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The effects of primary and secondary bacterial exposure on the seahorse (*Hippocampus erectus*) immune response

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ABSTRACT

Evolutionary adaptations in the Syngnathidae teleost family (seahorses, pipefish and seadragons) culminated in an array of spectacular morphologies, key immune gene losses, and the enigmatic male pregnancy. In seahorses, genome modifications associated with immunoglobulins, complement, and major histocompatibility complex (MHC II) pathway components raise questions concerning their immunological efficiency and the evolution of compensatory measures that may act in their place. In this investigation heat-killed bacteria (*Vibrio aestuarianus* and *Tenacibaculum maritimum*) were used in a two-phased experiment to assess the immune response dynamics of *Hippocampus erectus*. Gill transcriptomes from double and single-exposed individuals were analysed in order to determine the differentially expressed genes contributing to immune system responses towards immune priming. Double-exposed individuals exhibited a greater adaptive immune response when compared with single-exposed individuals, while single-exposed individuals, particularly with *V. aestuarianus* replicates, associated more with the innate branch of the immune system. *T. maritimum* double-exposed replicates exhibited the strongest immune reaction, likely due to their immunological naivety towards the bacterium, while there are also potential signs of innate trained immunity. MHC II upregulated expression was identified in selected *V. aestuarianus*-exposed seahorses, in the absence of other pathway constituents suggesting a possible alternative or non-classical MHC II immune function in seahorses. Gene Ontology (GO) enrichment analysis highlighted prominent angiogenesis activity following secondary exposure, which could be linked to an adaptive immune process in seahorses. This investigation highlights the prominent role of T-cell mediated adaptive immune responses in seahorses when exposed to sequential foreign bacteria exposures. If classical MHC II pathway function has been lost, innate trained immunity in syngnathids could be a potential compensatory mechanism.

1. Introduction

The vertebrate immune system consists of two interactive branches: the innate, characterised by rapid, non-specific responses, and the adaptive, which drives slower but highly specific pathogen recognition, assisted cellular activation, and immunological memory. (Bonilla and Oettgen, 2010; Medzhitov and Janeway Jr, 2000). These two facets are bridged and provided with further amplification potential by the complement system (Lo and Woodruff, 2020). Innate immune defences are conserved from invertebrates to vertebrates whilst the RAG1/RAG2 driven adaptive immune system, which evolved subsequently in gnathostomes, has become a hallmark of vertebrate evolution (Cooper and Alder, 2006; Flajnik and Kasahara, 2010; Fujita et al., 2004). Until recently, the concept of fundamental adaptive immune remodelling was inconceivable in gnathostomes due to the perceived ramifications that would drastically hinder immune function through the development of immunodeficiency and potential autoimmune conditions. However, this interpretation has softened following numerous discoveries of adaptive immune system restructuring and key gene losses in marine vertebrates (Buonocore and Gerdol, 2016; Dubin et al., 2019; Magadan et al., 2015; Roth et al., 2020; Star et al., 2011). The convergent loss of major histocompatibility complex II (MHC II) pathway components in codfishes, anglerfish, and the elephant shark have been identified, as well as in some syngnathids, which are in addition asplenic and miss crucial complement and immunoglobulin-related genes (Dubin et al., 2019; Liu et al., 2022; Roth et al., 2020; Star et al., 2011; Swann et al., 2020;

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Venkatesh et al., 2014). These instances not only confirm that immune systems have evolved with a high degree of flexibility including adaptive immune system remodelling, but are potentially commonplace among marine fishes, while also appearing to circumvent any debilitating immunological issues.

Previous work has suggested that the innate immune system could be of greater importance in teleosts compared with the slower-acting adaptive immune branch (Magnadóttir, 2010). In codfishes, the loss of MHC II, CD4, and the invariant chain (CD74) is theorized to be due to the MHC II pathway being surplus to requirements based on environmental and microbial demands, metabolic costs, and/or other historical disease-related events (Star et al., 2011; Malmstrøm et al., 2016). Reports have highlighted relatively slow adaptive immune response times in cold-blooded species compared with homeotherms (Abram et al., 2017; Scharsack and Franke, 2022). Slower adaptive immune activity could attributed in part to slower metabolic rates that exist among poikilothermic animals such as teleosts (Abram et al., 2017; Du Pasquier, 1982). Under hypothermic water conditions, innate immune system components have shown capabilities of enhancement following long-term exposure, compared with a more consistent suppression of the adaptive immune branch in similar conditions (Collazos et al., 1994; Le Morvan et al., 1997). Therefore, innate defences in fish consistently exposed to lower temperatures may compensate for other immunological deficits (Abram et al., 2017). So far, modifications to the RAG1/RAG2 adaptive immune system have been identified in the coelacanth, elephant shark, and a few teleost species, with little understanding of the driving forces behind these alterations (Buonocore et al., 2016; Dubin et al., 2019; Roth et al., 2020; Saha et al., 2014; Star et al., 2011; Venkatesh et al., 2014). A reliance on the innate immune system that led teleosts down an alternative evolutionary path to mammals, which rely heavily on the adaptive branch, may have rendered the adaptive immune system more evolutionarily flexible than the innate repertoire (Roth et al., 2020; Star et al., 2011). Innate immune derived memory coined as 'trained immunity' has been proposed in vertebrates and invertebrates (Kurtz, 2005; Netea et al., 2011), while pathogen-specific priming in invertebrates has also been observed (Roth et al., 2009; Sadd and Schmid-Hempel, 2006). These mechanisms, independent from the adaptive branch, highlight the complexities of immune system processes across the animal kingdom and should be considered when discussing immunological dependencies and the interpretation of functional importance. Immunological research on organisms with altered immune fundamentals should be encouraged as they can provide important insights into the evolutionary flexibility that can shape the immune system.

Syngnathid fishes (seahorses, pipefish, and seadragons) are the only group to have evolved the enigmatic male pregnancy and represent an intriguing study group when it comes to understanding the mechanisms of evolutionary innovation (Herald, 1959; Roth et al., 2020; Stölting and Wilson, 2007). Genomic modifications in some group members have led to a suggested functionally compromised MHC II (antigen presentation) pathway, as well as C4 (complement) and CD5 (B-cell function) gene absences (Haase et al., 2013; Liu et al., 2022; Luo et al., 2016; Roth et al., 2020). Gut-associated lymphoid tissue (GALT) is also deemed missing in seahorses and pipefish (Matsunaga and Rahman, 1998), while gill-associated lymphoid tissue (GIALT) is likely present based on studies pertaining to angiogenic and mucosal-related immune activities in the tissue (Luo et al., 2016; Parker and Roth, 2022). In turn, drastic evolutionary alterations and the genomic variability among syngnathids render them fascinating subjects for evolutionary immunology research. Since these discoveries, research has focused on exploring the potential evolutionary repercussions concerning the syngnathid immune capabilities, immune cell population dynamics, and the influence on male pregnancy evolution within the lineage (Haase et al., 2013; Keller and Roth, 2020; Parker et al., 2023a; Parker and Roth, 2022; Roth et al., 2020). Assessing the immunological vigilance of syngnathid fishes has often been limited to singular bacterial effectors or exposures (Beemelmanns and Roth, 2016b; Kang et al., 2022; Lin et al., 2016; Martins et al., 2010; Roth et al., 2012b). Understanding how alternative immune strategies with modified defence repertoires respond to sequential homologous and heterologous bacteria exposure, and how they evoke immune priming can provide an intriguingly different perspective on immune function. Moreover, disentangling how these unconventional model organisms approach and adapt to immune-deficiencies, could benefit the progression of contemporary vaccinations and autoimmune treatments.

This investigation aimed to assess the bacterial immune responses and priming capacities of the seahorse *Hippocampus erectus* upon exposure to heat-killed bacteria isolates. Unlike some of its pipefish relatives, the functionality and immunological relevance of the seahorse MHC II pathway is still largely undetermined. Potentially, the modification of CD74 in seahorses led to the dysfunctionality of the MHC II pathway (Roth et al., 2020). If the classical function of the MHC II pathway has not been conserved, it raises questions regarding alternative immune pathways accommodating this deficiency, as well as its capabilities in immune memory formation. Primary and secondary bacterial exposures to either heat-killed *Vibrio aestuarianus* or *Tenacibaculum maritimum* were conducted and individual immune response was assessed in the context of whether fish had been exposed previously or not, and to which bacteria combination. Adaptive and innate immune-related gene expression was assessed to elucidate the influence of bacterial specificity and exposure number. The first hypothesis of this study was that **(i)** treatment replicates cluster based on their overall gene expression profiles, with the largest disparities found between exposed and non-exposed treatments. In light of the fact that initial exposure to a bacterium elicits an innate immune response, it was hypothesized that **(ii)** fish not subjected to a primary exposure exhibit a greater innate immune response than fish with a homologous and heterologous double exposure. Secondary exposure to a bacterium was expected to be associated with an accelerated and stronger adaptive immune response owing to the establishment of immunological memory. In turn, the third hypothesis of this study was that **(iii)** homologous and heterologous double-exposed replicates, compared with single-exposed individuals, elicit a stronger adaptive immune response and that this is enhanced in homologous compared with heterologous treatments. Due to the deduced loss of MHC II functionality in *H. erectus*, it was hypothesized that **(iv)** the MHC I pathway is upregulated in exposed individuals as a compensatory consequence. Lastly, from what is known about syngnathid-bacterial co-evolutionary relationships, it was hypothesized that **(v)** *T. maritimum* evokes a stronger immune response overall than *V. aestuarianus* due to the immunological naivety of the host.

