statistically significant for both indices, independent of treatment (food amount). At the same time (15 dph), the bacterial communities of larvae and outflowing water were similar for the High and Low food treatments. However, significantly dissimilar bacterial communities were found between the outflowing (or rearing) water and larvae, independent of the amount of food added. On 25 dph, the larval bacterial community composition of Low and High food treatments was comparable. Interestingly, on 30 dph, a discrete grouping between the larval samples based on food amount was observed, where statistically significant differences in larval bacterial communities of Low and High food treatments were registered.

Discrete grouping of the inflowing water on 9 and 15 dph was mainly noticed along axis 2 (Fig. 5), where the compositions of bacterial communities of the inflowing water were significantly different between the two days (Table 3). Significantly different bacterial community compositions between pre-feeding larvae (on 9 dph) and feeding larvae on 15 dph were found only in the High food treatment. Regardless of the food amount fed, PERMANOVA based on Sørensen-Dice dissimilarities confirmed significant differences in bacterial communities between pre-feeding and feeding larvae on 25 dph, whereas no differences were found based on Bray-Curtis dissimilarities. Pre-feeding larvae and 30 dph feeding larvae had different bacterial communities, independent of the amount of food fed.

3.3.4 Differential abundance testing

Differences in larval bacterial community composition in response to amounts of food fed, were detected only on 30 dph. To investigate which ASVs contributed to this difference we performed a differential abundance testing. ASVs belonging to orders, potentially containing harmful members (e.g., Vibrionales, Rhodobacterales and Alteromonadales) were significantly more abundant in the larval bacterial community of the Low food treatment compared to the High food treatment (Fig. 6A). Among these, ASVs with sequence similarities to *Vibrio campbellii*, *V. harveyi* and *V. fluvialis*, were found by the RDP Sequence Match tool. Interestingly, ASVs belonging to the potentially beneficial orders of Lactobacillales and Bacillales, including matches to probiotics (e.g., *Bacillus cereus*, *Leuconostoc mesenteroides* and *Staphylococcus haemolyticus*), were significantly more abundant in the bacterial community of High food larvae than that of Low food larvae.

On 15 dph, DESeq2 analysis was carried out to compare the bacterial communities of inflowing vs. outflowing water and water vs. larvae in Low and High food treatments. An increase in abundances of ASVs belonging to the orders Rhodobacterales, Mycobacteriales, Lactobacillales and Bacillales was noticed in the bacterial communities of outflowing water compared to inflowing water in both, Low (Fig. 6B) and High (Fig. 6C) food treatments. Moreover, a decrease in abundance of ASVs belonging to the orders Oceanospirillales, Flavobacteriales and Alteromonadales was detected in the bacterial community of outflowing water compared to inflowing water in both treatments. Interestingly, the abundance of ASVs belonging to the Vibrionales order decreased in the bacterial community of the High food treatment. When comparing bacterial communities of outflowing water and larvae on 15 dph, we noticed differences of bacterial orders in their ability to colonise water and larvae. For instance, ASVs of the orders Rhodobacterales, Rhizobiales, Pirellulales and Mycobacteriales, Lactobacillales were differentially more abundant in outflowing water compared to larvae, regardless of the amount of food added (Figs. 6D and E). On the other hand, ASVs belonging to the Vibrionales order were differentially more abundant in the larval bacterial community than in water in both treatments.

DESeq2 analysis was carried out to compare the bacterial community of pre-feeding larvae (on 9 dph) to that of feeding larvae at different ages, which were found to have significantly different bacterial communities compared to pre-feeding larvae. In bacterial communities of feeding larvae on 15, 25 and 30 dph, the number of differentially more abundant ASVs of the potentially beneficial Lactobacillales order was higher compared to the pre-feeding stage, regardless of the amount of food fed (Figs. 7A – E). On the other hand, we noticed that feeding the larvae with High food amount initially inclined to select for potentially harmful bacteria in the larval bacterial community. For instance, a higher number of differentially more abundant ASVs belonging to

the potentially harmful orders Rhodobacterales, Oceanospirillales and Flavobacteriales was observed in the bacterial community of 15 dph larvae fed High amount of food, compared to pre-feeding larvae (Fig. 7A). On 25 dph, the numbers of differentially more abundant ASVs belonging to the potentially detrimental orders Rhodobacterales, Pseudomonadales and Alteromonadales were higher in feeding larval bacterial communities compared to pre-feeding larvae for both treatments. However, the number of differentially more abundant ASVs of the potentially detrimental Vibrionales order was higher in the feeding larval bacterial community of the High food treatment compared to pre-feeding larvae, only on 25 dph. Contrastingly, in the Low food treatment, the number of differentially more abundant ASVs of this order was higher in the pre-feeding larval bacterial community than in the feeding larval bacterial community on 25 dph (Fig. 7B and C). On 30 dph, in the bacterial community of Low food feeding larvae, there were more ASVs of the potentially detrimental orders Vibrionales, Rhodobacterales, Pseudomonadales, Flavobacteriales and Alteromonadales, compared to pre-feeding larvae, whereas the ASVs of these orders were not differentially more abundant (except for the Rhodobacterales order) in the larval bacterial community of High food larvae (Figs. 7D and E).

When comparing the larval bacterial communities between 15 and 30 dph, the numbers of differentially abundant ASVs belonging to the potentially detrimental orders Flavobacteriales and Alteromonadales decreased by 30 dph compared to 15 dph in both treatments. On the other hand, the numbers of differentially abundant ASVs of the potentially harmful orders Vibrionales and Rhodobacterales were reduced in the High food treatment on 30 dph, whereas the numbers remained unchanged or increased in the Low food treatment (Figs. 8A and B). Interestingly, the numbers of differentially more abundant ASVs of the potentially beneficial orders Lactobacillales and Bacillales were contrastingly higher in the larval bacterial community of the High food treatment on 30 dph (Fig. 8B).

4 Discussion

The present experiment aimed to identify the appropriate food amount that should be used to feed European eel larvae in order to gain potential benefits, such as improved feeding success and growth, but without compromising healthy larvae-bacterial interactions and larval wellbeing. The results suggest that the amount of food offered can affect efficiency of food intake, as indicated by higher gut fullness of eel larvae fed High amounts of food. Here, gut fullness of larvae was higher on 25 dph compared to 15 dph. This might be due to the larvae adapting their feeding behaviour and becoming more familiarised with their food and the feeding procedures by 25 dph, whereas on 15 dph, larvae were still at the learning phase of their foraging routine. Here, it is interesting to mention that at least beyond 20 dph, we observed that larvae swam towards the bottom of the tank and actively started searching for food as soon as the lights were turned on and

the flow of the tank was stopped, indicating that by this time the larvae had adapted to the feeding routine. On the contrary, even though larvae slowly swam or rather sank to the tank bottom already at the onset of feeding (on 10 dph), mainly due to the low salinity of the rearing water, but also to some degree due to the negative phototactic nature of eel larvae (Tsukamoto et al., 2009), they did not immediately start searching for food. Moreover, molecular analysis showed that the expression levels of *ghrl*, which encodes ghrelin (known as the "hunger hormone"), were higher in the Low food treatment by the end of the experiment (30 dph). This suggests that the larvae in the Low food treatment were starved compared to the High food treatment. A possible explanation for this is that the High food amount creates a bigger puddle of food, which allows larvae to feed inside the puddle of food for a longer time without being disturbed by other individuals. This behaviour was confirmed by our observations, where larvae tended to swim away from the food puddle when they were disturbed by their tank mates.

Nevertheless, the diet supplied during the experiment was generally accepted by most larvae, as indicated by successful feeding incidence of ~75%, irrespective of the food amount treatments. Here, food amount did not affect the chance of larvae to encounter the food within the range tested, as the increase in food amount increased the volume of the food puddle and not necessarily the tank bottom area covered by food. Therefore, larvae in both, Low and High treatments had equal opportunities to access and ingest the food. In fact, we observed a treatment specific feeding behaviour, where many larvae in the Low food treatment oriented themselves vertically (head down) into the food puddle and tried to forage while beating their tails to maintain their position. On the other hand, the High food treatment allowed many larvae to dive into the food puddle instead and feed while swimming through the food. In this regard, since the biting force of the first-feeding European eel larvae is low (Bouilliart et al., 2015) and the larval oesophagus has not developed mucous cells that facilitate food swallowing (Yoshimatsu, 2011), a vertical position during feeding might prevent efficient ingestion of the food. Contrarily, the ability to swim through the puddle of food, might facilitate more efficient ingestion. This potentially also explains why the different food amounts did not affect feeding incidence but affected gut fullness.

Moreover, feeding eel larvae with High food amount resulted in 3.7% faster growth (in terms of body area) compared to larvae fed Low food amount. This might be linked to the higher gut fullness observed in the High food treatment, but also to the high abundance of ASVs belonging to Gram-positive bacteria from the *Bacillus*, *Lactobacillus* and *Streptococcus* genera, which include the most tested probiotic strains in aquaculture. In this regard, probiotics, especially certain *Bacillus* strains are known to stimulate growth of fish (Austin and Sharifuzzaman, 2022). For instance, an ASV which showed a high sequence similarity to *Bacillus cereus*, a growth-stimulating probiotic (Wang et al., 2017), was more abundant in the bacterial community of larvae

fed High food amount compared to the Low food treatment on 30 dph. However, more research is needed to further investigate this in more detail. At this stage, it is also important to mention that despite larvae growing bigger in the High food treatment, larval body area did not increase beyond 15 dph for any of the food amount treatments. This might imply a general nutritional deficiency in the diet used during this experiment and emphasises the need for further optimisation of the larval diets.

In earlier studies, where the diet used in the current experiment was described (Bandara et al., 2023; Benini et al., 2023), two significant drops in survival of European eel larvae were observed during the first-feeding period. The first drop, which we also observed in the current study, occurs within the first few days after initiation of feeding and can be attributed to the challenging transition experienced by the larvae as they switch to exogenous feeding. The second drop in survival occurs between 20 and 24 dph. In previous research, where this was even more prominent, this drop was linked to the fact that larvae reached the so called "point-of-no-return" (Benini et al., 2023) and associated with detrimental bacteria interactions (Bandara et al., 2023). However, it is worth noting that this second drop in survival was not as pronounced in the present study compared to the earlier mentioned research. Intriguingly, we also did not observe the shift in larval bacterial community towards domination by potentially harmful or opportunistic bacteria that had previously coincided with the second drop in survival (Bandara et al., 2023; Benini et al., 2023). On the other hand, by the time the present experiment concluded (30 dph), the survival was ~10%, which represents an improvement compared to the previously reported survival rate of 4% at 28 dph for similarly captive-reared European eel larvae (Benini et al., 2023). This improvement can be partially attributed to a healthy bacterial community observed in the larvae during the current experiment. However, despite this modest improvement, the overall larval survival rate remains low from an aquaculture perspective. This suggests that the incomplete nutritional requirements, combined with suboptimal rearing conditions continue to be a challenge, emphasising the need for further optimisation in future research.

Regarding molecular analyses, food amount did not affect the expression of immune and stress related genes, which indicates that feeding eel larvae with High food amounts does not necessarily act as an additional stressor. An upregulation of *hsp90*, a gene related to the cellular stress response and repair mechanism, was noticed from 25 dph onward. In this regard, upregulation of heat shock proteins is associated with various biotic and abiotic stressors, including nutritional deficiencies (Roberts et al., 2010). Thus, the upregulation of *hsp*90 from the end of the first-feeding window might indicate that the larval diet used during the current experiment was suboptimal and did not completely fulfil the larval nutritional requirements. Moreover, we observed an upregulation of *tnfα*, which is normally encoding a cytokine important for

inflammation, apoptosis, cell proliferation, and innate immune responses. As previously described, it can be activated in fish by stimulants such as endotoxins, vaccinations, and pathogen-associated molecules (Lam et al., 2011). A possible explanation might be that certain molecular dietary components stimulated the *tnfa* expression. For instance, exposure to whey protein hydrolysate, which is an ingredient of the diet used in the present experiment, increased the *tnfa* levels in human monocytic leukemia cells (Ishikawa et al., 2022). However, we did not register an upregulation of other immune-related genes in response to the onset of feeding. As such, the reason behind the upregulation of this gene during the initial exogenous feeding period compared to the levels in pre-feeding larvae during the present experiment, is not clear.

Furthermore, bacterial community composition analysis showed that the different food amount treatments did not affect the alpha diversity of the larval bacterial community. ASV richness and the exp. Shannon were lowest in pre-feeding larvae (9 dph), but increased and remained higher throughout the feeding period, possibly due to new niches that can be occupied by new bacteria sourced from external sources (e.g., water and food). In this regard, an increase in diversity of bacterial communities was observed after the first feeding window also in other fish species and associated to the introduction of new bacteria through the food (Giatsis et al., 2015; Romero and Navarrete, 2006). Interestingly, when comparing bacterial communities of inflowing and outflowing water of the rearing tank on 15 dph, ASV richness remained unchanged in the Low food treatment, whereas it was reduced in the outflowing water in the High food treatment. This implies that selective forces (e.g., availability of DOM) were stronger in the High food treatment than in the Low food treatment and that certain ASVs were outcompeted from the system.

Under these circumstances, we speculate that feeding High amounts of such a liquid diet composed of ingredients with high leaching properties (in contrast to live feed, commonly fed to most other fish larvae in captivity), might perturb the microbial ecosystem due to the sudden increase in DOM supply, which can then allow for fast growth and selection towards opportunistic r-strategists. Our speculation is supported by the increase in ASVs belonging to potentially harmful orders such as Rhodobacterales, Oceanospirillales and Flavobacteriales, which were found in the larval bacterial communities of the High food treatment on 15 dph (compared to pre-feeding larvae). These bacterial orders have often been associated with stressed and diseased marine invertebrates (Bourne et al., 2008; Bourne and Munn, 2005; Meron et al., 2011; Sunagawa et al., 2009). Therefore, the lower survival observed after the initial onset of feeding in the High food treatment might be attributed to these potentially harmful bacteria that dominated the larval bacterial community.

On the other hand, feeding eel larvae with High food amounts benefited them during later ontogeny, where a healthier larval bacterial community was observed (30 dph). Here, ASVs of

the potentially beneficial orders Lactobacillales and Bacillales, including sequence matches to probiotics (e.g., *Bacillus cereus*, *Leuconostoc mesenteroides* and *Staphylococcus haemolyticus*) were more abundant compared to the Low food treatment. In this regard, previous studies demonstrated the ability of *B. cereus* to improve growth performance and intestinal health status of Pengze crucian carp, *Carassius auratus* (Yang et al., 2020, 2019), while *L. mesenteroides*, which is a lactic acid bacterium, inhibited the growth of pathogenic *Aeromonas hydrophila* (Allame et al., 2012). Moreover, *S. haemolyticus* was reported to produce bacteriocins (Suresh et al., 2014), ribosomally synthesised antimicrobial peptides, which have activity against a broad spectrum of Gram-positive pathogens (Cotter et al., 2005). On the other hand, the larval bacterial community of the Low food treatment on 30 dph was characterised by higher abundance of potentially harmful bacteria, including ASVs with high sequence similarity to *V. campbellii*, *V. harveyi* and *V. Fluvialis*, which are reported aquaculture pathogens (Austin and Zhang, 2006; Kumar et al., 2021; Kumara and Hettiarachchi, 2017).

Additionally, from the PCoA analysis, on 25 and 30 dph, we noticed a shift in the larval bacterial community of the High food treatment towards the food bacterial community. The bacterial community of the food was composed mainly of potentially beneficial orders such as Lactobacillales and Bacillales, which probably helped maintaining a healthier larval bacterial community by 30 dph in the High compared to the Low food treatment. Here, it needs to be mentioned that the food used during the present experiment contained whey protein, which has been reported to promote the proliferation and adhesion of *Lactobacillus* probiotics (Liu et al., 2022) and probably favoured the growth of Lactobacillales. As such, even though larval food is not considered a major determinant of the bacteria associated with fish larvae that are fed live feeds (Bakke et al., 2013), our results suggest that larval food can influence the bacterial community composition of European eel larvae to a large extent. This might be mainly due to the atypical feeding regime that is currently applied during captive rearing of eel larvae, where a formulated liquid diet is added to the tank bottom and larvae then forage by diving through this food puddle. Due to this feeding behaviour, bacteria from the food potentially colonise both, gut and skin, exerting a strong influence on the whole bacterial community of eel larvae.

5 Conclusion

In conclusion, feeding European eel larvae with High food amounts generally benefited the larvae as indicated by higher gut fullness, bigger larval size, and healthier larval bacterial community on 30 dph compared to larvae fed Low food amounts. Moreover, most of the larvae that were fed Low amounts of food were starving on 30 dph as indicated by the upregulation of *ghrl*, the gene encoding for ghrelin. Also, Feeding High amount of food did not trigger molecular mechanisms related to stress or immune response. Overall, in the present study, larval survival reached 10%

on 30 dph, which is a promising improvement compared to previously reported studies. However, larval biometry showed a cessation in larval growth, still indicating dietary imbalances. Therefore, further optimisation of the diet formulation, feeding regimes and rearing conditions (e.g., physio-chemical and microbial) as well as pre-feeding, gut-priming and/or immune-stimulation strategies are probably required to improve larval growth and survival during feeding culture.

CRediT author statement

All authors contributed to this manuscript.

Kasun A. Bandara: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. **Sebastian Nikitas Politis**: Conceptualization, Methodology, Investigation, Data curation, Validation, Visualization, Writing – review & editing, Supervision, Funding acquisition. **Sune Riis Sørensen**: Resources, Visualization, Writing – review & editing. **Elisa Benini**: Methodology, Investigation, Data curation, Writing – review & editing. **Jonna Tomkiewicz**: Conceptualization, Methodology, Resources, Data curation, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Olav Vadstein**: Conceptualization, Methodology, Resources, Data curation, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Table 1. Primers used for molecular analysis of immune, stress and appetite related gene expression (FW: Forward, RV: Reverse).

Function	Gene name	Abbreviation	Primer sequence		Accession number
Reference	Ribosomal protein S18	*rps18*	FW	ACGAGGTTGAGAGAGTGGTG	XM_035428800.1
			RV	TCAGCCTCTCCAGATCCTCT	
	Elongation Factor 1	*ef1*	FW	CTGAAGCCTGGTATGGTGGT	XM_035428274.1
			RV	CATGGTGCATTTCCACAGAC	
Appetite	Prepro-Ghrelin	*ghrl*	FW	TCACCATGACTGAGGAGCTG	XM_035381207.1
			RV	TGGGACGCAGGGTTTTATGA	
Stress / repair	Heat shock protein 90	*hsp90*	FW	ACCATTGCCAAGTCAGGAAC	XM_035392491.1
			RV	ACTGCTCATCGTCATTGTGC	
Pathogen recognition	Toll like receptor 18	*tlr18*	FW	TGGTTCTGGCTGTAATGGTG	XM035421803.1
			RV	CGAAATGAAGGCATGGTAGG	
Inflammatory response	Interleukin 10	*il10*	FW	CTCGACAGCATCATGACAACA	XM_035387988.1
			RV	CCAGAGGTTCAGTGTTTAGGC	
	Tumor necrosis factor α	*tnfa*	FW	CACCTCTCCTCTCCTCTCCT	XM_035428518.1
			RV	CTGGGACTGTTCTTTAGCGC	
Complement system	Complement component 1, Q subcomponent, C chain	*c1qc*	FW	TCTGCTGTCATGTTCACCCA	XM_035433127.1
			RV	CTTCTCGCCATCCCTTCCAT	

Table 2. PERMANOVA p-values based on Sørensen–Dice and Bray–Curtis indices for comparisons of bacterial communities of European eel (*A. anguilla*) larvae, inflowing and outflowing water, and food amounts (Low = 0.5 mL food / L water and High = 1.5 mL food / L water) at different ages. The significance level was set at <0.05 and significant p values are bolded and highlighted.

Age	Comparison	p values	
		Sørensen-Dice	Bray-Curtis
9 DPH	Inflowing water *vs*. larvae	**0.004**	**0.004**
15 DPH	Inflow *vs*. low food outflow	**0.012**	**0.019**
	Inflow *vs*. high food outflow	**0.006**	**0.003**
	Low food outflow *vs*. high food outflow	0.318	0.286
	Low food larvae *vs*. high food larvae	0.73	0.519
	Low food water (outflow) *vs*. larvae	**0.022**	**0.043**
	High food water (outflow) *vs*. larvae	**0.029**	**0.027**
25 DPH	Low *vs*. high food larvae	0.444	0.492
30 DPH	Low *vs*. high food larvae	**0.029**	**0.018**

Table 3. PERMANOVA p-values based on Sørensen–Dice and Bray–Curtis indices for comparisons of bacterial communities of inflowing water between 9 and 15 dph and of European eel (*A. anguilla*) larvae at different ages in reference to the bacterial community of pre-feeding larvae (on 9 dph). The significance level was set at < 0.05 and significant p values were bolded and highlighted.

Sample type	Comparison	p values	
		Sørensen-Dice	Bray-Curtis
Water inflow	9 *vs.* 15 dph	**0.002**	**0.001**
Low food larvae	9 *vs.* 15 dph	0.182	0.141
	9 *vs.* 25 dph	**0.018**	0.083
	9 *vs.* 30 dph	**0.031**	**0.039**
High food larvae	9 *vs.* 15 dph	**0.025**	**0.029**
	9 *vs.* 25 dph	**0.023**	0.068
	9 *vs.* 30 dph	**0.028**	**0.034**

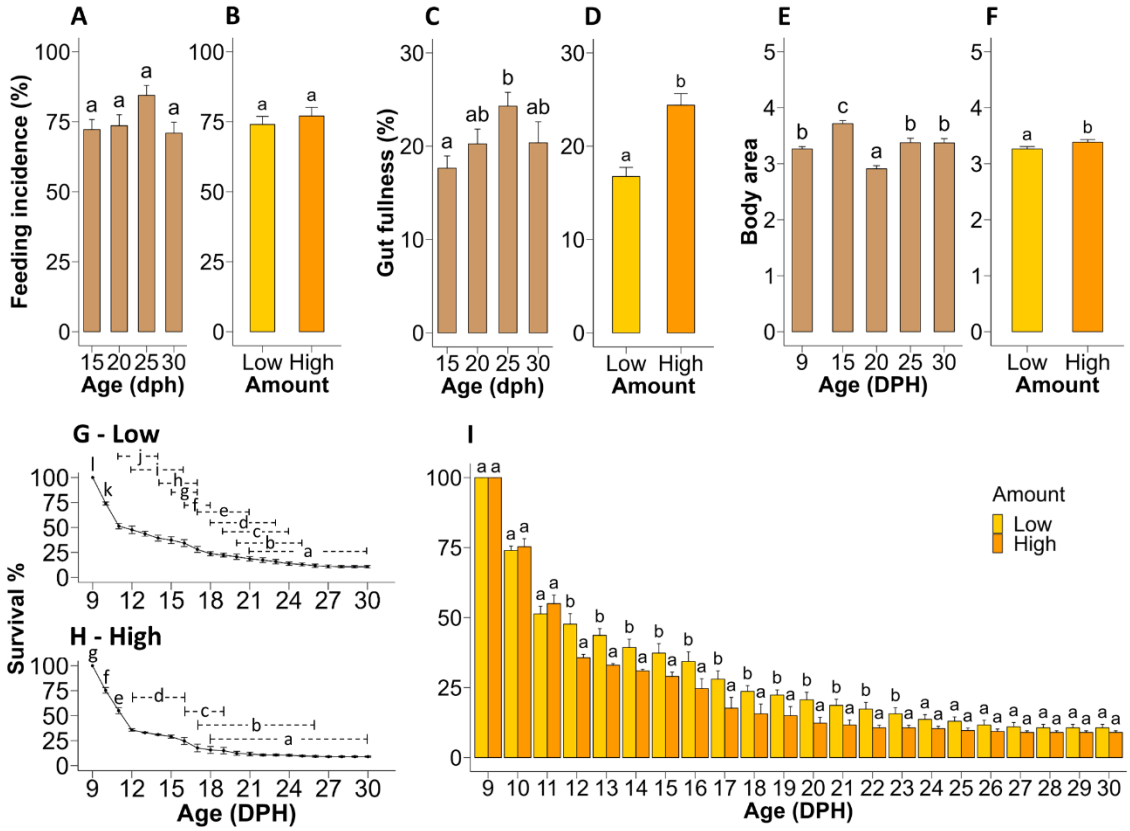

Fig. 1. Effect of age (DPH = days post-hatch) and amount of food treatment (i.e., Low = 0.5 mL food / L water and High = 1.5 mL food / L water) on feeding incidence (A-B), gut fullness (C-D), body area presented in mm^2 (E-F) and survival (G-I) of European eel (*A. anguilla*) larvae. Graphs A, C and E show the data as a function of age, whereas graphs B, D and F show the effect of food amount fed. Graphs G and H show the change in larval survival over time in Low and High food groups, respectively, whereas graph I shows the effect of food amount at each age. Values represent means (± SEM) for replicate tanks (n = 3). Different lower-case letters represent significant differences ($P < 0.05$).

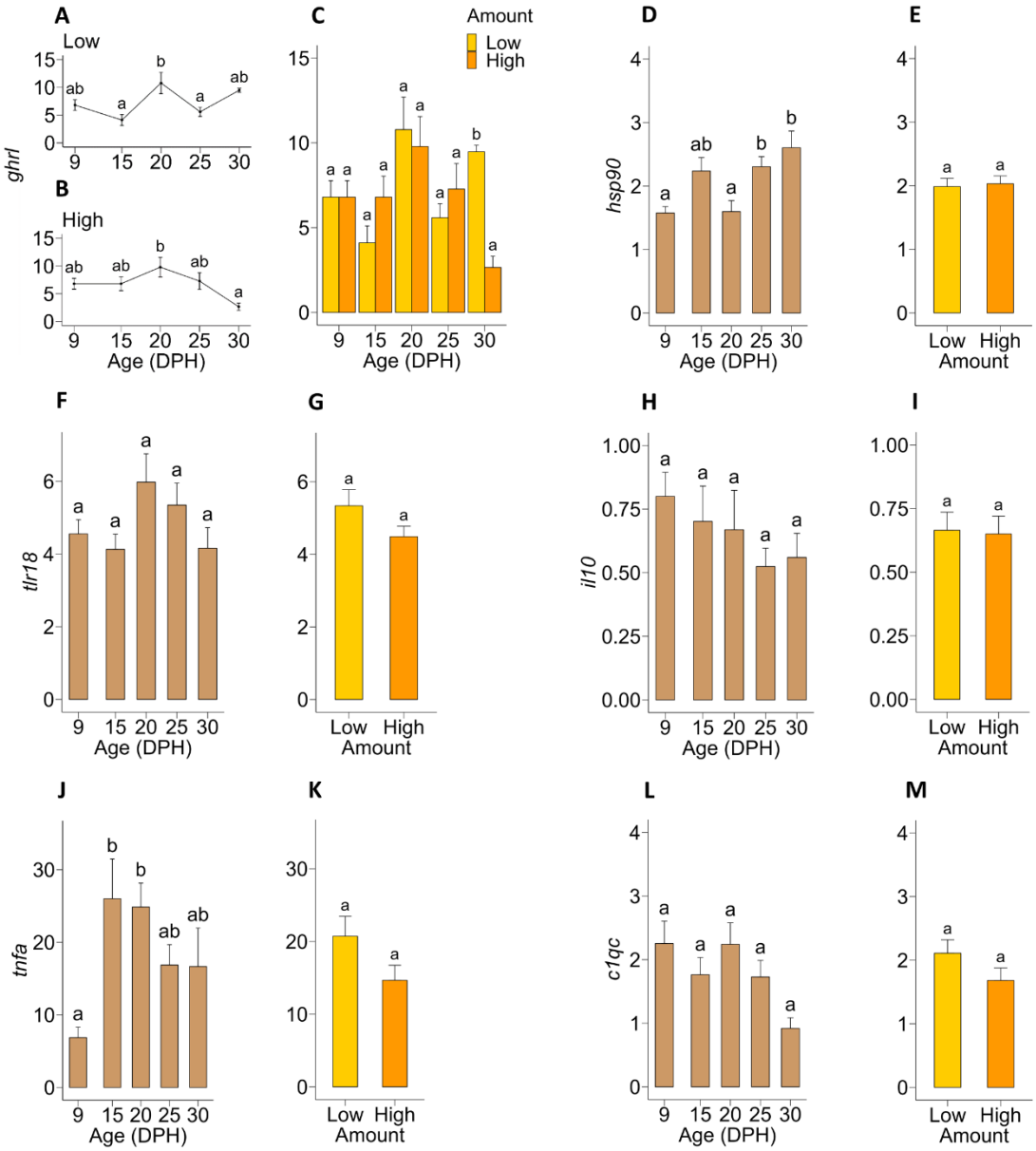

Fig. 2. Expression of genes related to food intake (*ghrl*), stress/repair mechanism (*hsp90*) and immune response (*tlr18*, *il10*, *tnfa* and *c1qc*) in European eel (*A. anguilla*) larvae as a function of age (DPH = days post-hatch) and in response to the amount of food (i.e., Low = 0.5 mL food /L water and High = 1.5 mL food /L water), relative to the expression levels at hatching (0 dph). Graphs A, B, D, F, H, J and L show the changes in relative expression levels of genes over time, whereas graphs C, E, G, I, K and M show the effect of food amount fed on the relative expression of each gene. Values represent means (± SEM) for replicate tanks (n=3) and different lower-case letters represent significant differences ($P < 0.05$).