2. Material & methods

2.1. Ethics statement

This study was conducted according to German animal welfare law and the ethical approval afforded by the Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Ditgitalisierung (MELUND) Schleswig-Holstein (Permit no: V24257982/2018). No endangered fish species caught from the wild were used in this study.

2.2. Fish

Captive-bred *Hippocampus erectus* males were initially obtained from qualified aquarium breeders (Seepferdchen24, Meeresaquaristik GmbH, Ottersberg), then bred for several generations and maintained at 25 °C in the GEOMAR aquaria facilities. Fish were kept in tanks containing Baltic Sea water and supplemented with additional marine salt to increase salinity to 30% (Qin et al., 2020). Seahorses were fed live mysids from aquarium stocks and frozen mysids (Fischfutter Erdmann, Germany) twice a day.

2.3. Bacterial strains and culture preparation

Vibrio spp. are a diverse group of opportunistic Gram-negative bacteria omnipresent in the Baltic Sea, as well as one of the most prevalent bacteria in the natural biome and seahorse gut (Colwell and Grimes, 1984; Pappert et al., 2023; Thompson et al., 2004). While often harmless, under certain conditions *Vibrio* strains can become pathogenic and instigate disease development in seahorses (Alcaide et al., 2001; Qin et al., 2017; Xie et al., 2020) and pipefishes (Goehlich et al., 2021; Haase et al., 2013; Roth et al., 2012a), especially within the fish trade (Kang et al., 2022). The strain used in this investigation was *V. aestuarianus*, a known occupant of Baltic Sea brackish waters (Eiler et al., 2006).

T. maritimum is a marine bacterium implicated in skin and mucosal-related infections in fish such as *Pagrus major* and *Acanthopagrus schlegeli* of the Pacific Ocean (Avendaño-Herrera et al., 2006; Hikida et al., 1979; Masumura and Wakabayashi, 1977). *T. maritimum* has been used previously in syngnathid immune challenge experiments (Beemelmanns et al., 2019; Beemelmanns and Roth, 2017) despite the strain, to the best of our knowledge, not having come into contact previously with fish of the Baltic Sea region or those within our aquaria system.

Cryocultures of *V. aestuarianus* and *T. maritimum* originally extracted from healthy broad-nosed pipefish (*S. typhle*) and pacific seabream, respectively (Beemelmanns and Roth, 2017; Roth et al., 2012a; Suzuki et al., 2001), were thawed and allowed to grow in Medium101 (0.5% (w/v) peptone, 0.3% (w/v) meat extract, 1.5% (w/v) NaCl in MilliQ water). Following exponential growth, bacteria were harvested in marine broth by centrifugation (5 min, 5000 rpm) and washed twice using sterile PBS buffer. Estimates for cell counts were determined using two dilution series (*V. aestuarianus*: 3×10^8 cfu mL^{-1}; *T. maritimum*: 2.4×10^7 cfu mL^{-1}). Cultures were washed with 30 mL of sterile PBS buffer and heat-killed (*V. aestuarianus*: 75 min at 65 °C; *T. maritimum*: 75 min at 60 °C). To confirm the heat-killing process, products were plated on Medium101 agar and assessed. Resultant heat-killed cultures were stored at −20 °C in preparation for the experimentation.

2.4. Experimental design and sampling

Seven treatments were used in this investigation consisting of primary and secondary exposure, with either heat-killed *V. aestuarianus* and/or *T. maritimum* being used, while naïve phases represented those without bacterial introduction. Exposure programmes were as follows: naïve/naïve (control) **(*NN*)**, *Vibrio*/*Vibrio* **(*VV*)**, *Tenacibaculum*/*Tenacibaculum* **(*TT*)**, *Vibrio*/*Tenacibaculum* **(*VT*)**, *Tenacibaculum*/*Vibrio* **(*TV*)**, naïve/*Vibrio* **(*NV*)** and naïve/*Tenacibaculum* **(*NT*)** (Fig. 1). The sequential exposures were separated by a 5-week period.

Eight seahorse males were used for each treatment and each fish was weighed and measured (mean: 22.5 g; 15.7 cm) in order to determine the appropriate pathogen load to use. Fish of the same treatment were kept in 20 L tanks for the duration of the experiment. 20 μL per gram body weight of bacteria was the ratio used to establish the appropriate dosage size, resulting in a final injection volume of ~150 μL per seahorse. This dosage ratio is in line with a previous immunological study conducted by Peuss et al. (2020). Fish were monitored for fatalities and behavioural changes during the five weeks leading up to the second exposure phase. Four days after the second treatment phase, all fish were euthanized with an excess of MS-222 (500 mg/L, Sigma-Aldrich), prior to the gills being sampled, preserved in RNAlater, and initially stored at 4 °C. Samples were relocated to −20 °C after 2 weeks.

2.5. RNA extraction and sequencing

Samples were thawed and homogenized prior to RNA extraction using the RNeasy 96 Kit (Qiagen, Hilden Germany). To ensure that the extracted RNA was of sufficient quality prior to library preparation, a NanoDrop-1000 spectrophotometer (NanoDrop) and Fragment Analyzer (Agilent Technologies) were utilised. Library preparation was carried out at the Beijing Genomics Institute (BGI), as well as paired-end RNA sequencing (RNA-seq) via the DNBseq platform (2 × 150 bp reads).

2.6. Orthologous gene identification

Orthofinder (v2.4.0) (Emms and Kelly, 2015, 2019) was utilised to identify orthologous genes from 11 fish species acting as references (supplementary; Table S1). DIAMOND (v0.9.21) (Buchfink et al., 2015) and MAFFT (v.7.475) (Katoh and Standley, 2013) were used for multiple sequence alignment prior to adopting FastTree for maximum-likelihood trees.

2.7. Read processing and principal component analyses

Raw reads were adapter trimmed and quality pruned using Fastp (v0.20.1) (Chen et al., 2018) before being aligned and mapped to the genome using STAR (v2.7.9a) (Dobin et al., 2013). Resultant counts were obtained and consolidated using the TPMCalculator (v0.0.3) (Vera Alvarez et al., 2019).

Principal component analysis (PCA) was carried out on all replicates to determine whether replicate group clustering could be observed. Scaled TPM (Transcripts per Million) values were used and filtered for genes that showed expression in at least three replicates. Following the all-replicate comparisons, replicates relating to *V. aestuarianus* (*NN*, *NV*, *VV* & *TV*) and *T. maritimum* exposure (*NN*, *NT*, *TT* & *VT*) were divided for separate PCAs. Multivariate analysis of variance (MANOVA) followed

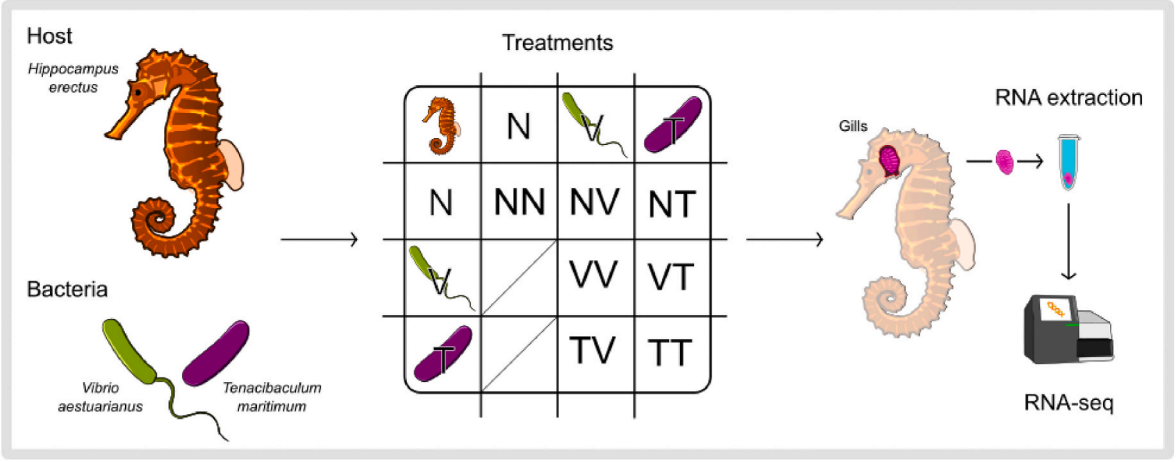

Fig. 1. Schematic diagram depicting the treatment assignments and workflow used in this investigation.

with treatment as a predictor variable (PC1-PC5), to assess which principal components associate with which treatment type for analysis. The PC1-PC5 range was used because it represents the largest combined degree of variance and, therefore, should be considered when attempting to discern differences between sample groups, driven by this variation. In order to determine where differences lie between treatment groups, analysis of variance (ANOVA) was carried out on the scores of the most influential PCs (PC1-5), followed by post-hoc Tukey-tests to identify specific treatment differences. PCs with the greatest significant treatment differences (MANOVA; $P < 0.05$) were used for the visual PCA representations.