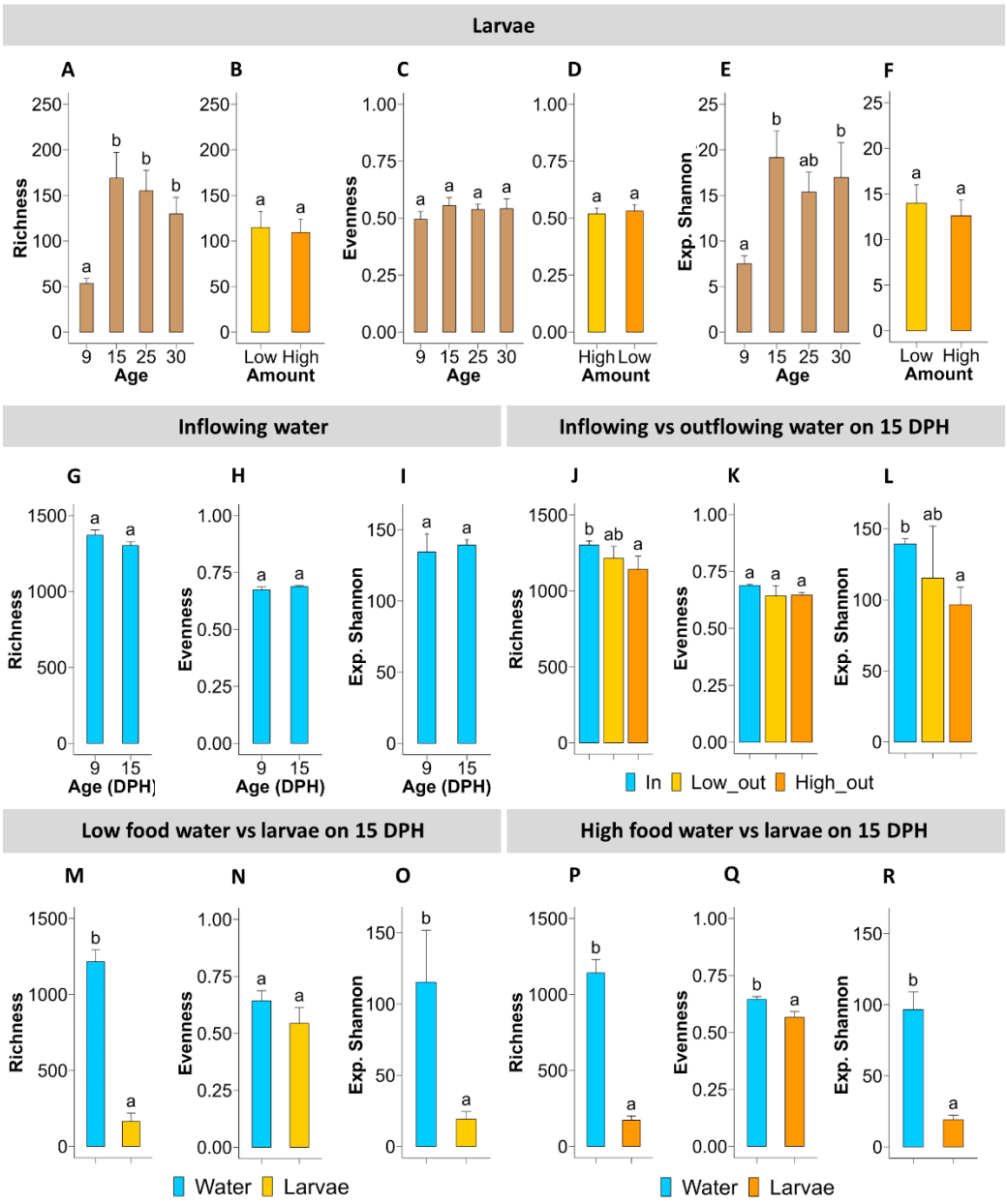

Fig. 3. Different alpha diversity indices (i.e., ASV richness, evenness, and exponential Shannon) in bacterial communities of European eel (*A. anguilla*) larvae and rearing water. Graphs A, C and E show alpha indices for the bacterial community of larvae as a function of age (DPH = days post-hatch), while graphs B, D and F, in response to the amount of food (i.e., Low = 0.5 mL food / L water and High = 1.5 mL food / L water). Graphs G - I show alpha indices for bacterial communities of inflowing water on 9 and 15 dph, while graphs J - L compare the alpha indices for communities of inflowing and outflowing water of Low and High food treatments on 15 dph. Comparisons of alpha indices in bacterial communities between rearing water and larvae on 15 dph are shown in graphs M to O for the Low food treatment and graphs P to R for the High food treatment. Values represent means (± SEM) for replicate tanks (n = 3) and different lower-case letters represent significant differences ($P < 0.05$).

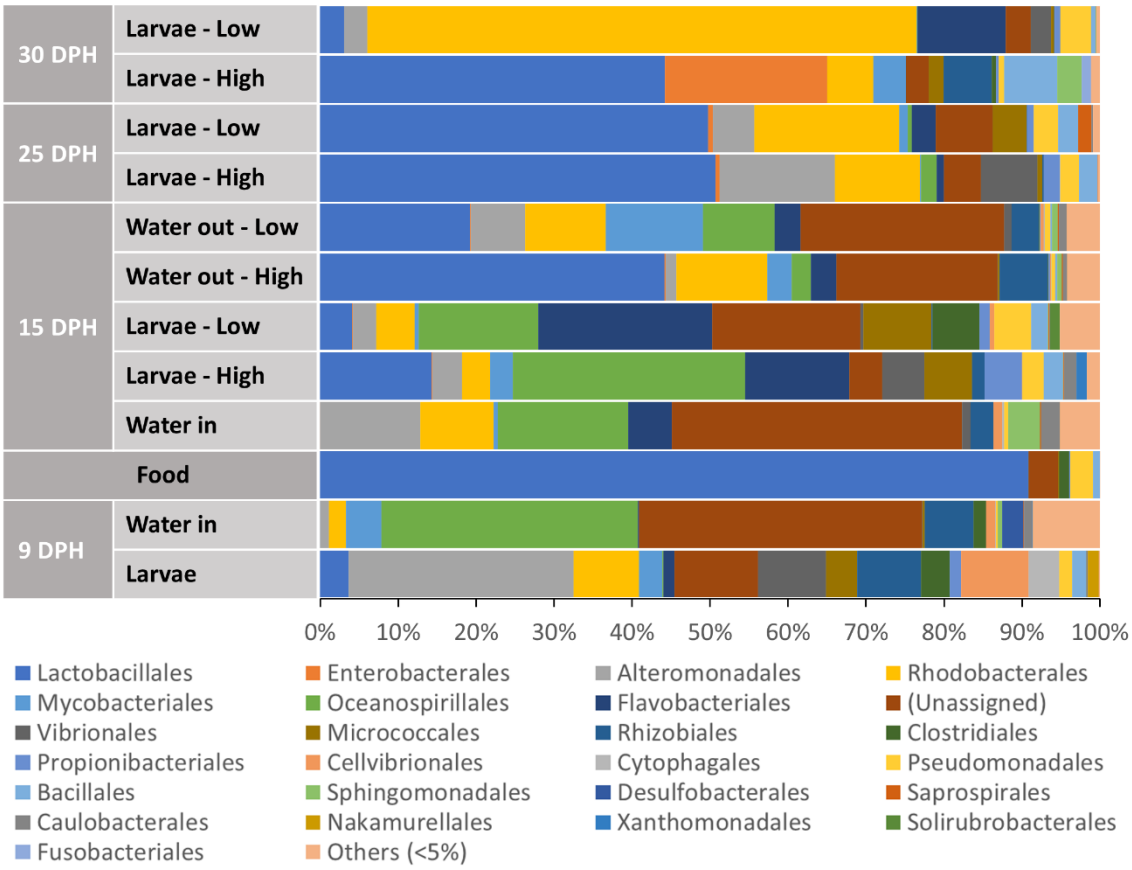

Fig 4. Relative abundances of the bacterial orders detected in European eel (*A. anguilla*) larvae, rearing water, and feed as a function of food amount (Low = 0.5 mL food / L water and High = 1.5 mL food / L water) and age. Each stacked bar represents the mean (n = 4) relative abundances of bacterial orders detected in each sample. "Unassigned" stands for ASVs that could not be classified reliably at the order level, while DPH stands for days post-hatch.

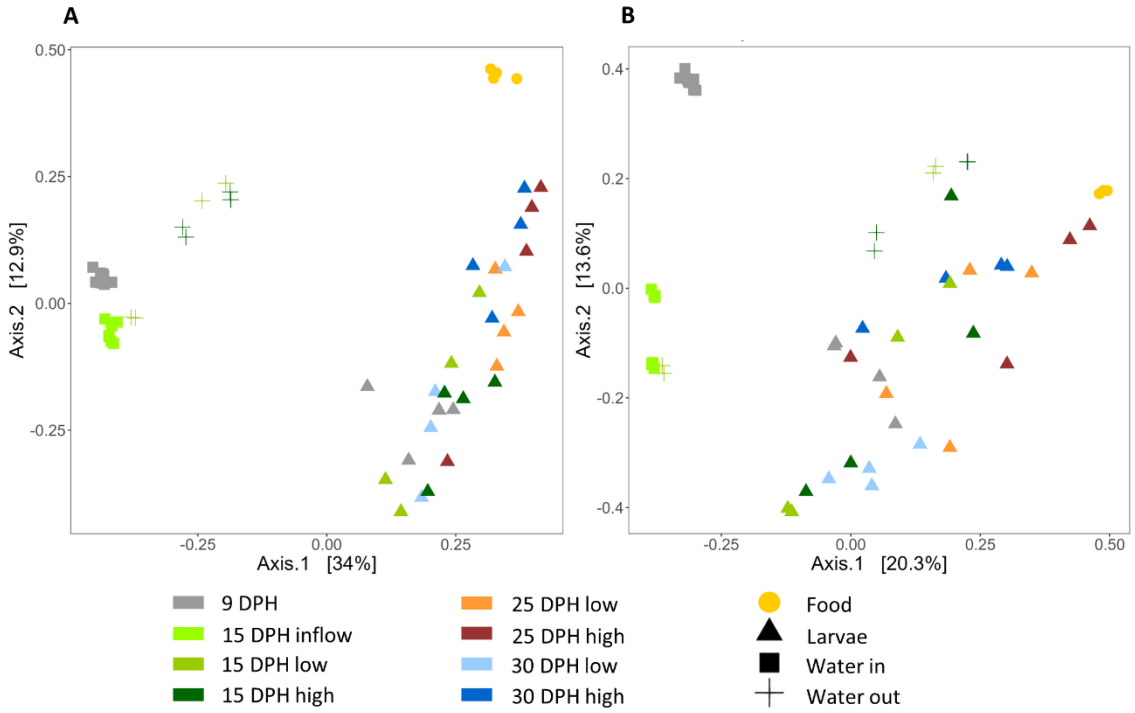

Fig. 5. PCoA ordination plots based on Sørensen–Dice (A) and Bray–Curtis (B) indices for comparison of bacterial communities of European eel (*A. anguilla*) larvae, water, and food as a function of age and food amounts (Low = 0.5 mL food / L water and High = 1.5 mL food / L water). DPH stands for the days-post hatch.

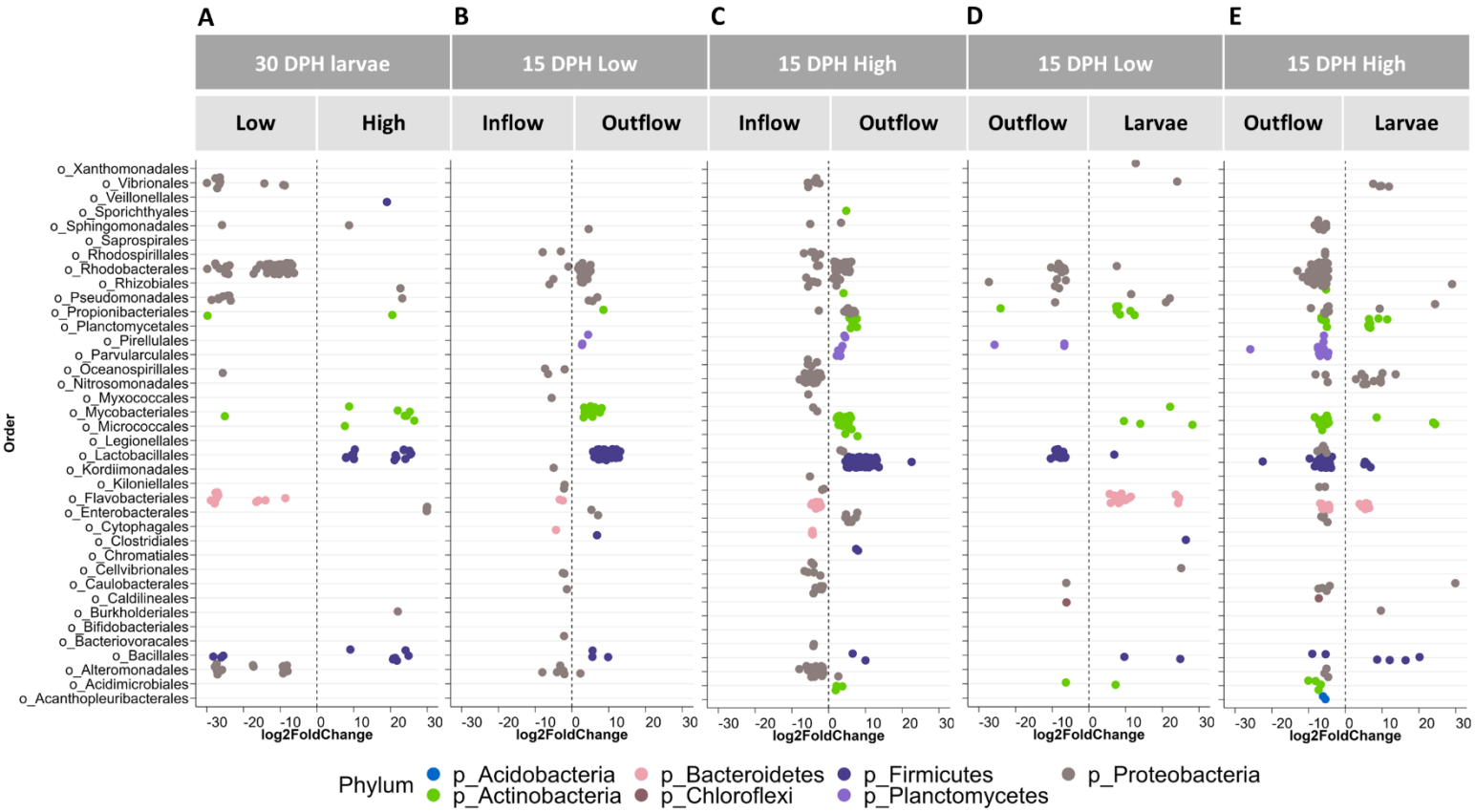

Fig. 6. Results for DESeq2 analysis to determine differentially abundant taxa in European eel (*A. anguilla*) larvae fed Low (ref.) *vs.* High food amounts on 30 DPH (A), in inflowing (ref.) *vs.* outflowing water on 15 DPH in Low food (B) or High food (C) treatments, and in outflowing water (ref.) *vs.* eel larvae on 15 DPH in Low food (D) or High food (E) treatments. Each dot represents an ASV, while log2-fold difference in the abundance of each ASV compared to the reference (ref.) sample is shown. In DESeq2 analysis, only ASVs that are statistically significant ($p < 0.05$) are reported. DPH stands for days post-hatch. Food amounts were: Low = 0.5 mL food / L water and High = 1.5 mL food / L water.

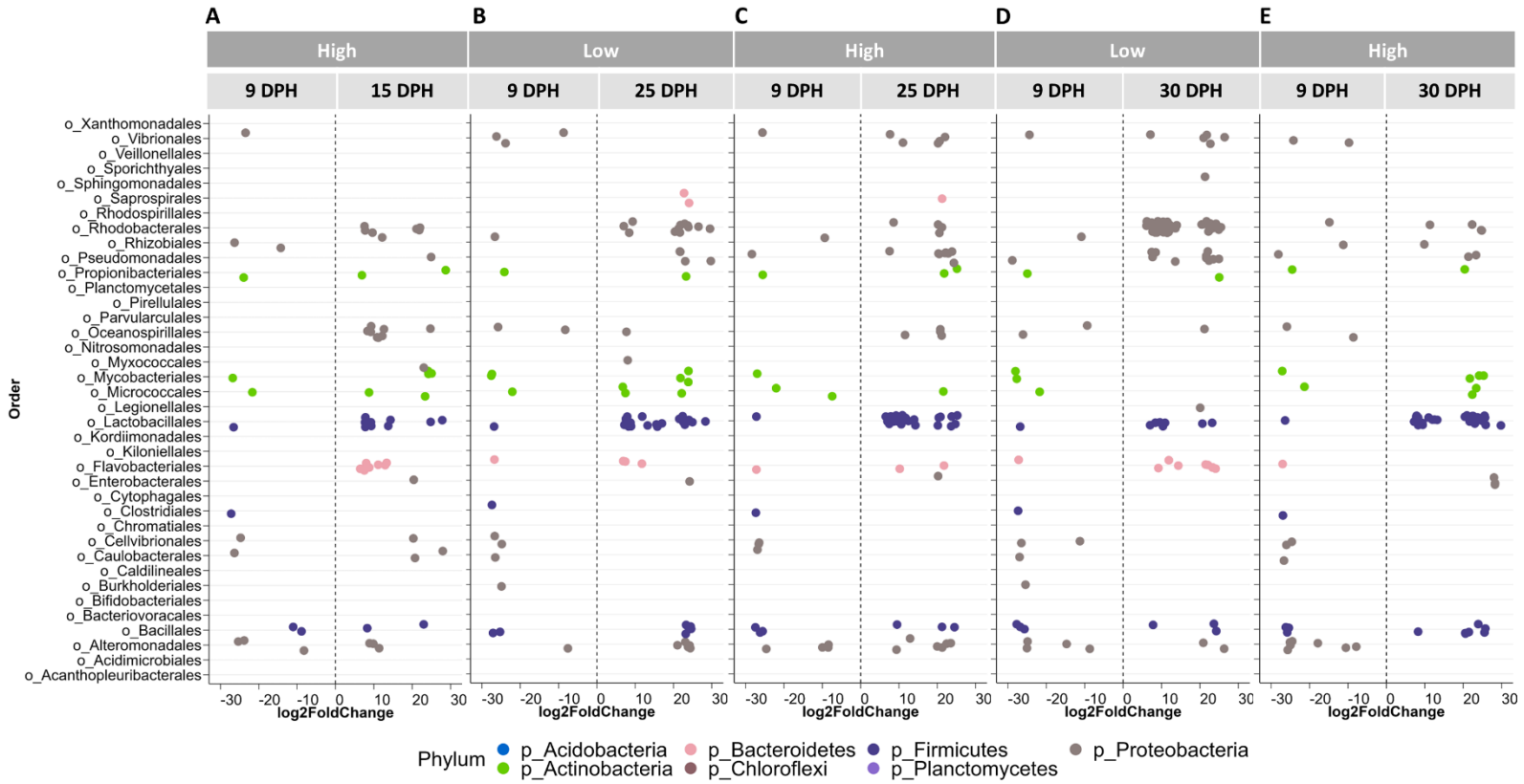

Fig. 7. Results for DESeq2 analysis to determine differentially abundant taxa of pre-feeding (on 9 DPH) (ref.) *vs*. feeding European eel (*A. anguilla*) larvae of different ages (15, 25 and 30 DPH) (graphs A – E). Each dot represents an ASV, while log2-fold difference in the abundance of each ASV compared to the reference (ref.) sample is shown. In DESeq2 analysis, only ASVs that are statistically significant ($p < 0.05$) are reported. Comparison between 9 and 15 DPH larval bacterial communities for Low food treatment, which was not significantly different are not shown. DPH stands for days post-hatch. Food amounts were: Low = 0.5 mL food / L water and High = 1.5 mL food / L water.

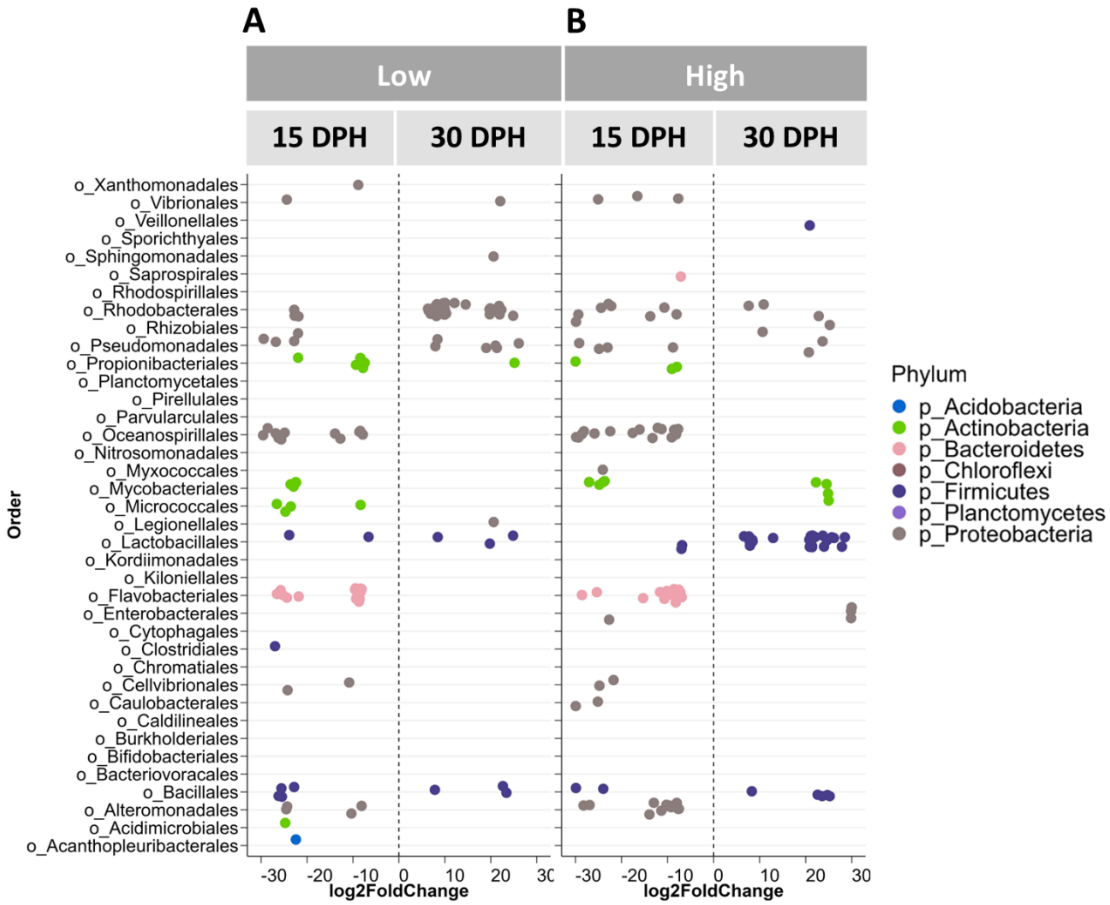

Fig. 8. Results for DESeq2 analysis to determine differentially abundant taxa of 15 (ref.) *vs*. 30 days post-hatch (DPH) old European eel (*A. anguilla*) larvae fed Low (A) or High (B) food amounts. Each dot represents an ASV, while log2-fold difference in the abundance of each ASV compared to the reference (ref.) sample is shown. In DESeq2 analysis, only ASVs that are statistically significant ($p < 0.05$) are reported. Food amounts were: Low = 0.5 mL food / L water and High = 1.5 mL food / L water.

Study 4:

β-Glucan enhances development and modulates the bacteriome and immuno-stress response of European eel larvae

Kasun A. Bandara, Elisa Benini, Sune Riis Sørensen, Jonna Tomkiewicz, Olav Vadstein, Sebastian N. Politis

Manuscript

β-Glucan enhances development and modulates the bacteriome and immuno-stress response of European eel larvae

Authors:

Kasun A. Bandara[1,2 *], Elisa Benini[1], Sune Riis Sørensen[1], Jonna Tomkiewicz[1], Olav Vadstein[2], Sebastian N. Politis[1]

Affiliation:

[1]Technical University of Denmark, National Institute of Aquatic Resources, Kgs. Lyngby, Denmark

[2]Department of Biotechnology and Food Science, NTNU - Norwegian University of Science and Technology, 7491 Trondheim, Norway

Abstract

Closing the life cycle of the European eel (*Anguilla anguilla*) in captivity is indispensable to ensure the sustainability of aquaculture and the conservation of natural populations through restocking of this critically endangered species. Research-based advancements in assisted reproduction and hatchery rearing techniques have led to a stable production of European eel offspring that reach the feeding stage in captivity. However, the early larval rearing phase is characterised by substantial mortalities hampering the progression of closing the life cycle in captivity. To a large extent, detrimental larval-bacteria interactions and an early undeveloped immune system of the larvae aggravate these mortalities. In the present study, we tested the application of β-glucan, a known immunostimulant and bacterial community modulator in aquaculture, as a prophylactic measure during the early larval rearing phase of European eel. For this, yolk sac larvae were either reared under the common (control) hatchery conditions or treated with Yeast β-1,3/1,6-glucan (BG; MacroGard®) added to the rearing water at a concentration of 5 mg/L from 5 until 9 days post-hatch (dph) when the larvae were close to reaching the exogenous feeding stage. Thereafter, both groups of larvae were fed according to the common hatchery protocol until 20 dph. The experiment was repeated 4 times, each time using offspring from different parental crosses. Measurements included survival, growth, occurrence of deformities, expression of immune and stress related genes, as well as bacteriomes of larval and rearing water (16s rRNA gene amplicon sequencing). No effect of BG treatment on larval survival and growth was detected. However, we found an effect of BG treatment on larval development, immune and stress response, as well as larval and water bacteriome. Interestingly, the occurrence of all types of deformities studied (pericardial edema, emaciation, deformed neurocranium, spinal curvature and deformed jaw) was significantly lower in the BG-treated larvae. For the immunological analysis, *il10* ($p = 0.01$), which encodes for an anti-inflammatory cytokine and *hsp90* ($p = 0.02$), a gene related to the cellular stress/repair mechanism, were both downregulated in BG-treated larvae at the end of the treatment period (9 dph). Moreover, the BG treatment altered the alpha diversity of bacteria, resulting in significantly different compositions of bacteriomes in both, larvae and water at the end of the treatment period (9 dph). Strikingly, 80 ASVs of the genus *Vibrio* were significantly more abundant in the water bacteriome of the control compared to the BG treatment. On the other hand, we observed that bacteria of Unassigned taxa (relative abundance of ~69%) dominated the bacteriome of BG-treated water, suggesting that the BG treatment selected for a more specialised (probably K-selected) water bacterial community. All the beneficial effects of BG treatment, however, disappeared ~10 days post-treatment (by 20 dph). In conclusion, the treatment of European eel yolk sac larvae with BG (applied in rearing water) proved beneficial as manifested by the significantly reduced occurrence of deformities, and reduced cellular stress response, as well as improved bacterial communities of rearing water and larvae through a significant reduction in *Vibrio* spp. Despite these benefits, BG treatment did not improve larval survival and growth during the present experimental window and thus, further research is encouraged to refine applications and improve the offspring culture of European eel.

Keywords: *Anguilla anguilla*, immunostimulation, deformities, Immune system ontogeny, larval and water bacteriome

1 Introduction

European eel (*Anguilla anguilla*) has been a commercially important species for fisheries and aquaculture in Europe (Nielsen and Prouzet, 2008). Currently, the commercial exploitation of European eel, both from fisheries and aquaculture, totally depends on the wild stock (Podda et al., 2021). The European eel is defined as critically endangered on the IUCN red list as the stock has declined sharply for several decades (Pike et al., 2020). Thus, the establishment of hatchery techniques and technology for this species is indispensable to ensure the sustainability of aquaculture, sustain fisheries and support conservation management through stock enhancement. Advancements in research and technology have led to a stable production of European eel offspring, opening new avenues to conduct larval culture research (Tomkiewicz et al., 2019). However, the early larval-rearing phase of the European eel is still characterised by substantial mortalities. Among other reasons, these mortalities can be attributed to a poorly developed immune system during early life stages (Miest et al., 2019) and negative larval-bacteria interactions (Bandara et al., 2023; Sørensen et al., 2014).

Generally, negative larval-bacteria interactions result in dysbiosis, leading to poor performances (e.g., growth and survival) during fish larval culture (Vadstein et al., 2013). Sørensen et al. (2014) demonstrated that the survival of European eel larvae was affected by bacterial interference during the early yolk-sac phase, where higher longevity of larvae reared under bactericidal conditions (i.e., in a mixed-antibiotic solution) compared to bacteriostatic conditions was reported. Further, Bandara et al. (2023) showed that the mortalities observed at the end of the first feeding window were partly attributed to the detrimental larval-bacteria interactions. Moreover, there is no doubt that the larval immune system plays a vital role in combatting pathogenic and opportunistic microorganisms. However, the early stages of most marine fish larvae are considered immunocompromised because the adaptive arm of the immune system is not fully developed. Thus, the immune function of early larvae relies entirely on the innate immune arm during this period (Vadstein et al., 2013). During the larval pre-feeding period, Miest et al. (2019) identified a period during this early development of European eel with relatively low expression of immune-related genes. This potentially immuno-compromised period coincided with a sharp drop in larval survival leading to only ~35% of larvae reaching the feeding stage. Therefore, it seems to be beneficial to explore measures that can be applied during early larval rearing to manage bacterial communities and strengthen larval immunity. However, such measures also bring limitations when applied in the hatchery. For instance, the heavy and prophylactic use of antibiotics has become problematic and should be banned as a microbial management tool (Cabello, 2006). On the other hand, the lack of a fully developed adaptive immune function in the early larval stage limits the applicability of tools available for immunisation such as vaccination. Therefore,

alternative approaches such as immunostimulation (i.e., stimulation of the innate immune system; Vadstein, 1997) and prebiotics should be considered to improve health and survival during the early larval rearing phase of European eel.

In this regard, β-glucans (BGs), polymers comprised of repeating units of glucose and linked by β-glycosidic bonds, are among the most tested compounds as an immunostimulant and prebiotic in aquaculture (reviewed in Meena et al., 2013). The reported beneficial effects of β-glucans include potentiation of immune cell resilience, reduction of stress, protection of teleost fish against subsequent challenge (reviewed in Petit and Wiegertjes, 2016), potential to enhance bone structure and prevent malformations (Miest et al., 2016), and alteration of bacterial community composition (Baumgärtner et al., 2022; Kühlwein et al., 2013; Miest et al., 2016). Naturally, BGs occur as a cell wall compound in yeast, plants, seaweeds, mushrooms, and bacteria (reviewed in Meena et al., 2013). Their immunostimulatory function depends on the source, solubility, molecular mass, tertiary structure, and degree of branching (reviewed in Vetvicka et al., 2013). Different application strategies of BG such as oral administration, intraperitoneal injection, and bath treatment (immersion) have been explored for different fish species (reviewed in Petit and Wiegertjes, 2016). However, the applicability of the administration strategy also largely depends on the targeted developmental stage of the fish.

The pathways of how BGs induce innate immunity in fish are not fully understood. However, due to the well-conserved nature of the innate immune arm, the immunostimulatory mechanism in fish has so far been assumed to be like that of mammals (Rodrigues et al., 2020). Induction of the cellular immune response initiates when BG binds to one or more specific cell surface receptors, such as toll-like receptor 2 (TLR-2; Underhill et al., 1999), dectin-1 (Brown et al., 2003), complement receptor 3 (CR3; Elder et al., 2017), lactosylceramide (Zimmerman et al., 1998), and less defined scavenger receptors. Nonetheless, the status of fish and their ability to recognise BG is not well-defined. For instance, there has not been a definite discovery of homologues to dectin-1 in fish. Conversely, there is speculation that receptors from the C-type lectin receptors (CLRs) family might be involved in beta-glucan recognition in fish (Rodrigues et al., 2020). When BG receptors are engaged by BG, a myriad of immune responses are initiated leading to phagocytosis (Gantner et al., 2003; Herre, 2004; Mueller et al., 2000), the release of certain cytokines, production of Reactive Oxygen Species (ROS; Del Fresno et al., 2018) and antigen presentation (Raa, 2000).

In the present study, we tested the potential of yeast-derived β-1,3/ 1,6-glucan (commercially available as MacroGard®) as a prophylactic measure that can be incorporated into the current larval culture protocol of European eel. We hypothesised that BG could modulate the larval innate immune response and bacteriomes (of larvae and water), resulting in improved viability of

European eel yolk sac larvae during the previously identified immunocompromised period (Miest et al., 2019). For this, we treated European eel larvae with BG by adding to rearing water from 5 dph (evident mouth opening) until 9 dph (just before reaching the feeding stage) and compared them to the control group. From 10 dph and onwards, both groups were offered the same exogenous food until the end of the experiment on 20 dph. We explored the expression patterns of genes involved in innate immunity and the stress/repair mechanism and investigated the bacterial community composition (of larvae and water). Throughout the experiment, larval survival and growth were followed. Moreover, we also examined eel larval deformities, inspired by the observation of Miest et al., (2016), where the effect of BG on the expression of genes related to skeletal development in turbot (*Scophthalmus maximus*) larvae was described, suggesting that BG might have the potential to reduce malformations.

2 Materials & methods

2.1 Ethics statement

All fish were handled according to the European Union regulations concerning the protection of experimental animals (Dir 2010/63/EU). Experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2020-15-0201-00768). Broodstock was anaesthetised individually before tagging, biopsy, and stripping of gametes, while euthanised after stripping (females) or at the end of the experiment (males) by submergence in an aqueous solution of ethyl p-aminobenzoate (benzocaine, 20 mg/L, Sigma Aldrich, Germany). Larvae were anaesthetised and euthanised using tricaine methanesulfonate (MS-222, Sigma Aldrich, Germany) at a concentration of 7.5 and 15 mg/L, respectively.