Influential genes characterising each respective PC were investigated and loadings were displayed to help interpret the drivers. The most influential genes with the greatest loading values (top 10%) were investigated in depth for proposed roles in immune function and physiology. Immune genes were then displayed on each respective PCA representation, to provide an overarching view of the immune system's influence in shaping the dispersal of the treatment clusters. The percentage of genes (10%) was in line with a similar previous study (Parker et al., 2023a) and chosen to provide a clearer view of the most important genes charged with contributing to treatment differences.

2.8. Pairwise differential gene expression analyses

DESeq2 (v.1.36.0) (Love et al., 2014) was adopted for differential gene expression (DGE) analyses in R (v4.2) (R Development Core Team, 2013). Pairwise comparisons were carried out between selected replicate groups. Single-exposure replicates were compared with the naïve (***NN/NT*** & ***NN/NV***), homologous double-exposed (***NT/TT*** & ***NV/VV***), and heterologous double-exposed (***NT/VT*** & ***NV/TV***) replicates. Homologous double-exposed groups were also compared with heterologous replicate groups (***TT/VT*** & ***VV/TV***), while also being compared with naïve (***NN/TT, NN/VV, NN/VT*** & ***NN/TV***). The Benjamini and Hochberg method was used for multiple testing *P*-value correction (Benjamini and Hochberg, 1995). Genes with an absolute log-2 fold change (log2FC) expression >0.5 or < -0.5, and an adjusted *P*-value <0.05 were used.

2.9. Functional group enrichment analyses

Separate gene ontology (GO) functional group analyses were conducted on differentially expressed and PCA gene data sets. For the PCA functional enrichment analysis, the top 10% most influential genes were used. Each gene possesses a positive or negative loading value, which relates to its influence on driving differences between treatments for each PC. Therefore, those genes with the most positive/negative values were assessed for each respective PC. For the DGE data sets, genes with the same expression direction (i.e., upregulated or downregulated; log2FC) for each pairwise comparison were analysed concurrently. The bioinformatics resource tool, DAVID (v2021) (Huang et al., 2009; Sherman and Lempicki, 2009), was used to extract GO_FAT biological process functional groups that combine gene pathways into broader functional categories. For background gene references the zebrafish (*Danio rerio*) library was utilised, while the high DAVID stringency setting was adopted to support group establishment. Each resultant group was given an FDR-corrected value and a functional enrichment score. Gene ontology bubble plots were created for data sets with strongly enriched findings, incorporating gene log2FC, average expression, and adjusted *P*-values to provide a weighted visual assessment.

3. Results

3.1. Read and ortholog abundances

A total of ~145–233 M paired-end reads were attained per treatment group with samples ranging from 25.6 to 29.6 M reads and averaging at 29 M, while 16,938 ortholog gene matches (*D. rerio*) were identified.

3.2. Principal component analyses

Replicates were conducive to PCA and demonstrated treatment-specific clustering, and an overall difference between all groups was confirmed following MANOVA (PC1:PC5) (supplementary; Table S2). Moreover, individual treatment differences were identified using ANOVA and post-hoc Tukey testing (supplementary; Tables S3–4). The majority of significant treatment differences were found within PC1, which accounted for 41% of the variation within the dataset. In general, PC1 distinguished *NT*, *TV*, *VV,* and *NN* (positive leaning) from *NV*, *VT,* and *TT* (negative leaning) from each other (Fig. 2). The most significant differences were found between the following treatment pair comparisons: *NV/NT*, *TT/NT*, *VT/NT* and *TV/NV*. In fact, *NT* replicates were found to be significantly different from every other treatment type. Intriguingly, the two treatment groups that exhibited the greatest significant difference when compared with *NN* were both groups that only underwent a single exposure, *NT* and *NV*. *TT* replicates also matched this trend; however, *TV*, *VT,* and *VV* were all shown to have statistically similar expression dynamics to *NN* when looking at PC1. ANOVA also highlighted significant overall differences in gene expression between the two homologous (*VV* and *TT*) and between the heterologous vaccinated treatments (*TV* and *VT*).

Separated group PCA plots gave a clearer clustering outlook for each bacteria treatment individually in *H. erectus* and provided bacteria-specific platforms to assess trend differences between treatments. MANOVA confirmed that within PC1-5 significant differences exist between *V. aestuarianus* treatment groups (supplementary; Table S5). PC1 (53%) for all replicates that received *V. aestuarianus* as their final exposure, showed separation of *NV* from all the other replicates including the control (*NN*) (Fig. 3). Furthermore, each difference was confirmed to be highly significant using ANOVA and Tukey testing (supplementary; Tables S7–8). PC2 (12%), on the other hand, showed a statistically significant separation of *NV*, *TV*, and *VV* replicates from *NN*. This trend was supported further when looking at PC2 and PC3 (8%) in combination. Incidentally, PC3 also showed a significant difference between *VV* and *NV*, along with an even stronger distinction between *VV* and *TV*. Significant differences for all *V. aestuarianus* treatment comparisons could therefore be explained by PC1-PC3.

As with the *V. aestuarianus* treatments, MANOVA highlighted the presence of an overall expression profile difference between *T. maritimum* treatment groups (supplementary; Table S5). The *T. maritimum*-related treatments also yielded similar clustering patterns, with PC1 (52%) accounting for differences between *NT* and the two double exposure treatments (*VT* and *TT*); the *NN* clustering distinction from *NT* was less pronounced than it was from *NV* (Fig. 4; supplementary; Tables S6 and S8). In addition, *NN-TT* and *NN-NT* comparisons also yielded strong significant differences along PC1, along with the *NN* and *VT* clusters, but to a lesser degree. Differences between treatment clusters were also observed in PC2 (8%), with *VT* expression significantly contrasting both *NN* and *TT*, while PC3 (6%) only explained differences between *NN* and the single and double homologous *T. maritimum* treatments (*NT* and *TT*). Overall, when considering just PC1 and PC2 significant differences were found between all *T. maritimum* treatment comparisons.

3.2.1. PCA influential genes and functional enrichment

A total of 55 genes were shown to be significant in inferring differences between *V. aestuarianus*-related replicates (Fig. 5; supplementary; Table S9). As the number of total influential genes was limited, all genes were represented in the *V. aestuarianus* PCA representations; however, functional enrichment analysis was not carried out for these treatments due to insufficient gene numbers. A large proportion of these genes are positively associated with the *NV* treatment (PC1). Based on the PCA of PC1 and PC2, three genes are associated with the double exposure replicates (*VV* and *TV*), namely *capza1a* (actin organization activity),

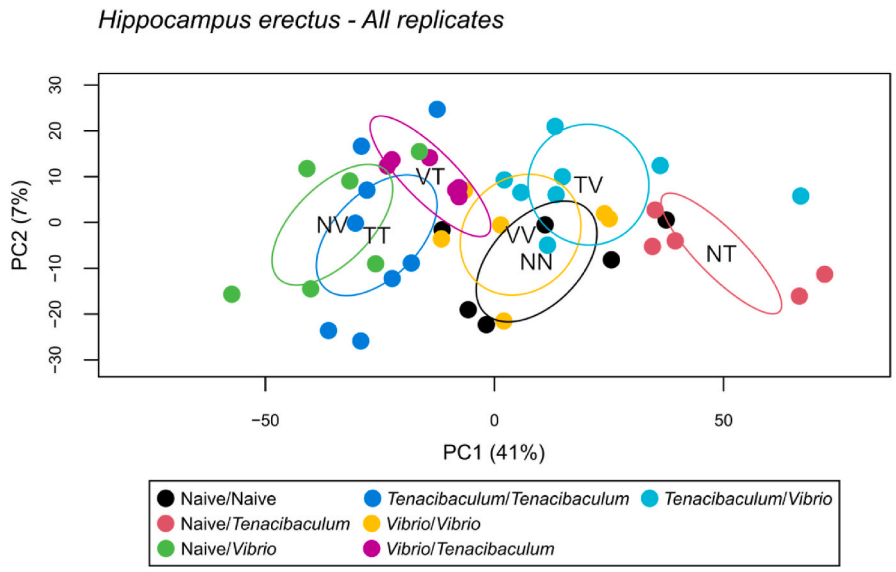

Fig. 2. Principal component analysis of normalized TPM values for all *Hippocampus erectus* treatment replicates (naïve/naïve (*NN*), naïve/*Vibrio* (*NV*), *Tenacibaculum/Vibrio* (*TV*) and *Vibrio/Vibrio* (*VV*), naïve/*Tenacibaculum* (*NT*), *Vibrio/Tenacibaculum* (*VT*) and *Tenacibaculum/Tenacibaculum* (*TT*). Ellipses represent 70% confidence.