2.2 Experimental animals

The offspring of European eel were obtained from EEL-HATCH, an experimental facility of the Technical University of Denmark. Gametes originated from farmed broodstock (Royal Danish Fish, Hanstholm, Denmark) reared at 20ºC, by applying previously described assisted reproduction protocols for the induction of gametogenesis and final maturation (Kottmann et al., 2020). Eggs from each female (n = 4) were mixed with a sperm pool from several males (Benini et al., 2018), applying standardised fertilisation procedures (Butts et al., 2014; Sørensen et al., 2013). The fertilised eggs were incubated following previously described protocols (Politis et al., 2018b, 2017, 2014) and embryos hatched at ~56 hours post-fertilisation.

2.3 Experimental design

2.3.1 Exposure of larvae to MacroGard® during pre-feeding

The experiment was repeated 4 times, each time using offspring from different parental crosses. Newly hatched larvae from each parental cross (n = 4) were equally distributed into two 80 L tanks at a density of 311 ± 50 larvae/L. The tanks were connected to a recirculating aquaculture system (RAS), consisting of a biofilter, trickle filter, protein skimmer (Turboflotor 5000 single 6.0, Aqua Medic GmbH, Bissendorf, Germany) and UV light (11 W, JBL ProCristal, Neuhofen, Germany) as previously described in (Bandara et al., 2023). On 4 dph, the larval tanks were randomly allocated to control and treatment groups. The tanks housing the control group remained connected to the same RAS used to rear the larvae until they reached 4 dph, whereas the tanks housing the treatment group were connected to an identical, but separate RAS. In both RAS units, larvae were reared in total darkness and monitored under low-intensity illumination (Politis et al., 2014). The temperature was ~20°C, salinity ~36 PSU, and flow rates ~ 12 mL/s. From 5 until 9 dph, the treatment group received Yeast β-1,3/1,6-glucan in the commercially available form as MacroGard® (Biorigin, Brazil) at a concentration of 5 mg/L (Piaget et al., 2007). MacroGard® is an insoluble form of β-1,3/1,6-glucans derived from *Saccharomyces cerevisiae*, containing a minimum of 60% β-glucans. A stock solution of the MacroGard® was prepared daily by dispersing the powder in ~36 PSU saline water, obtained from the same RAS that supplied water to the corresponding tanks. During the exposure period, the water flow into the rearing tank was stopped and the pre-calculated volume of the freshly prepared MacroGard® stock solution was added to the treatment tank. After ~30 min exposure time, rearing tanks were disconnected from the RAS and water flow was started, so that the MacroGard® particles were flushed out from the tank. After letting water flow through for ~30 min, tanks were re-connected to the RAS. Larvae were exposed to MacroGard® five times/day at 09:00, 11:00, 13:00, 15:00 and 17:00. The control tanks were sham handled by similar handling procedures, except for the addition of MacroGard®.

2.3.2 *Larval rearing during exogenous feeding*

On 9 dph, the salinity was reduced to ~18 PSU (Politis et al., 2018a; 2021) by connecting the tanks to a similar (see 2.2.1) RAS that supplied water at this salinity. Thereafter, larvae were moved to replicated (n = 2) 8 L Kreisel tanks, which were connected to new RAS units (previously described in Benini et al., 2023), where larvae were reared until 20 dph. The temperature was ~20°C, salinity ~18 PSU and flow rate ~ 420 mL/min. Larvae were reared in darkness except during feeding when lights (~500 lx) were turned on. A slurry-type diet, which has previously been described by Benini et al. (2023) was offered following the described feeding protocol. The larvae were moved to clean tanks after the last feeding, each day.

2.4 Sampling and data collection

2.4.1 Larval biometry and classification of deformities

For morphometric measurements and classification of deformities, samples (n = 3) containing ~10 larvae each, from each parental cross (n = 4) were randomly sampled from the control and treatment groups, at 4 and 9 dph, while samples of ~10 larvae from each rep (n = 2), parental cross (n = 4), and treatment (n = 2) were randomly sampled at 20 dph. Sampled larvae were anaesthetised prior to being photographed using a digital camera (Digital Sight DS-Fi2, Nikon Corporation, Japan) mounted to a stereo microscope (SMZ 1270, Nikon Corporation, China) and euthanised thereafter. Imaged larvae were later analysed for biometrics and deformities using NIS-Elements Analysis D software (Version 5.20.00). Larval body area at each sampling point was measured as a proxy of larval growth. For larval deformities, the images taken were inspected to identify six types of deformities (pericardial edema, emaciation, deformed neurocranium, spinal curvature and jaw deformity) following classification according to Kurokawa et al., (2008).

2.4.2 *Survival*

Larval survival was estimated by the end of the BG exposure period (9 dph), at first feeding (10 dph), and during the feeding window (15 dph) via counts of larvae from known volume subsamples (n = 5) taken from the rearing tanks. Additionally, all larvae at the end of the experiment (20 dph) as well as those sampled from each experimental unit, were enumerated. Larval survival was then calculated as a percentage of initial numbers at each period, separately for the BG exposure and the feeding period.

2.4.3 *Sampling*

For molecular analysis, samples (n = 3) containing ~10 larvae each were collected from the control and treatment groups, for each parental cross (n = 4) on 9 dph (at the end of the BG exposure). On 20 dph, samples of ~10 larvae were collected from each replicated (n = 2) tank of the control and treatment groups for each parental cross (n = 4). Sampled larvae were immediately euthanised, preserved in RNAlater (Sigma-Aldrich St Louis, USA) and stored at -20°C for later analysis (Politis et al., 2017).

For the bacterial community composition analysis, larval samples (n = 4) containing ~10 larvae each were collected from the control and treatment groups for each parental cross (n = 4) on 4 and 9 dph. Also, samples (n = 2) of ~10 larvae were collected from each replicated (n = 2) tank of the control and treatment groups for each parental cross (n = 4) on 20 dph. Sampled larvae were immediately euthanised, rinsed and stored at -20°C for later analysis. To investigate the influence of BG treatment on the water bacterial community, rearing water samples (n = 4) were

collected from the tank outlets from the control and treatment tanks for each parental cross (n = 4) before (4 dph) and after (9 dph) the exposure period. For this, 250 mL of water from each sample was vacuum filtered through 0.22 µm white gridded filters (diameter = 47 mm; Merck KGaA, Darmstadt, Germany) using a Büchner funnel and the filters were stored in sterile cryotubes at −20°C until processing (Bakke et al., 2013).

2.4.4 Gene expression analysis

Total RNA from samples was extracted using the NucleoSpin (Mini) RNA isolation kit, following the protocol provided by the supplier (Macherey-Nagel GmbH & Co. KG, Düren, Germany). RNA concentration (148.9 ± 89.7 ng/mL) and purity (260/280 = 2.15 ± 0.02, 230/260 = 1.78 ± 0.56) were determined by spectrophotometry using Nanodrop ND-1000 (Peqlab, Germany). The concentration was normalised to 100 ng/mL with HPLC water. From the resulting total RNA, 450 ng was reverse transcribed using the qScript™ Ultra SuperMix cDNA Synthesis Kit (Quantabio, Germany) according to the manufacturer's instructions, including an additional gDNA wipe-out step before transcription [PerfeCtaR DNase I Kit (Quantabio, Germany)].

Expression levels of 8 target and 2 reference genes were determined by quantitative real-time PCR (qRT-PCR). Primers were designed using primer 3 software v 0.4.01 based on sequences available in Genbank databases (Table 1). All primers were designed for an amplification size ranging from 75 to 200 nucleotides. The expression of genes in each larval sample was analysed in technical replicates (n = 3) using the QuantStudio5 (Applied Biosystems, Thermo Fisher Scientific, USA) qPCR system. The qPCR assays were performed in a final volume of 10 µL reaction mixtures, containing 4 µL of cDNA template, 0.5 µL of UltraPure™ DNase/RNase-free distilled water (Thermo Fisher Scientific, USA), 6 µL of PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific, USA) and 0.5 µL of each primer (Table 1). The mixture was vortexed and distributed in low-profile 0.2 ml optical 8-tube strips (BIO-RAD, USA), covered with flat optical 8-cap strips (BIO-RAD, USA), and kept on ice until placed in the real-time PCR thermal cyclers. Here, the following PCR thermal profile was used: initial denaturation at 95°C for 2 min, followed by 40 amplification cycles (at 95°C for 15 s, at 60°C for 1 min and at 90°C for 15 s) and a final step at 60°C for 1 min and 90°C for 15 s (melting curve). Ct values and quality of the run were then visualised with Design and Analysis Software version 2.5.1 (Thermo Fisher Scientific, USA). Ribosomal protein S18 (*rps18)* and elongation factor 1a (*ef1a*) were chosen as reference genes, as they have been suggested to be the most stable in fish larvae and are thus, reliable reference genes (McCurley and Callard, 2008). The relative quantity of target gene transcripts (ΔCT) was normalised to the geometric mean of the two reference genes. The coefficient of variation (CV) of technical replicates was calculated and checked. Further analysis of gene

expression was carried out according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), to calculate the expression of targeted genes relative to the expression levels in the control group.

2.4.5 Characterisation of bacterial community composition by amplicon sequencing

DNA from larvae and water were isolated using the MagAttract PowerSoil Pro DNA Kit (Qiagen, Germany) following the protocol developed by the supplier for automated high-throughput isolation of DNA with the Thermo Scientific® KingFisher® Flex platform. Briefly, samples (pools of ~10 larvae or filter papers) were homogenised in bead-beating tubes containing ~0.55 g of 0.1 mm glass beads (Bertin Technologies, France) and 800 µL of lysis buffer, using a Precellys 24 tissue homogeniser (Bertin Technologies, France) at 5500 rpm for two times 30 s with a 15 s break in between. The tubes containing the lysates were centrifuged at 15000 g for 1 min, and the supernatants were transferred into 1.5 mL Eppendorf tubes. Then, 300 µL of CD2 solution was added to each Eppendorf tube, vortexed to mix and centrifuged at 15000 g for 1 min. Prepared lysates i.e., supernatants from the previous step, were transferred to the KingFisher Flex platform (Thermo Fisher Scientific), where total genomic DNA was captured on specialised magnetic beads in the presence of buffers, washed on the beads and then eluted.

The V3 and V4 regions of the bacterial 16S rRNA gene were amplified from the extracted DNA using the forward primer, Ill-341F_K1 (5′- NNNNCCTAC GGGNGGCWGCAG -3′) and the reverse primer, Ill805R (5′- NNNNGACTACNVGGGTATCTAAKCC-3′) (Klindworth et al., 2013). Each PCR reaction contained 0.02 U/µL Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific), 0.2 mM of each dNTP (VWR), 0.3 µM of each primer (SIGMA), 1x Phusion HF buffer (containing 7.5 mM $MgCl_2$) (Thermo Fisher Scientific) and PCR grade water (VWR) up to a total reaction volume of 25 µL, and 1 µL of DNA extract as a template. The PCR reactions were run with 35 cycles (T100TM Thermal Cycler, Bio-Rad) (Bugten et al., 2022). The PCR amplicons were purified and normalised using SequalPrep Normalisation Plate (96) kit (Invitrogen, USA), following the protocol provided by the supplier. Using the Nextera®XT DNA Sample Preparation Kit (Illumina), a unique pair of index sequences that represented the PCR amplicons, originating from each sample, was added by an additional PCR step with 10 cycles. The indexed PCR products were purified and normalised using the SequalPrep Normalisation Plate (96) kit (Invitrogen, USA). Finally, the samples were pooled and concentrated with AmiconUltra 5.0 Centrifugal Filter (Merck Millipore, Ireland) following the manufacturer's protocol. The amplicon library was sequenced in a MiSeq run (Illumina, San Diego, CA) with V4 reagents (Illumina) at the Norwegian Sequencing Centre (NSC), University of Oslo.

The Illumina sequencing data were processed using the USEARCH utility (version 11) (https://www.drive5. com/usearch/). Merging the paired reads, trimming off primer sequences and

filtering out reads shorter than 380 base pairs were carried out using the command "Fastq_mergepairs". The "Fastq_filter" command (with an expected error threshold of 1, was used for further processing, including demultiplexing, removal of singleton reads, and quality trimming. The "Unoise3" command was used for chimaera removal and generation of amplicon sequence variants (ASVs) (https://drive5.com/usearch/manual/ cmd_unoise3.html). Taxonomy was assigned by applying the "SINTAX" script (Edgar, 2016), with a confidence value threshold of 0.8 against the RDP reference data set (version 18). Before analysing the data, ASVs representing eukaryotic amplicons (e.g., algae, fish DNA), Archaea and Cyanobacteria/Chloroplast were removed from the ASV table. Moreover, ASVs that were highly abundant in the DNA extraction kit blank and reported as common contaminants were removed.

2.5 Statistical analysis

2.5.1 Larval survival, body area, deformities, and expression of immune- and stress-related genes

R studio statistical analysis software (version 4.2.0) was used for all statistical analyses. Residuals were evaluated for normality and homoscedasticity (plot of residuals vs. predicted values) to ensure that they met model assumptions. Data were transformed appropriately to meet these assumptions when necessary. Alpha was set at 0.05 for testing the main effects and interactions. To compare larval survival at the end of the BG treatment (on 9 dph) and relative expression levels of genes related to stress and immune response between the control and treatment groups on 9 and 20 dph two-sample t-tests were used. Larval survival during the feeding period, body area, and deformity data were analysed using a series of mixed model ANOVAs, where the main model variables were treatment (control *vs.* BG treatment) and age, whereas families were considered random. The initial model tested included an interaction effect between treatment and age. The model was reduced, when possible, and means were contrasted using Tukey's honestly significant difference test (Tukey's HSD).

2.5.2 Measures of microbial diversity

Different packages developed for R statistical software (version 4.2.0) were used to calculate diversity indices and perform statistical analyses. Data were transformed appropriately to meet these assumptions when necessary. Beta-diversity analyses were performed on the ASV table that had been filtered to remove any ASVs that had less than 2 counts in at least two samples and rarefied by sub-sampling ten times at 20,000 reads per sample. Ordination by principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarities (Bray & Curtis, 1957) was used to visualise differences in microbial community composition between different groups of samples using the function "plot_ordination" within the "phyloseq" package (version 1.40.0). Permutational

multivariate analysis of variance (PERMANOVA) (Anderson, 2001) based on the Bray-Curtis dissimilarities was used to test for differences in community composition (beta diversity) as a function of treatment and age. Pairwise differences were tested using the function "pairwise.adonis2" in the "vegan" package (version 2.6.2). Alpha-diversity measures include diversity numbers of order 0 (ASV richness), 1 (exponential Shannon/exp. Shannon) and evenness (Hill, 1973). These indices were calculated using the vegan community ecology package (version 2.6.2). Alpha diversity indices in the larval and water bacterial communities were analysed using a series of mixed model ANOVAs, where the main model variables were treatment (control and treatment) and age, whereas families were considered random. The model was reduced, when possible, and means were contrasted using Tukey's honestly significant difference test (Tukey's HSD). To compare the alpha diversity indices between the water and larval bacterial communities on 4 and 9 dph two-sample t-tests were used. Residuals were evaluated to ensure that they met model assumptions. The package "DESeq2" (version 1.36.0) was used on the unrarefied ASV table to assess the differential abundance of ASVs between the samples of interest. DESeq2 includes a model based on the negative binomial distribution and Wald's post hoc test for significance testing. The P-values adjustment method used was the Benjamin and Hochberg method (Benjamini and Hochberg, 1995), which accounts for multiple comparisons.

3 Results

3.1 Survival and body area

No effect of BG treatment was detected on larval survival neither at the end of the exposure period on 9 dph (Figure 1 A) nor at the end of the experiment on 20 dph [Figure 1B(i)]. However, a significant reduction in larval survival was observed with age throughout the feeding period [Figure 1B(ii)]. An increase in larval body area throughout ontogeny indicated larval growth [Figure 1C(i)], but no effect of BG was detected [Figure 1C(ii)].

3.2 Deformities

In our analysis, there was no significant age × treatment interaction observed for three of the deformity types: pericardial edema, emaciation, and neurocranium deformities (Figures 2A–C). Interestingly, the occurrence of pericardial edema was ~86% higher ($p = 0.01$) in the control group compared to the BG-treated group [Figure 2A(i)]. Moreover, a significant effect of age on pericardial edema was found, which was not detected on 4 dph, but increased to ~9% occurrence on 9 and 20 dph [Figure 2A(ii)]. A significant treatment effect was also detected for emaciation, where ~81% more ($p = 0.03$) emaciated larvae were detected in the control group than in the treated group [Figure 2B(i)]. Moreover, a higher occurrence of emaciation was detected on 20 dph than on 4 dph [Figure 2B(ii)]. For deformation of the neurocranium, ~80% more ($p = 0.03$)

larvae with this type of deformity were detected in the control group than in the BG-treated group [Figure 2C(i)], while the occurrence was not affected by age [Figure 2C(ii)]. For spinal curvature and jaw deformity, a significant age × treatment interaction was found (Figures 2D and E). The occurrence of both types of deformities was significantly higher in the control group compared to the BG treatment on 20 dph [Figure 2D(i) and E(i)]. Moreover, in the control group, both types of deformities occurred at significantly higher percentages in older larvae (9 and 20 dph old) than in 4 dph old larvae [Figure 2D(ii) and E(ii)]. However, their occurrence did not increase with age in the BG-treated group [Figure 2D(iii) and E(iii)]. Overall, our results of deformity analysis indicated that the occurrence and/or detectability of deformities increased with larval age and that the BG treatment reduced the occurrence of larval deformities compared to the control group.

3.3 Bacterial community composition analysis

3.3.1 Alpha diversity: Diversity within samples

When analysing the alpha diversity indices of larval bacterial communities, a significant age × treatment interaction was detected for ASV richness and exp. Shannon index, but not for evenness. BG treatment increased ($p = 0.01$) the ASV richness of the larval bacterial community by ~30% compared to the control group at the end of the exposure period on 9 dph [Figure 3A(i)]. However, the effect was diminished by 20 dph, where ASV richness was comparable between treatment groups. While ASV richness remained unchanged throughout ontogeny in the control group [Figure 3A(ii)], it decreased significantly in the bacterial community of feeding larvae (on 20 dph) compared to pre-feeding larvae (on 4 and 9 dph) in the treatment group [Figure 3A(iii)]. On the other hand, evenness within the bacterial communities was neither affected by treatment nor larval age [Figure 3B (i) and (ii)]. As observed for the ASV richness, BG treatment increased ($p = 0.005$) the diversity of the larval bacterial community as indicated by a ~40% higher exp. Shannon index in the treatment group than in the control group at the end of the exposure period on 9 dph [Figure 3C(i)]. Moreover, the exp. Shannon index in the larval bacterial community did not change with age in the control group [Figure 3C(ii)], whereas it decreased significantly in the bacterial community of feeding larvae compared to that of the pre-feeding larvae in the treated group [Figure 3C(iii)]. Overall, BG treatment increased the diversity of the larval bacterial community, mainly by increasing the bacterial richness.

For all three alpha diversity indices of rearing water bacterial communities, the age × treatment interaction was significant. ASV richness in bacterial communities of water was similar between the control and the treatment tanks before the BG treatment on 4 dph [Figure 3D(i)]. However, the water bacterial community of the control had a higher ($p = 0.02$) ASV richness on 9 dph compared to the BG treatment. While ASV richness in the water bacterial community of the

control increased ($p < 0.001$) with age, it remained unchanged in the treatment group [Figures 3D(ii) and (iii)]. Both evenness and exp. Shannon index were higher in the water bacterial community of the treatment before the BG treatment on 4 dph [Figures 3E(i) and F(i)]. Interestingly, both alpha diversity indices decreased significantly in the water bacterial community of the treatment compared to the control after the BG treatment on 9 dph. Moreover, both evenness and exp. Shannon index increased significantly with age in the bacterial community of the control [Figures 3E(ii) and F(ii)], whereas both indices were significantly lower in the bacterial community of BG-treated water [Figures 3E(iii) and F(iii)]. In contrast to the higher alpha diversity observed in the bacterial community of the BG-treated larvae, we observed that BG treatment lowered the alpha diversity of the bacterial community of the rearing water.

When comparing the bacterial communities of rearing water and larvae on 4 dph, all alpha diversity indices were significantly higher in the rearing water than in the larvae for both control and treatment groups [Figures 3G to I]. On 9 dph, ASV richness was significantly higher in the bacterial community of rearing water compared to that of larvae in both the control and treatment groups [Figures 3J (i) and (ii)]. Moreover, the rearing water bacterial community was more even ($p < 0.01$) than the larval bacterial community in the control [Figure 3K (i)], whereas evenness was comparable between water and larvae in the BG treatment [Figure 3K (ii)]. However, the water bacterial community was more diverse than the larval bacterial community in both groups, with a significantly higher exp. Shannon index in the bacterial community of rearing water [Figure 3L (i) and (ii)]. Overall, we observed a more diverse bacterial community in the rearing water than in the larvae in both control and treatment groups.

3.3.2 Beta diversity: Comparison of samples

PCoA based on Bray–Curtis distance was performed to evaluate the β-diversity. The two axes of the PCoA plot based on the Bray-Curtis distances captured 24.2% of the variability in the data (Figure 4). Strikingly, the PCoA show a clear effect of BG treatment on the larval and water bacterial communities. Before the BG treatment (on 4 dph), samples (larvae or water) of the control and treatment groups clustered together, indicating that they had similar bacteriomes. After the BG treatment (on 9 dph), a clear separation between the samples of the control and treatment groups was observed for both larval and water samples. However, the differences disappeared by 20 dph, as indicated by the lack of separation of the two groups in the PCoA plot. On both 4 and 9 dph, discrete clustering of larval and water samples can be seen. On 4 dph, larval and water samples separated mainly along axis 1 for both control and treatment. On 9 dph, larval and water samples still separated mainly along axis 1 for the control group, but the separation between larval and water samples was mainly along axis 2 for the treatment group (Figure 4).

Moreover, PERMANOVA tests based on Bray–Curtis distances were carried out to extract significant differences (Table 2). Before the BG treatment (on 4 dph), both rearing water and larval bacteriomes of the treatment group were comparable to those of the control group. Interestingly, at the end of the BG treatment (on 9 dph) both rearing water and larval bacteriomes were significantly different between the groups, indicating the effect of BG treatment. However, 10 days post-treatment (on 20 dph), the effect of BG on bacterial communities diminished, leading to similar larval bacteriomes in both experimental groups. Moreover, rearing water and larval bacterial communities were significantly different throughout the experiment in both control and treatment groups (Table 2).

3.3.3 Relative abundances at the order level and differential abundance analysis

Before the BG treatment (on 4 dph), bacterial communities of water from both, control and treatment groups were mainly composed of bacteria belonging to the following orders (relative abundances (%) for control and treatment, respectively): "Unassigned" (16.7 and 21.2), Vibrionales (17.2 and 20.3), Alteromonadales (13 and 11), Oceanospirillales (14 and 14.5), Flavobacteriales (6.2 and 3.7), Rhodobacterales (15.5 and 13.4) and Clostridiales (13.2 and 3.9) (Figure 5). Additionally, the Burkholderiales order contributed (5.8%) to the water bacterial community of the treatment group. As for the pre-treated larvae (4 dph), bacterial communities were dominated by the following orders (relative abundances (%) for control and treatment, respectively): Burkholderiales (12.4 and 9.2), "Unassigned" (11.1 and 13.4), Pseudomonadales (11.3 and 6.3), Vibrionales (12.4 and 6.7), Alteromonadales (7 and 13.6), Oceanospirillales (12.6 and 17.7), Rhodobacterales (6.7 and 8.5), and Cellvibrionales (9.7 and 8.5) as shown in Figure 5.

On 9 dph, the bacterial community of rearing water of the treatment was composed mainly of "Unassigned" taxa (69.3%), whose contribution to the water bacterial community of the control group was only 22%. On the other hand, the water bacterial community of the control group contained considerable proportions of other orders such as Vibrionales (12.4%), Alteromonadales (13.4%), Oceanospirillales (17.5), and Rhodobacterales (26.9%). The contribution of these orders to the water bacterial community of the treatment group was less than 10%. At the ASV level, DESeq2 analysis revealed that clearly more ASVs belonging to the orders Vibrionales, Alteromonadales and Oceanospirillales were significantly more abundant in the water bacterial community of the control group than in the treatment group (Figure 6A). Strikingly, 80 ASVs of the Vibrionales order were significantly more abundant in the water bacterial community of the control group compared to the BG treatment, whereas only 2 ASVs of this order were significantly more abundant in the water bacterial community of the BG treatment group (Figure 6A). Regarding the larval bacteriome, it is noteworthy that two ASVs belonging to the Vibrionales order showed significantly higher abundance in the control group. In contrast, none of the ASVs

from this order displayed a significant abundance difference in the treatment group (Figure 6B). These results suggest that BG treatment selected for a more specialised (potentially K-selected) water bacterial community, as indicated by the dominancy of "Unassigned" taxa (~69%) in the bacterial community and they displaced potentially harmful bacterial taxa such as Vibrionales.

For the larval bacterial community composition, the selection for specialised bacterial taxa and displacement of Vibrionales was noticed at the end of the exposure period (9 dph), but to a lesser extent than in the water bacterial communities. For instance, the contribution of "Unassigned" taxa for the bacterial community of the treatment group was 28.9%, whereas its contribution to the bacterial community of the control group was only 13.3% (Figure 5). On the other hand, the Vibrionales order had a relative abundance of 13.3% in the bacterial community of the control larvae, while it was only 4.1% in the bacterial community of the BG-treated larvae. Further, DESeq2 analysis revealed that two ASVs of the Vibrionales order were significantly more abundant in the bacterial community of the control larvae compared to the BG-treated larvae (Figure 6). On the other hand, ASVs of the following orders: Oceanospirillales, Cellvibrionales and Alteromonadales were significantly more abundant in the bacterial community of the BG treatment group compared to the control group. However, the differences caused by the BG treatment in the larval bacterial communities diminished by 20 dph.

3.4 Gene expression analysis

Expression levels of stress and immune related genes between treatments were statistically tested at the end of the BG treatment (on 9 dph) and at the end of the experiment (on 20 dph). At the end of the BG treatment, *hsp90*, a gene related to the stress/repair mechanism, was downregulated in the BG-treated larvae (Figure 7A). Likewise, *il10*, the gene encoding the anti-inflammatory cytokine interleukin 10, exhibited downregulation when subjected to BG treatment (Figure 7B). The other immune related genes tested, *tlr18*, *tnfa*, *c1qc*, *irf3*, *irf7* and *lysc,* were not differentially expressed between the groups at the end of the BG treatment (Figure 7C-H). On 20 dph, expression levels of all the genes tested were comparable between the control and BG treatment groups (Figure 7I-P). Overall, the results of the molecular analysis indicate that BG treatment provided the larvae with stress protection as indicated by lower expression levels of *hsp90 and* had an immunomodulatory effect through the downregulation of *il10*. However, these effects were not detected 10 days post-treatment.

4 Discussion

The present study demonstrated the modulatory effect of BG on eel larval performance and their associated immune and stress response as well as on bacterial communities of water and larvae for the first time. our initial hypothesis that BG can improve the survival and growth of the European eel yolk-sac larvae, was not supported. These findings contradict previous results showing the positive effect of BG on survival and growth of first-feeding turbot (*S. maximus*) larvae (Miest et al., 2016) and blue-fin porgy (*Sparidentax hasta*) larvae (Al-Gharabally et al., 2013), as well as improved growth performance in Persian sturgeon (*Acipenser persicus*) juveniles (Aramli et al., 2015). On the other hand, there have been documented instances of BG treatment showing no impact on larval size in turbot (*S. maximus*) juveniles (Fuchs et al., 2015), and in some cases, even exhibiting a detrimental effect on growth in Atlantic cod (*Gadus morhua*) larvae (Jensen et al., 2008). This suggests that the influence of BG treatment differs across species and developmental stages (or possibly also experimental setups).

Strikingly, our results demonstrated the ability of BG treatment to reduce the occurrence of deformities in eel larvae. A previous study suggested the potential of using BG as a measure to prevent malformations based on their observations of increased expression of osteocalcin, a gene responsible for bone mineralisation in BG-treated turbot (*S. maximus*) larvae (Miest et al., 2016). In the present study, we analysed different types of larval deformities and noticed that the occurrence of all types studied was significantly lower in the BG-treated larvae compared to the control. However, the present study does not have molecular evidence to elucidate possible mechanisms behind the observed positive effect of BG on malformations. BGs are polymers comprised of repeating units of glucose, which is a precursor of monomers (glycosamine and glucuronic acid) of hyaluronic acid (HA) (Roseman et al., 1953a, 1953b). HA is one of the three major classes of Glycosaminoglycans (GAGs), the main composite of the transparent gelatinous matrix of the body of eel larvae (Pfeiler, 1999; Pfeiler et al., 2002). Therefore, BG might be available for the formation of larval body structure, probably through a bacterial lysis step (e.g., with the involvement of β-glucanase), of which the resulting product mainly is glucose (Wu et al., 2021). The resulting glucose dissolved in the rearing water might be available to larvae through drinking (Reitan et al., 1998). As this is purely speculatory, further work needs to investigate the mechanisms behind the ability of BG to improve the development of eel larvae.

Moreover, we observed that the older the larvae the higher the occurrence of deformities (except for deformed neurocranium). There are different possible explanations for this observation. In some cases, the detectability of deformity increases with age, e.g., jaw deformities are not quite visible during the pre-feeding stage because their jaws are not fully developed. Similarly, the edematous state of the heart is not clearly visible in the yolk sac larvae because of the close

arrangement of the heart and the yolk sac. The occurrence of emaciation was significantly higher in the feeding larvae compared to the yolk sac larvae probably indicating nutritional deficiencies of the larval diets used during the experiment. In the treated group, the occurrence of jaw deformities and spinal curvature did not increase with age, whereas in the control group, both types of deformities occurred at higher percentages on 9 and 20 dph compared to 4 dph. This again suggests that BG potentially provides structural components for larval body formation and thus, reduces the occurrence of malformations.

BG treatment also altered the bacterial community compositions of both, larvae and water, with significantly different bacterial communities at the end of the treatment (9 dph). A higher diversity was observed in the bacterial community of the treated larvae compared to the control, mainly due to higher ASV richness. The present analysis involved pooled samples of entire larvae, but information regarding the impact of BG supplementation on the bacterial communities of entire larvae is generally limited. In accordance with our findings, a study on whole turbot (*S. maximus*) larvae revealed increased richness in bacterial communities of larvae fed rotifers (*Brachionus plicatilis*) enriched with BG compared to the control group (Miest et al., 2016). Nonetheless, several studies that exclusively examined gut samples have documented changes in gut microbiota composition in response to dietary BG supplementation. However, these alterations appear to lack a consistent and clearly discernible pattern across different studies. For instance, dietary supplementation of BG reduced species richness and diversity in the intestinal microbial communities of carp, *C. carpio* (Kühlwein et al., 2013), whereas another study of the same species found increased diversity due to increased richness of operational taxonomic units (Jung-Schroers et al., 2016). In Nile tilapia (*Oreochromis niloticus*), Chao Richness of the gut bacterial community increased after 15 days of BG treatment (in water at 0.1 mg/L; Souza et al., 2020). On the other hand, BG reduced bacterial diversity in the Senegalese sole (*Solea senegalensis*) gut, manifested as reduced species richness and Shannon diversity when administered by oral intubation (Carballo et al., 2019).