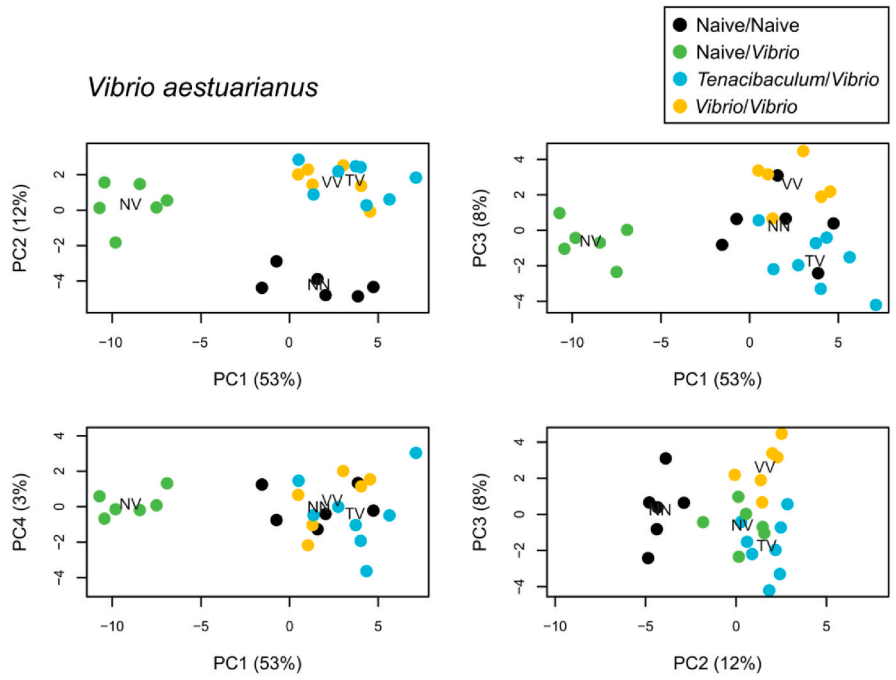

Fig. 3. Principal component analysis for naïve/naïve (*NN*), naïve/*Vibrio* (*NV*), *Tenacibaculum/Vibrio* (*TV*) and *Vibrio/Vibrio* (*VV*) *Hippocampus erectus* treatment replicates. Letter labels indicate respective treatment means.

c1galt1c1 (transmembrane chaperone) and *mgst3a* (glutathione peroxidase activity). The same PCA highlighted six genes with immunological function; *bach2b* (lymphocyte maturation) and *grem2b* (inflammation) associated with *NN*, while *huwe1* (inflammation), *hivep3b* (immune response), *sema7a* (immune modulation/inflammation) and *wdr36* (T-cell activation) associated with *NV*.

For the *T. maritimum* PCA assessments, a total of 1386 genes were shown to be influential in driving differences between treatments. Considering the top 10% of genes for each PC (139 genes), 85 across all PCs were found to have an immune-related function (Fig. 6, supplementary; Table S10). Unlike in the *V. aestuarianus* assessment, influential immune genes associated with differences along the PC1 axis in *T. maritimum* were almost exclusively linked to the homologous and heterologous treatments (*TT* and *VT*) and not the single-exposed replicates (*NT*). In the PC1-2 combination, no immune genes were strongly associated with *NT* replicates. A notable number of genes involved in macrophage (*ncor2*, *letm1* & *tjp1a*), autophagy (*usp19*), neutrophil and NK-cell function (*nbeal2* & *rfx7b*) were strongly associated with *VT* and *TT* replicates, along with several other innate immunity genes (*sorl1*, *itga1*, *atf7a* & *huwe1*).

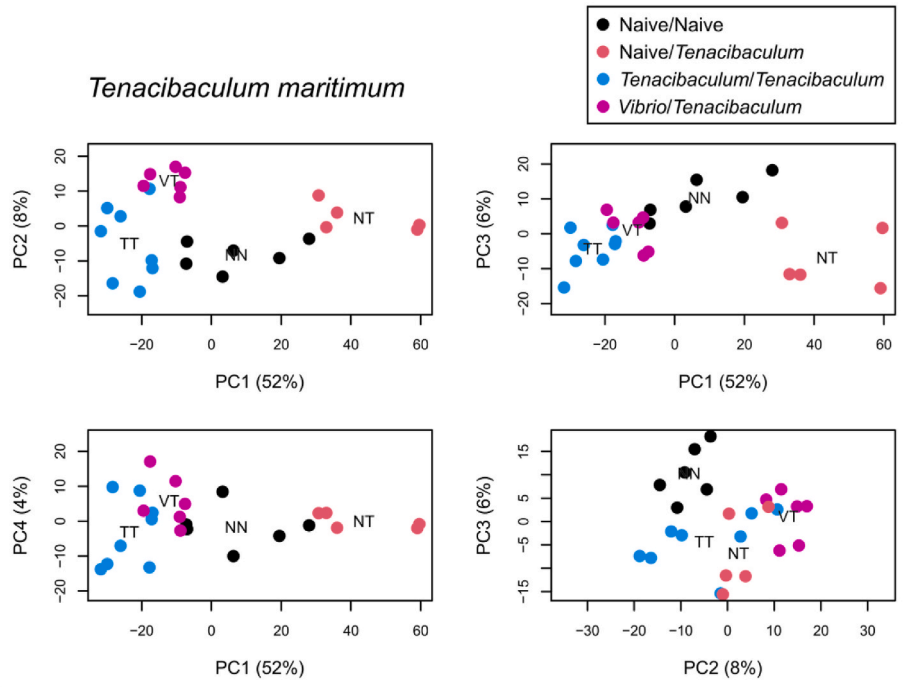

Fig. 4. Principal component analysis for naïve/naïve (*NN*), naïve/*Tenacibaculum* (*NT*), *Vibrio*/*Tenacibaculum* (*VT*) and *Tenacibaculum*/*Tenacibaculum* (*TT*) *Hippocampus erectus* treatment replicates. Letter labels indicate respective treatment means.

GO functional enrichment analysis was carried out on the influential genes for each principal component, separating genes based on their PC loading direction. Due to the limited number of influential genes driving the *V. aestuarianus* treatment differences, no functional enrichment was identified following DAVID GO analysis. For the *T. maritimum* replicate analysis, the vast majority of genes influencing PC1 had a negative loading value and are therefore heavily associated with *TT* and *VT* replicates rather than *NN* and *NT*. Chromatin modification, RNA biosynthesis, catabolism, haematopoiesis, and immune system development were all functionally enriched pathways associated with influential genes characterising *TT* and *VT* (supplementary; Table S11). Other principal components failed to yield any additional significant functional pathway information (supplementary; Tables S12–17).

3.3. Differential gene expression analysis and functional enrichment

Pairwise comparisons between treatments yielded 726 and 1467 differentially expressed transcripts found in *V. aestuarianus* and *T. maritimum* comparisons, respectively (supplementary; Table S18). Among the *V. aestuarianus* pairwise assessments, *NV/TV* yielded the largest number of differentially expressed genes. Interestingly, 25 differentially expressed genes are shared between *NN/VV* and *NN/TV*, while *NN/NV* only shares two in total (supplementary; Fig. S1). In the equivalent *T. maritimum* comparison, over half of *NN/VT* differentially expressed genes are shared with *NN/NT*. However, *NN/TT* only accrued a small number of differentially expressed genes overall so little overlap was observed. Differences between single-exposed and double-exposed replicates in *T. maritimum* comparisons were the greatest, producing the largest number of differentially expressed genes. *VV/TV* only yielded three significant differentially expressed genes.

3.3.1. Vibrio aestuarianus treatments

***NN/NV*:** The *NN/NV* pairwise comparison produced few immune-related differentially expressed genes, aside from the upregulation of *thbs1b* (inflammation), *plgrkt* (inflammation regulation), and the fish disease implicated *abi1b* in *NV* (Zhou et al., 2017).

***NN/VV*:** All but one gene (*adcy2a*) was shown to be downregulated in *VV* when compared with *NN*. Downregulated genes in *VV* involved in immune system function include *sh2d1aa* (lymphocyte activation), *ptges* (inflammation), *itgbl1* and *gapdh* (immune regulation), as well as the angiogenic factor *angpt1*.

***NN/TV*:** The adaptive immune gene *lpar1* was downregulated in *TV* compared with *NN*, while most genes with a positive immune function such as *havcr1* (T- and B-cell activity), *spp1* (T-cell activation) *usp13* and *si:dkey-26c10.5* (innate immunity) were also shown to be downregulated in *TV* compared with *NN*. However, a number of immune suppressive/modulatory (*itgbl1*, *gapdh*, *prdm16* & *sfrp5*) genes were also strongly downregulated in *TV* replicates. Two BMP signalling (*sfrp5* & *grem2b*) and several angiogenic genes (*jcada*, *nme2b.1*, *tspan12*, *ptprb* & *angpt1*) were downregulated in *TV* in comparison with *NN*.

***NV/TV*:** Pairwise comparisons between *NV/TV* yielded a number of differentially expressed genes, with two of the most notable coding for CD40 and MHC II (*mhc2dca*) (antigen presentation), both of which showed upregulated expression in *TV* compared with *NV*. Conversely, three genes with putative roles in T-cell response inhibition (*ildr2*, *pcdh18a* & *lrrc8c*) were downregulated in the same comparison. Abundant genes coding for immune-related integrin pathway components (*yrk*, *fn1b*, *itga4*, *loxl3a*, *itga3a*, *itga1*, *sema7a* & *itgbl1*) were also identified in *NV/TV* comparison, all of which exhibited downregulated expression in *TV*. Interestingly, innate immune genes (*blk*, *colec12*, *inavaa*, *tnc*, *ptk2ab*, *sema3d*, *yrk*, *gapdh*, *thbs4b* & *yes1*) and most genes involved in inflammation were found in this comparison to be downregulated in *TV* compared with *NV*. In line with the *NN/TV* comparison, all BMP signalling genes (*tgfb1b*, *tbx2b*, *sost* & *grem2b*) were shown to have downregulated expression in *TV* compared with *NV*. Moreover, the same downregulated expression trend concerning angiogenesis genes (*serpinf1*, *cdh5*, *ppp1r16b*, *reck*, *ptk2ab*, *fli1a*, *ackr3b*, *amot*, *tnn*, *jcada*, *adamts3*, *mmp2*, *kdrl*, *robo4*, *pappa2*, *clec14a*, *calcrlb*, *ptprb* & *sox18*) and integrin signalling genes (*itga1*, *yrk*, *itga3a*, *itga4*, *itga5*, *itga9*, *sema7a* & *itgbl1*) was also evident in *TV* replicates compared with *NV*.

***NV/VV*:** As with the *NV/TV* comparison, genes coding for MHC II (*mhc2dca*) were upregulated in *VV* compared with *NV*, while *mpx* (peroxidase) was the only other notable upregulated immune gene. An intriguing number of genes with suppressive roles were identified to be

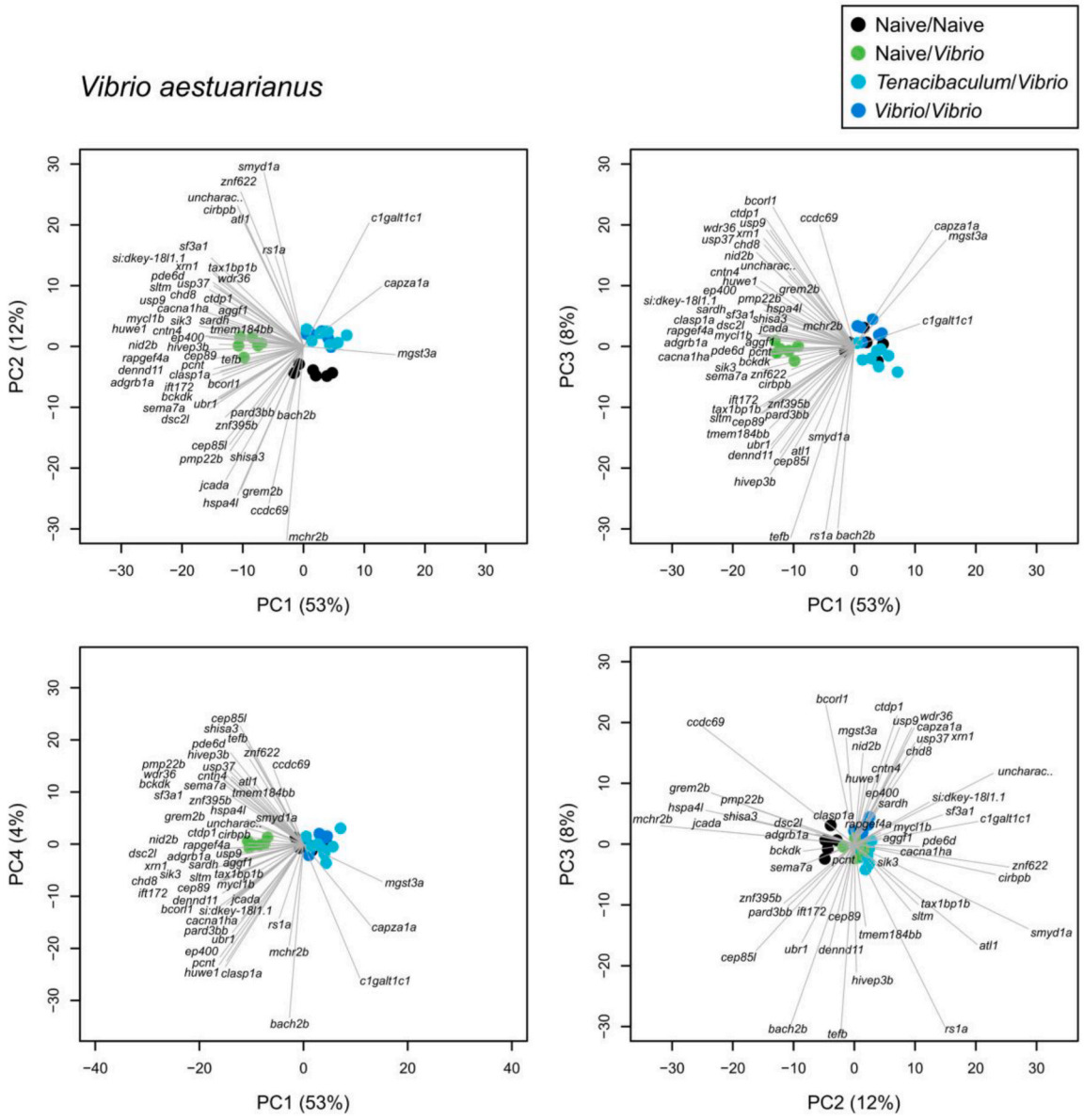

Fig. 5. Principal component analysis plots for naïve/naïve (*NN*), naïve/*Vibrio* (*NV*), *Tenacibaculum*/*Vibrio* (*TV*) and *Vibrio*/*Vibrio* (*VV*) *Hippocampus erectus* treatment replicates. Based on most influential genes (55 genes).

downregulated in *VV* compared with *NV* including, *rgs13* (B-cell inhibition) *hivep1*, *fgfr4* (negative NF-κB regulation), and *tgfb1b* (immune suppression). Additional immune genes that were downregulated in *VV* included *cxcl12a* (neutrophil activity), *aplp2* (MHC I interaction), *thbs1b,* and *mrvi1* (inflammation). Once again, BMP signalling related genes (*tgfb1b*, *bmp16* & *sost*) exhibited downregulated expression in *VV* compared with *NV*, in line with the previously mentioned homologous and heterologous *V. aestuarianus* comparisons. *Fermt2* was the only gene with putative angiogenic function to be differentially expressed in the *NV/VV* comparison, matching the downregulated trend in *VV* found in previous comparisons in this study.

VV/TV: Only three genes were differentially expressed in the VV/TV comparison, all three were upregulated in TV when compared with VV. These include *ptdss1* (biosynthesis), *klf9* (RNA synthesis), *and cbln2b* (synapse function).

3.3.2. *Tenacibaculum maritimum* treatments

***NN/NT*:** Differentially expressed immune genes in *NN/NT* were spread when it came to functionality, with a number of inflammatory genes showing upregulated (*zgc:195173*, *CU571081.1* & *si:ch73-160i9.3*) and downregulated expression (*agtr1a*, *cxcl19* & *panx3*) in *NT* compared with *NN*. Similarly, T- and B-cell activation genes also appeared to split with some exhibiting positive expression differences (*mcl1a*, *si:dkey-70p6.1* & *prkcab*), while others were downregulated (*lpar1* & *plcl2*) in *NT* compared with *NN*. Only two genes with BMP signalling activity were identified in *NN/NT*, with both *tgfb2* and *fstb* being downregulated in *NT*.

***NN/TT*:** Only six genes were differentially expressed in the *NN/TT* comparison. The most notable downregulated gene in *TT* compared with *NN* was *chia3* (immune modulation and tolerance) which codes for a chitinase.