A very important finding in our study was that BG treatment significantly reduced the abundance of ASVs of the genus *Vibrio*, especially in the rearing water. This genus includes many members that are detrimental in aquaculture (Austin and Zhang, 2006; Kumar et al., 2021; Kumara and Hettiarachchi, 2017). Reduction of ASVs of the genus *Vibrio* in response to BG was also observed in the larval bacteriome, but to a lesser extent during the present study. While the effect of BG on the rearing water bacteriome has hardly been addressed elsewhere, the effect of BG on gut-associated bacteria of the genus *Vibrio* has been reported. A significant decrease ($P < 0.05$) in the abundance of bacteria from the genus *Vibrio* in response to yeast β-glucans treatment was observed in the intestine of juvenile sole, *S. senegalensis* (Carballo et al., 2019) and common

carp, *C. carpio* (Jung-Schroers et al., 2016). Dietary BG supplementation also reduced intestinal *Vibrio* counts in pompano fish, *Trachinotus ovatus* (Do Huu et al., 2016). While the exact mechanism by which BG regulates *Vibrio* spp. remains unclear, there may be a connection to the bacterial breakdown of BG into short-chain fatty acids (SCFAs), which are recognised for their bioactive properties in combating *Vibrio* spp. In fact, bacterial degradation of non-starch polysaccharides such as BG resulted in the production of SCFAs (Kihara and Sakata, 2002, 1997), which decreased the total count of intestinal *Vibrio* spp. (Chuchird et al., 2015) and exhibited strong inhibitory capacity against *Vibrio* spp. in marine shrimp, *Litopenaeus vannamei* (da Silva et al.2013). Moreover, dietary inclusion of SCFAs lowered total viable bacteria and presumptive *Vibrio* spp. counts in the rearing water of tiger shrimp, *Penaeus monodon* (Ng et al., 2015). We hypothesised that the reduction in ASVs of the genus *Vibrio* in the bacteriome of water and larvae during the present experiment was due to bacterial degradation of BG to SCFAs. In addition to the reduced abundance of *Vibri*o spp., BG treatment led to a shift in the water and larval bacterial communities towards communities dominated by "Unassigned" taxa, reflecting most probably a K-selected community composed of diverse and specialised bacterial taxa.

Interestingly, we observed an effect of BG on molecular immune response as indicated by the downregulation of the gene encoding anti-inflammatory cytokine IL10, which exerts a conserved role in dampening inflammatory responses (Sakai et al., 2021). However, we did not see an effect of BG treatment on *tnfa*, which is a gene that encodes a pro-inflammatory cytokine. In puffer fish, a study by Sakai et al. (2021) demonstrated time-dependent fluctuations in the expression of both, pro-inflammatory and anti-inflammatory cytokines when exposed to immunostimulation by lipopolysaccharides (LPS). In that study, initially, there was an upregulation of pro-inflammatory cytokines such as TNF-α and IL-1β, which was followed by a subsequent downregulation, eventually returning to pre-stimulation levels. Concurrently, there was an upregulation of anti-inflammatory cytokines like IL-10 (Sakai et al., 2021). During the present study, the pro-inflammatory *tnfa* gene could have been upregulated during the initial phase of BG exposure, but the temporal resolution of sampling possibly did not detect it. In previous studies on different fish species, both pro-inflammatory (Biswas et al., 2012; Zhang et al., 2009) and anti-inflammatory (Falco et al., 2012; Miest et al., 2016) effects of BG have been reported. In the present study, apart from *il10*, the other studied immune related genes (*tr18, tnfa, c1qc, irf3, irf7 and lysc*) were not affected by the BG treatment. This is partly consistent with Miest et al. (2016) who reported that BG did not modulate the expression of some of these genes (encoding for the bactericidal enzyme, *lysc* and pattern recognition receptor, *tlr3*) in turbot (*S. maximus*) larvae. Contrary to our findings, they reported a modulatory effect of BG on proinflammatory cytokine, *tnfa* and complement component, *c3* (Miest et al., 2016). Moreover, our findings contrast the findings of some other studies conducted on fish juveniles (Falco et al., 2014, 2012; Fredriksen et al., 2011), highlighting

that the immunomodulatory effect of BG also depends on the source of BG, administration route, species, developmental stage and even on the organ (e.g., gut, head kidney, gills etc.).

During the present study, we observed significantly lower expression levels of *hsp90* at the end of the BG treatment in the treatment group. In line with our findings, the ability of dietary BG supplementation to prevent stress has been reported in larvae of turbot, *S. maximus* (Miest et al., 2016) and rainbow trout, *Oncorhynchus mykiss* (Jeney et al., 1997). However, the expression of heat shock proteins can be upregulated in response to the presence of biotic stresses such as harmful microorganisms (Roberts et al., 2010). Moreover, in the present study, we observed a significantly higher abundance of ASVs of genus *Vibrio* in the bacterial communities of the control group. Thus, higher expression levels of *hsp90* observed in the control group might also be attributed to the higher abundances of *Vibrio* spp. However, our results demonstrated that BG-treated larvae were less stressed either due to the direct effect of BG treatment on the cellular stress response or due to modulation of the bacterial communities through the displacement of *Vibrio* spp.

The observed effect of BG (added to rearing water) on the immune and stress/repair response as well as the bacterial community composition was, however, not long-lasting. This was indicated by the diminishing of differences in expression levels of immune and stress related genes and bacterial community composition between control and treatment groups 10 days after the termination of the treatment (on 20 dph). While the “short-lived” effect of BG on immunity (intestinal and systemic) has been described before (reviewed in Dalmo and Bøgwald, 2008), there is a potential for obtaining long-lasting beneficial effects of BG by the phenomenon called “trained immunity”. Trained immunity which in contrast to immunostimulation (i.e., short-term, specific enhancements of the immune response) represents a more long-lasting and broadly adaptive reprogramming or "training" of the innate immune system in response to various cues. (Petit and Wiegertjes, 2016; Zhang et al., 2019). As such, further investigations for potential applications for European eel larvae are strongly encouraged.

5 Conclusion and future perspectives

Based on the results of the present study we can conclude that the exposure of European eel yolk sac larvae to β-1,3/ 1,6-glucan in rearing water is beneficial. This is indicated by the significantly reduced occurrence of deformities, improved bacterial communities of rearing water and larvae (reduced abundance of *Vibrio* spp. and increased abundance of K-strategists), and reduced cellular stress in the BG-treated larvae. However, the modulatory effect of β-1,3/ 1,6-glucan on the bacterial communities or stress and immune response diminished shortly after the termination of the treatment. Moreover, the positive effects on microbial and immune modulation were not

reflected in larval growth and survival, at least not during the period covered by the present experiment. In conclusion, treatment with β-1,3/ 1,6-glucan (added to rearing water) can serve as a prophylactic measure that can be incorporated during the rearing of pre-feeding European eel larvae. However, further work needs to be performed to refine the application protocol in terms of dose and duration. Also, our results encourage testing β-glucan treatments on other developmental stages of European eel, potentially as a dietary supplement for feeding larvae and/or developing a treatment for broodstock fish to enhance parentally transferred immunity to the offspring.

CRediT author statement

All authors contributed to this manuscript.

Kasun A. Bandara: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. **Elisa Benini**: Methodology, Investigation, Data curation, Writing – review & editing. **Sune Riis Sørensen**: Resources, Visualization, Writing – review & editing. **Jonna Tomkiewicz**: Conceptualization, Methodology, Resources, Data curation, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Olav Vadstein**: Conceptualization, Methodology, Resources, Data curation, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Sebastian Nikitas Politis**: Conceptualization, Methodology, Investigation, Data curation, Validation, Visualization, Writing – review & editing, Supervision, Funding acquisition.

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Table 1. Primers used for molecular analysis of immune and stress related gene expression (FW: Forward, RV: Reverse).

Function	Gene name	Abbreviation	Primer sequence		Accession number
Reference	Ribosomal protein S18	*rps18*	FW	ACGAGGTTGAGAGAGTGGTG	XM_035428274.1
			RV	TCAGCCTCTCCAGATCCTCT	
	Elongation factor 1	*ef1a*	FW	CTGAAGCCTGGTATGGTGGT	XM_035428800.1
			RV	CATGGTGCATTTCCACAGAC	
Stress/ repair	Heat shock protein 90	*hsp90*	FW	ACCATTGCCAAGTCAGGAAC	XM_035392491.1
			RV	ACTGCTCATCGTCATTGTGC	
Pathogen recognition	Toll-like receptor 18	*tlr18*	FW	TGGTTCTGGCTGTAATGGTG	XM035421803.1
			RV	CGAAATGAAGGCATGGTAGG	
Inflammatory response	Interleukin 10	*il10*	FW	CTCGACAGCATCATGACAACA	XM_035387988.1
			RV	CCAGAGGTTCAGTGTTTAGGC	
	Tumor necrosis factor α	*tnfa*	FW	CACCTCTCCTCTCCTCTCCT	XM_035428518.1
			RV	CTGGGACTGTTCTTTAGCGC	
Complement system	Complement component 1, Q subcomponent, C chain	*c1qc*	FW	TCTGCTGTCATGTTCACCCA	XM_035433127.1
			RV	CTTCTCGCCATCCCTTCCAT	
Induce type I Interferon (ant-viral)	Interferon regulatory factor 3	*irf3*	FW	GAAGAGGTGGCAGCAAAATC	XM_035405523.1
			RV	GGAAAAAGAGGGGGATTCAC	
	Interferon regulatory factor 7	*irf7*	FW	TTCCTTGGAAGCACAACTCC	XM_035396683.1
			RV	TGTCGTTCGGATTCTCTCTG	
Antibacterial response	Lysozyme type C	*lysc*	FW	ACGGCATCTTCCAGATCAAC	AZBK01554584
			RV	TGGAGCACGGGATATTACAG	

Table 2. PERMANOVA p-values based on Bray–Curtis dissimilarities for comparisons of bacterial communities of European eel (*Anguilla anguilla*) larvae and water between control and treatment (treated with a concentration of 5 mg β-glucan /L of water from 4 to 9 DPH) groups at different ages. The significance level was set at <0.05 and rows containing significant comparisons were bolded.

Comparison		R^2 value	P value
4 DPH	Water: Control *vs*. Treated	0.069	0.061
	Larvae: Control vs. Treated	0.039	0.227
	Control: Water *vs*. Larvae	**0.234**	**0.001**
	Treated: Water *vs*. Larvae	**0.222**	**0.001**
9 DPH	**Water: Control *vs*. Treated**	**0.435**	**0.001**
	Larvae: Control *vs*. Treated	**0.096**	**0.002**
	Control: Water *vs*. Larvae	**0.299**	**0.001**
	Treated: Water *vs*. Larvae	**0.188**	**0.001**
20 DPH	Larvae: Control *vs*. Treated	0.042	0.368

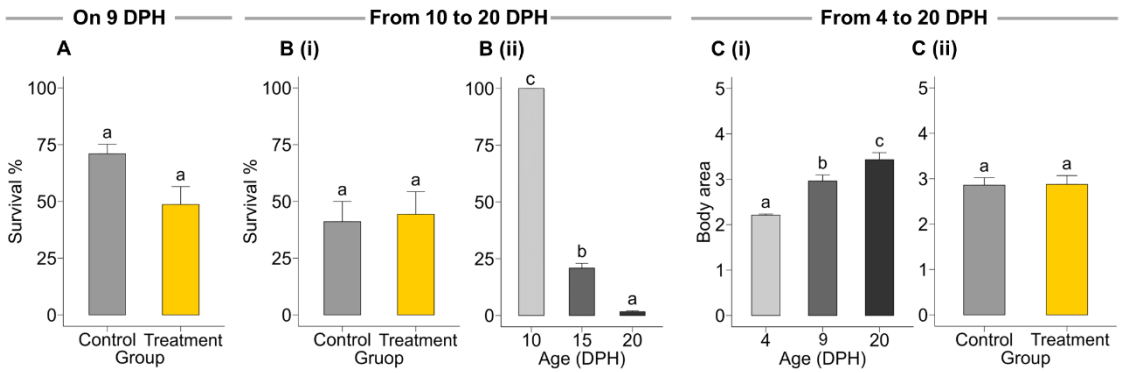

Figure 1. Survival of European eel (*Anguilla anguilla*) larvae at the end of the β-glucan exposure period on 9 DPH (A). Effect of treatment [B(i)] and age [B(ii)] on survival at the end of the experiment on 20 DPH. Effect of age [C(i)] and treatment [C(ii)] on body area (mm^2). Larvae were treated with a concentration of 5 mg β-glucan / L of water (Treatment) from 5 to 9 DPH and reared until 20 DPH compared to the control group. DPH stands for days post-hatch. Values represent means (± SEM) and different lower-case letters represent significant differences ($P < 0.05$).

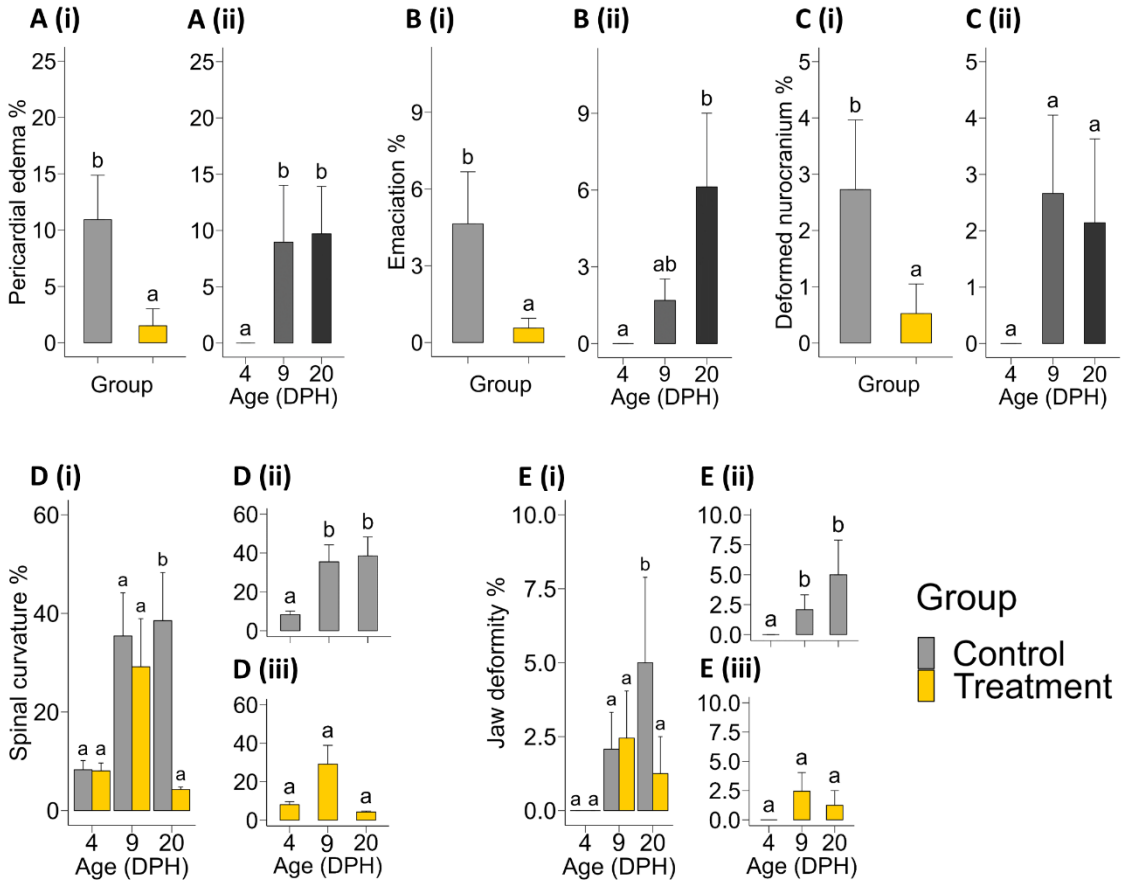

Figure 2. Effect of β-glucan treatment on the occurrence of different types of deformities in European eel (*Anguilla anguilla*) larvae. Effect of β-glucan treatment [A(i), B(i) and C(i)] or age [A(ii), B(ii) and C(ii)] on pericardial edema, emaciation, and deformed neurocranium, respectively. Effect of treatment at each age [D(i) and E(i)] and effect of age at each treatment [D(ii-iii) and E(ii-iii)] on spinal curvature and jaw deformity, respectively. Larvae were treated with a concentration of 5 mg β-glucan / L of water from 5 to 9 DPH and reared until 20 DPH compared to the control group. DPH stands for days post-hatch. Values represent means (± SEM) and different lower-case letters represent significant differences ($P < 0.05$).