***NN/VT*:** A number of immune regulatory genes were shown to be downregulated in *VT* compared with *NN*, namely, *hsp11* (immune cell infiltration), *sema3e* (macrophage regulation), *itgbl1* (NK-cell inhibition) and *prdm16* (hematopoietic cell regulation). Incidentally, *itgbl1* and *prdm16*, along with *gapdh* (inflammation), *usp13,* and *si:dkey-26c10.5*

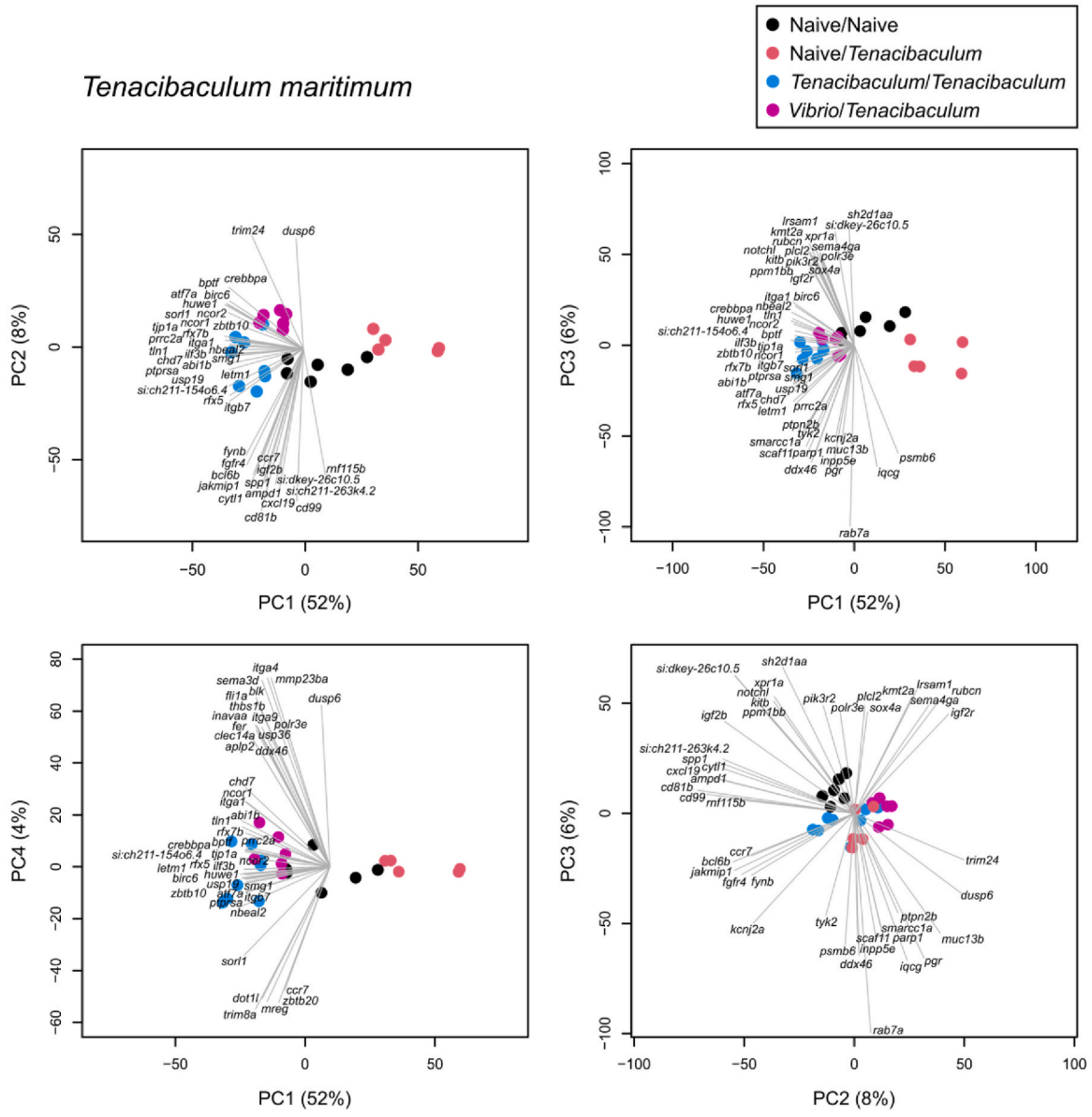

Fig. 6. Principal component analysis plots for naïve/naïve (*NN*), naïve/*Tenacibaculum* (NT), *Vibrio*/*Tenacibaculum* (*VT*) and *Tenacibaculum*/*Tenacibaculum* (*TT*) *Hippocampus erectus* treatment replicates. Based on the top 10% most influential genes (139 genes). Genes displayed with loadings have exhibited an immune function.

(innate immunity) expression in *VT* matches the same downregulated expression in *TV* when compared with *NN*. No BMP signalling genes were differentially expressed, however, all genes involved in angiogenesis or its regulation (*sema3e*, *tspan12*, *angpt1* & *nme2b.1*) were strongly downregulated in *VT* compared with *NN*.

***NT*/*TT*:** MHC II was not differentially expressed in the *T. maritimum* treatments, however, the MHC II regulator (*rfx1a*) and MHC I (*mhc1uka*) were found to be highly upregulated in *TT* compared with *NT*. Incidentally, a number of genes linked to T-cell activities (*lpar1*, *si:ch211-232i5.3*, *sema5a*, *spp1*, *lrrc8c*, *pdgfra*, *stap2a*, *nfatc4*, *cd40*lg & *jakmip1*) and antigen binding (*cd81b*) were also upregulated in *TT* compared with *NT*, while *cd59* which is purported to interfere with antigen binding was shown to be conversely downregulated (Michielsen et al., 2018). In addition, B-cell-related genes such as *plcl2* (B-cell activation), *cd40*lg (B-cell maturation), and *lpar1* (B-cell proliferation) were also upregulated in *TT* when compared with the non-vaccinated *NT* replicates. In total, 28 genes with roles in inflammation were identified with the vast majority exhibiting upregulated expression in *TT* compared with *NT*, these included three different metallopeptidases (*adamts7*, *adamts15a* & *adamts1*). Similarly, 8 upregulated genes involved in integrin signalling pathways, including *itga3a*, *itga5,* and *itgb1a*, were also all upregulated in *TT*. Interestingly, eight upregulated immune genes found in *NT*/*TT* were shown to be all downregulated in the *NV*/*VV* comparison, including *fgfr4* (immune modulation), *cxcl12a* (neutrophil activity), *thbs1b* (inflammation), *bcl6b* (inflammation regulation), *pdgfra* (growth factor), *mrvi1* (inflammation), *tgfb1b* (immune suppression), *aplp2* (MHC I interaction). As with the *NN*/*TT* comparison, the chitinase gene, *chia3*, was also strongly downregulated in *TT* compared with *NT*. A mix of BMP regulator (*fstb*) and negative regulator genes (*ccn6*, *sost*, *fstb*, *sostdc1a*, *zgc:113531* & *fstl1a*) were shown to be upregulated in *TT* compared with *NT*. Unlike the comparisons with *NN* replicates, angiogenesis-related genes were universally upregulated in *TT* compared with *NT*, with *angpt1, angptl1b,* and *robo4* exhibiting the highest expression.

***NT*/*VT*:** The *NT*/*VT* pairwise comparison shared a number of

similarly expressed immune genes with the *NT/TT* comparison, these included the upregulation of *rfx1a* (MHC II regulation), *pcl2* (B-cell activation), *lrrc8c* (adaptive immunity) and *pdgfra* (growth factor) in *VT* compared with *NT*. However, unlike the *NT/TT* comparison, genes coding for MHC I were not differentially expressed in the *NT/VT* assessments. BMP signalling components (*sost*, *tbx2b*, *pnpla6* & *acvrl1*), as with *NT/TT*, were all shown to be upregulated in *VT* compared with *NT*, while positive angiogenic gene expression in *VT* also matches the trend shown in *TT* when compared with *NT*.

TT/VT: Among the seven upregulated genes in *VT* compared with *TT* include *chia3* (immune regulation), *dusp2, and dusp1* (innate immunity), while most upregulated genes in *TT* compared with *VT* matched those found in the *NT/TT* comparison. These included immune genes such as *jakmip1* (T-cell activity), *gapdh* (inflammation), and *bcl6b* (inflammation regulation, adaptive immunity).

3.3.3. Functional enrichment

Gene ontology functional enrichment analysis on upregulated and downregulated gene sets for each pairwise comparison was conducted. In some cases, gene abundances were not sufficient to carry out robust functional enrichment analysis. Significant enrichment observations are reported henceforth (supplementary; Tables S19–33).

All downregulated genes in *TV* when compared with *NN*, highlighted a number of enriched functional pathways including those associated with muscle, blood circulation, and metabolism. Cell morphogenesis, growth, and neuron development, as well as heparin-binding processes, were all significantly enriched in upregulated *VV* genes when compared with *NV*. Genes downregulated in *TV* compared with *NV* exhibited angiogenesis, cell morphogenesis, and cell migration pathway enrichment.

In *NN/NT*, downregulated genes in *NT* were significantly enriched when it came to muscle-related pathways, cell morphogenesis, and vascular development. The most prominently enriched pathways were attributed to upregulated genes in *TT* in the *NT/TT* pairwise comparison. These included pathways involved in angiogenesis and vascularization, muscle and neuron morphogenesis, cell migration, and lymphangiogenesis (Fig. 7). In line with *NT/TT*, upregulated genes in the *NT/VT* data set were also associated with vascularization and blood vessel development.