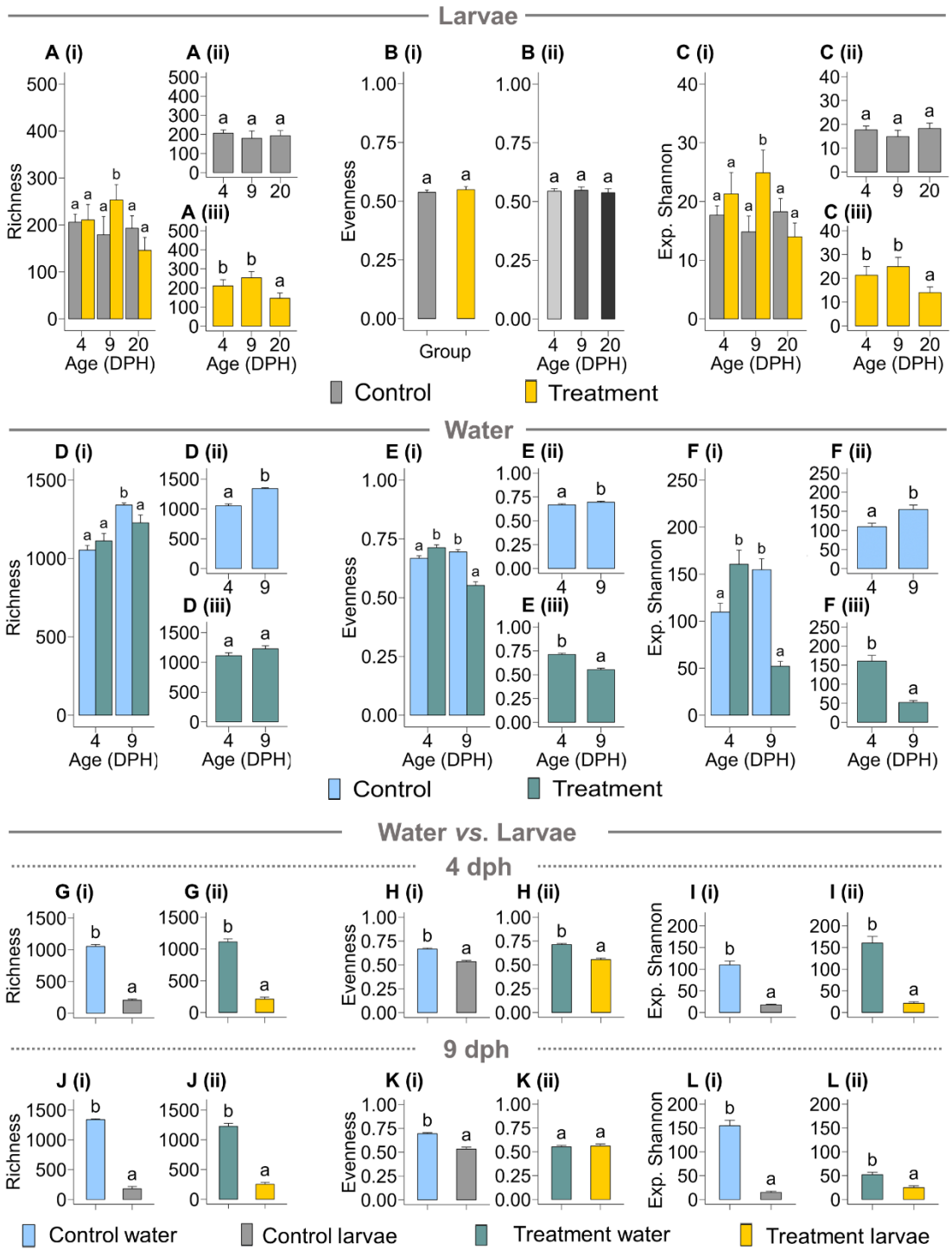

Figure 3. Alpha diversity measures, including ASV richness, evenness, and exponential Shannon index (Exp. Shannon) analysed for bacterial communities of European eel (*Anguilla anguilla*) larvae and rearing water of the control (clean) and treatment (treated with a concentration of 5 mg β-glucan / L of water from 5 to 9 DPH) groups before (4 DPH) and after (9 DPH) the treatment. DPH stands for days post-hatch. Values represent means (± SEM) and different lower-case letters represent significant differences ($P < 0.05$).

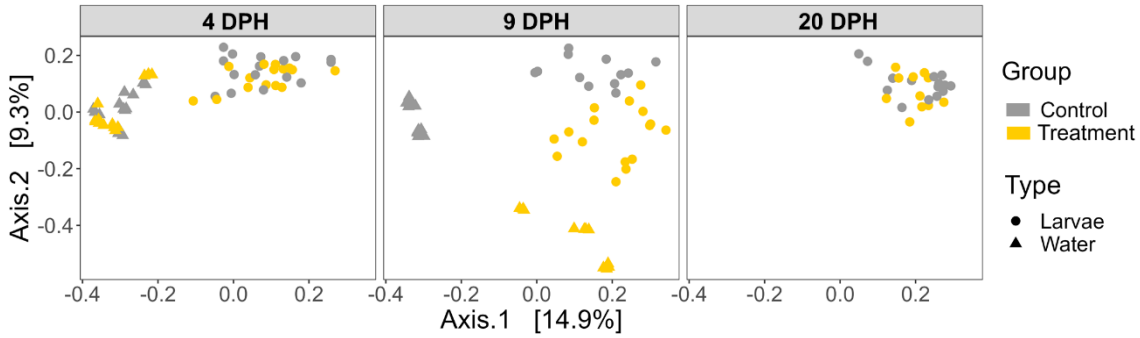

Figure 4. PCoA ordination plots based on Bray–Curtis dissimilarities for comparison of the bacterial communities of European eel (*Anguilla anguilla*) larvae and rearing water. The same ordination was plotted independently for each age (DPH = days post-hatch), where colours indicate the control and treatment (treated with a concentration of 5 mg β-glucan /L of water from 4 to 9 DPH) groups and shapes the type of sample (i.e., larvae or rearing water).

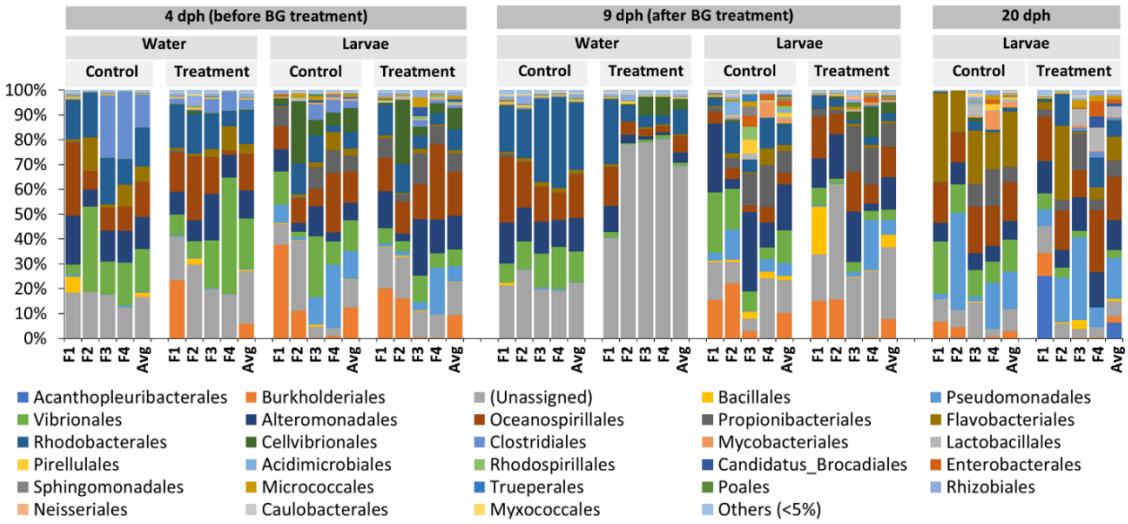

Figure 5. Relative abundances of the bacterial orders detected in European eel (*Anguilla anguilla*) larvae and rearing water of the control and treatment (treated with a concentration of 5 mg β-glucan /L of water from 4 to 9 dph) groups before (on 4 dph), and after the β-glucan treatment (on 9 dph) and at the end of the experiment (20 dph). The stacked bar for each family (F1 to F4) represents the mean relative abundances of bacterial orders detected in the replicated samples (n = 4) and each average (Avg) stacked bar represents the mean relative abundances of bacterial orders over the families (n = 4). "Unassigned" is ASVs that could not be classified reliably at the order level. (dph = days post-hatch, BG = β-glucan)

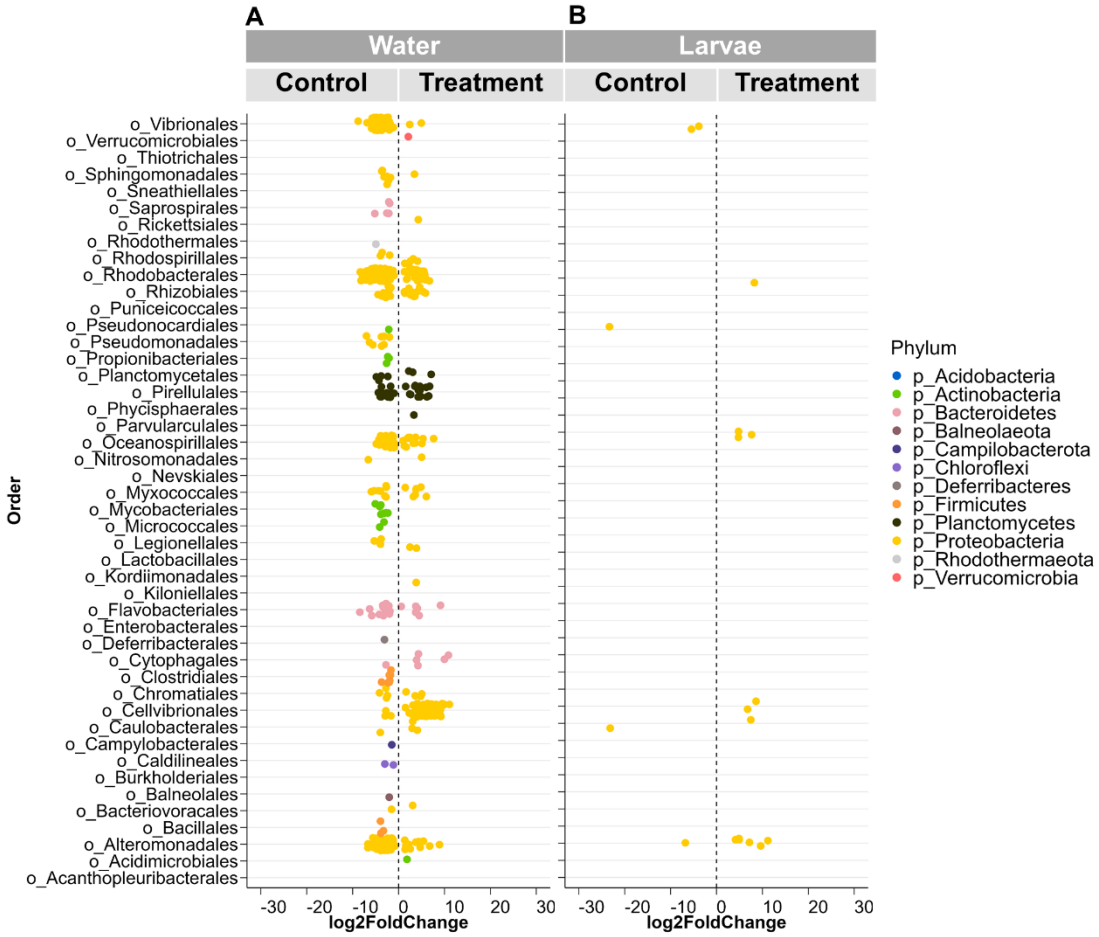

Figure 6. Results for DESeq2 analysis to determine differentially abundant taxa in bacterial communities of European eel (*Anguilla anguilla*) larval rearing water (A) and larvae (B) of control (ref.) and treatment (treated with a concentration of 5 mg β-glucan /L of water from 4 to 9 days post-hatching) groups at the end of the treatment period. Each dot represents an ASV and a log2-fold difference in the abundance compared to the reference (ref.) sample. In DESeq2 analysis, only ASVs that are statistically significant ($p < 0.05$) are reported.

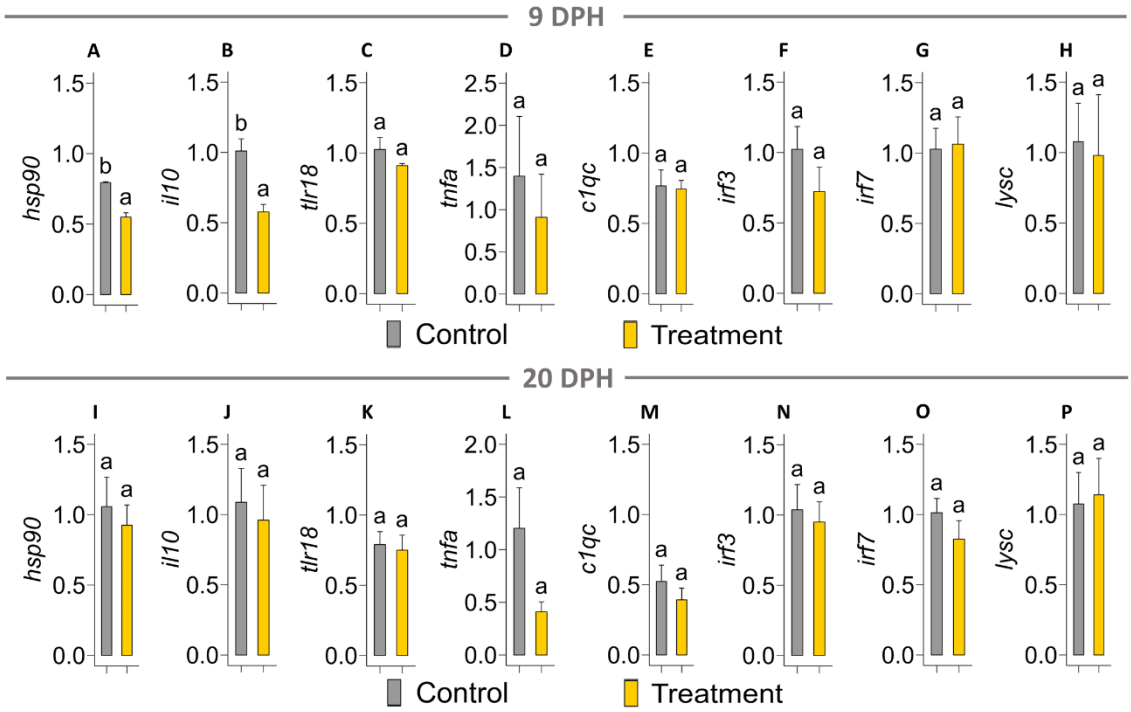

Figure 7. Relative expression of genes related to stress/ repair mechanism (*hsp90*) and immune response (*il10, tlr18, tnfa, c1qc, irf3, irf7 and lysc*) in European eel (*Anguilla anguilla*) larvae of control and treatment (treated with a concentration of 5 mg β-glucan / L of water from 4 to 9 days post-hatch/ DPH) at the end of the β-glucan treatment (on 9 DPH) and on 20 DPH relative to the expression levels in the control group at each age. DPH stands for days post-hatch. Values represent means (± SEM) and different lower-case letters represent significant differences ($P < 0.05$).

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Kasun Anuruddha Bandara

September 2023

Technical University of Denmark

National Institute of Aquatic Resources
Kemitorvet
DK-2800 Kgs. Lyngby

www.aqua.dtu.dk