4. Discussion

The immune response characteristics and transcriptome assessments

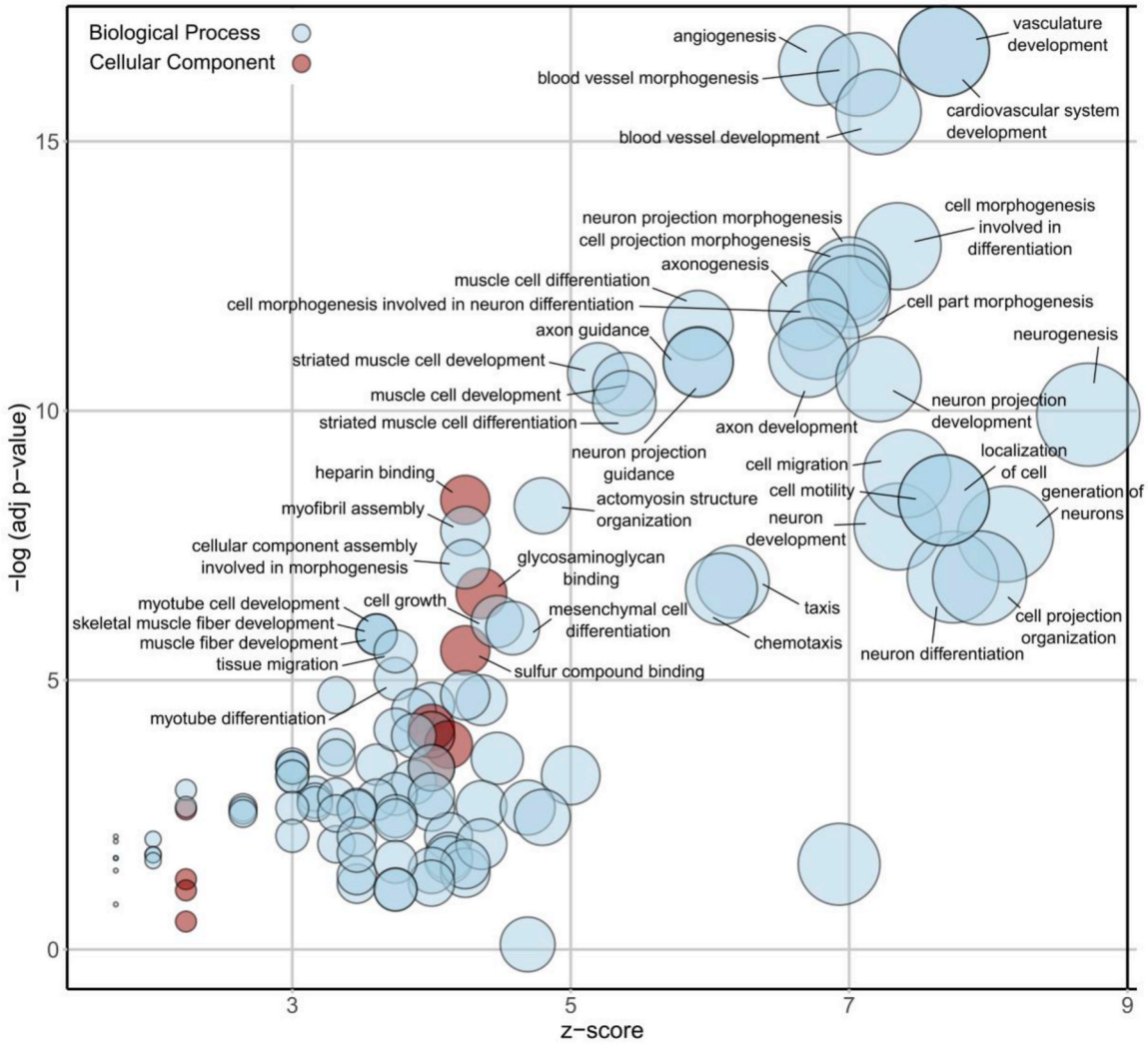

Fig. 7. Gene ontology bubble plot depicting biological and cellular processes upregulated in the double exposed *Tenacibaculum maritimum* treatment (*TT*) compared with unvaccinated *Tenacibaculum maritimum* replicates (*NT*) (66 genes) in *Hippocampus erectus*. Bubble size correlates with gene abundances associated GO term. Labelled bubbles depict the most enriched processes with (-log (adjusted *P*-value >5). GO terms selected based on their DAVID clustering enrichment score (>2.5) (Sherman and Lempicki, 2009).

of primary and secondary bacterial exposures in fish have been well researched, largely due to the drive to improve aquaculture practices of commercially important fish through vaccination development (Espelid et al., 1991; Irie et al., 2005; Lenz et al., 2013; Midtlyng et al., 1996; Raida and Buchmann, 2008; Toranzo et al., 1997). Novel insights into the teleost immune system, concerning genomic rearrangements, losses, and gene expression changes, have highlighted the plastic potential of the teleost immune system (Dubin et al., 2019; Roth et al., 2020; Solbakken et al., 2016; Star et al., 2011). Giving focus to peculiar evolutionary instances can provide an understanding of alternative immune strategies and their limitations, while also shedding light on mechanisms or drivers of evolutionary change. By utilising whole-transcriptome profiling, this investigation aimed to elucidate which innate and adaptive immune system pathways are activated following exposure to heat-killed bacteria in *H. erectus*, building on previous syngnathid immune studies (Parker et al., 2023b; Roth et al., 2012a, 2012b). The immunological ambiguity that surrounds seahorses, particularly in relation to the MHC II pathway, renders them arguably the most interesting of syngnathid sub-groups to study. The activation and memory-forming potential of the seahorse MHC II pathway are yet to be understood due to the functional loss of the crucial invariant chain (CD74) (Roth et al., 2020). This study brought to light new information regarding the seahorse adaptive, innate, and memory-inducing immune activities, providing some additional context to the discussion surrounding MHC II functionality and the potential compensatory mechanisms that may be at work in its absence.

PCAs highlighted that gene expression profiles differ between single (*NV & NT*) and their double-exposed counterparts (*VV*, *TV*, *TT & VT*), while the highest number of differentially expressed genes as well as the greatest disparities, were also associated with comparisons between single-exposed and double-exposed individuals (*NV/TV & NT/TT*). In turn, both these observations provide strong support for the first hypothesis of this study **(i)**. In the context of immunity, immune system development enrichment was significant in its influence when discerning *VT* and *TT*, from *NT*. Genes such as *itgb7* and *itga1,* are linked with lymphocyte migration and motility in humans (Butcher et al., 1999; Reilly et al., 2020), and are associated more with double-exposed replicates. This is the first indication that prior bacterial experience and the establishment of immunological memory are occurring in seahorses involved in this investigation. However, acquired immune memory stimulated by epitope-specific recognition via the MHC II pathway, in theory, should only be activated in homologous double-exposed replicates. Taken that the functional role of MHC II, if any, in seahorses is ambiguous, the inferred presence of immunological memory in *VT* could be down to alternative machinery.

4.1. Innate immunity

Both innate and adaptive immune systems are characterised by a variety of pathways, interacting mechanisms, and immune cell constituents, with the innate immune system tasked with mounting a primary response following bacterial exposure (Akira and Takeda, 2004; Raida and Buchmann, 2009; Rodriguez et al., 2005). Upregulated innate immune responses have been recorded previously in syngnathids following exposure to Gram-negative bacteria (Beemelmanns and Roth, 2016a; Jiang et al., 2022; Roth et al., 2012a; Roth et al., 2012b; Samaraweera et al., 2019). Some indications of elevated innate immune activity were also observed here in *NV* when compared with *VV* and in particular *TV*. These processes including inflammation, macrophage, and neutrophil-related activity are crucial innate immunity components and have been identified as such in fish species previously upon exposure to *Vibrio* bacteria (Beemelmanns and Roth, 2016a; Chaves-Pozo et al., 2005; Magnadóttir, 2006; Wang et al., 2016). However, this trend was not consistent in *TT* and *VT* replicates when compared with *NT*. Most inflammation and traditionally associated innate immune-related expression, in some cases the same genes, exhibited upregulated expression in double-exposed individuals. Inflammation represents one of the first immunological steps upon infection, in addition to sequestering the invasion and activating the adaptive immune system (Joffre et al., 2009; Newson et al., 2014). The prominent expression of genes, such as *clec14a, sema3d, adamts7, and inavaa*, which are typically associated with innate and inflammatory processes (Luong et al., 2018; Kanth et al., 2021; Zelensky and Gready, 2005; Zhang et al., 2015), could be a sign of the innate immune system assisting the adaptive branch. Nevertheless, these differential gene expression findings match the patterns displayed by the PCA assessments (PC1) which associate innate immune genes strongly with *TT* and *VT* but not *VV* and *TV*. These discrepancies may also have been influenced by the activation response times required following exposure to novel pathogens, i.e., *T. maritimum*, and may have benefited from a more delayed sampling point.

4.2. Trained immunity

The innate branch has traditionally been described as a general, non-specific immune response to infection largely irrespective of the bacterial type, hindering the ability for immune memory establishment. However, for some years now it has been suggested that a degree of bacterial specificity and memory does exist in the innate immune system (Bannerman et al., 2004; Blischak et al., 2015; Huang et al., 2001; Jenner and Young, 2005; Nau et al., 2002). The term 'trained immunity' is used to describe immunological memory derived from the innate defence repertoire, separate from the adaptive branch (Netea et al., 2020). This term was coined following a number of reports advocating the innate immune system's ability to establish and utilise prior infection knowledge to combat specific and general future pathogen encounters, a trait typically associated with the adaptive immune system (Cheng et al., 2014; Cooper and Eleftherianos, 2017; Netea et al., 2011). Adaptive immune memory begins through antigen-presentation-mediated T-cell activation and extends beyond eradication through small populations of persisting lymphocytes, specific to the invading entity (Chaplin, 2010). Contrastingly, trained immunity is orchestrated by a range of innate immune cell types (myeloid, NK-cells, macrophages, dendritic cells, innate lymphoid cells, and monocytes) with different origins and effector functions, and does not rely on the same gene rearrangements and calculated cell differentiation steps (Netea et al., 2020; Vetvicka et al., 2021). Instead, defences are mediated by expression modulation via chromatin remodelling and other restructuring of epigenetic motifs (Quintin et al., 2012; Saeed et al., 2014). Histone modification and chromatin modification processes were found to be the most enriched GO terms associated with PC1 in the *T. maritimum* treatments assessed in this study. Specifically, this enrichment aligned with *TT* and *VT* treatment replicates; fish subjected to secondary exposures. Another trained immunity indicator comes in the form of upregulated *jakmip1* expression in *TT* replicates, which was previously marked for NK-cell-related innate immune memory related to the human cytomegalovirus (Rückert et al., 2022).

In mammals, trained immunity provides a less specific, broader memory-driven defence than the adaptive equivalent (Vetvicka et al., 2021). Moreover, it can be triggered and afford protection against homologous and heterologous bacterial exposures (Netea et al., 2011, 2016); which potentially explains the close association with *VT* and *TT* replicates. This could be an indication that trained immunity through the innate immune system is an active part of the seahorse defence system following sequential infections. It also offers an explanation for upregulated innate gene expression in treatments where adaptive immune expression indicators were expected to dominate. The adoption of alternative immune strategies would be in line with other peculiar adaptations that have been proposed in seahorses, such as the immunogenetic losses of *c4* (complement) and *cd5* (B-cell function), as well as the loss of spleen function. Assessing the role of trained immunity in the context of the functional ambiguity of the MHC II pathway in seahorses is intriguing to contemplate, as from an immune memory standpoint it may provide some compensation for the proposed loss of adaptive

immune memory function. Alternatively, possessing an effective and robust trained immunological memory may have rendered the MHC II pathway surplus to requirements, and contributed to its eventual loss or a shift in function. These proposals are in need of further functional clarification; however, indications here suggest that trained immunity dynamics could be an interesting path for exploration in syngnathids in the future. Similar expression patterns were not observed in the *V. aestuarianus* treatments due to the lack of significant influential genes required for analysis. A less aggressive seahorse immune response to *V. aestuarianus* may explain this in part, prompted perhaps by a greater natural familiarity between the host and bacteria. In order to understand the differences between *V. aestuarianus* and *T. maritimum* immune responses, strain-specific immune response triggers, host-bacteria evolutionary history, and familiarity need to be considered.

4.3. Bacterial familiarity

As mentioned, there are reports pertaining to specific bacterial response processes being adopted in the innate immune system (Bannerman et al., 2004; Blischak et al., 2015; Huang et al., 2001; Jenner and Young, 2005; Nau et al., 2002). Differences in how the innate defences respond to *V. aestuarianus* exposure compared with *T. maritimum* are therefore plausible. For example, Gram-negative bacterial triggers can vary and induce different inflammatory reactions depending on the bacterial antigen and would likely also vary between bacterial strains (Heumann and Roger, 2002). Additionally, these expression dissimilarities are likely due, in large part to the differences in the bacterial familiarity with the host. *T. maritimum* replicates exhibited a far greater number of differentially expressed genes than *V. aestuarianus* exposed fish, which could be an indication that transcriptionally, more was required to combat the unfamiliar bacterial exposure. To our knowledge, *T. maritimum* has never encountered *H. erectus* prior to this experiment and unlike *V. aestuarianus* is not a resident of Baltic Sea waters. For this reason, the innate immune responses likely differ between microbes, explaining in part the difference between the findings observed here. Consequently, the second **(ii)** hypothesis of this investigation, that individuals without a primary exposure treatment show a greater innate immune response than homologous and heterologous double-exposed fish, is only supported by the *V. aestuarianus*-related replicates.

4.4. Adaptive immunity

Adaptive immune responses and immunological memory are driven primarily by T- and B- lymphocyte subsets activated by circulating antigen-presenting cells (Bonilla and Oettgen, 2010). Unlike trained immunity, adaptive immunity is highly specific and utilises immunoglobulin gene recombination processes to establish long-lasting immunological memory (Danilova, 2012). Specific antigen receptors corresponding to invading pathogen strains are established during infection; the persistence of these specific receptors provides the foundation for adaptive immunological memory and rapid response capacity upon re-infection (Janeway et al., 2001). Indications of adaptive immune activity were observed in a number of pairwise comparisons in this investigation. In particular, in *TT* where T- and B-cell activation and proliferation genes were upregulated compared with *NT*. This was one of the key signs of a stronger adaptive immune response in double-exposed individuals compared with single-exposed. In addition, *TT*'s upregulated expression of MHC I transcripts suggests $CD8^+$ cytotoxic T-cell-related activity upon secondary exposure to *T. maritimum*. Previous research has propounded that cytotoxic T-cell subsets could be a crucial immune system constituent in syngnathids and in particular the MHC II deficient *S. typhle* (Parker et al., 2022; Parker and Roth, 2022). Additional evidence in this investigation that suggests upregulated adaptive immune activity comes in the form of downregulated *chia3* (immunological tolerance) expression in *TT* compared with *NN*.

Gene expression patterns in *VV* and *TV* replicates were less defined in terms of an acquired immune response, triggered by secondary exposure. On the one hand, downregulated expression in *VV* compared with *NV* of *rgs13*, *nfil3-6,* and *tgfb1b*, with potential roles in dictating B-cell responsiveness (Shi et al., 2002), regulatory T-cell regulation and T-cell inhibition (Kim et al., 2019; Wahl et al., 1988), respectively, suggest a reaction to re-exposure. However, similar observations were not evident in *VV* when compared with *NN*, with most immune genes exhibiting downregulated expression in *VV*. In addition, alternative immune genes were downregulated in *VV* compared with *NV*, further confusing the general immunological status of the *VV* double-exposed individuals. The lack of a clear-cut immunological direction in *V. aestuarianus* replicates could be due to the seahorse's regular prior experiences with *V. aestuarianus* derived antigens in the environment. Also consider that *Vibrio* strains, including *V. aestuarianus,* which are commonly isolated from fish and mollusc microbiomes, have been suggested to be capable of existing in a mutualistic state without imparting harm on the host (Halpern and Izhaki, 2017; Tison and Seidler, 1983; Senderovich et al., 2010). It is therefore possible that a mutual relationship exists between *V. aestuarianus* and the *H. erectus* used in this study, which may have been rooted in a coevolutionary history that led to immunological tolerance. Moreover, the conversely heightened immune status in *TT* supports the relevance of immunological naivety in its contribution to the response dynamics of fish in this investigation. In turn, *TT* replicates support the third hypothesis of this study that homologous double-exposed replicates elicit stronger adaptive immune responses than single-exposed (*NT*) fish **(iii)**. Conversely, *VV* observations were not in line with this prediction likely due to the seahorses' high familiarity with *V. aestuarianus*. While limited, upregulated immune genes of *VT* when compared with homologous replicates (*TT*), were shown to be linked more with innate functioning dual-specific phosphatases (*dusp1* and *dusp2*). *Dusp1* has been linked with innate immune regulation, while *dusp2* has been suggested to play a role in toll-like receptor function (Abraham and Clark, 2006; Chen et al., 2002; Lang et al., 2006). Conversely, adaptive immune activity in the form of *bcl6b* (T_h2 response) and *jakmip1* (T-cell and NK-cell activity) upregulation in *TT* compared with *VT* could be an indicator of adaptive immune potential (Bilic and Ellmeier, 2007; Libri et al., 2008; Rückert et al., 2022). Moreover, both *blcb6b* and *jakmip1* have been implicated in $CD8^+$ T-cell-related adaptive immune memory function in mice (Libri et al., 2008; Manders et al., 2005). While requiring further functional clarification in syngnathids, the presence and activation of these genes encourage the adoption of adaptive immune recollection. They also support in part the fourth hypothesis of this investigation, that heterologous (*VT*) double-exposed replicates elicit a weaker adaptive immune response than homologous (*TT*) replicates **(iii)**.

MHC II presents exogenous antigenic peptides to $CD4^+$ T-lymphocytes and its upregulated expression is indicative of an activated adaptive immune system (Neefjes et al., 2011). MHC II gene upregulation in *TV* and *VV* compared with *NV* was observed here in *H. erectus* and could indicate MHC II pathway activity and functionality in the species. However, individuals with elevated *mhc II* expression do not exhibit upregulated expression of other key MHC II pathway constituents, such as the transactivator (*ciita*), activation-induced cytidine deaminase (*aicda*), the invariant chain (*cd74*), and T-cell co-receptor (*cd4*). The lack of a functional CD74 has been deduced previously and its absence is the foundation for the MHC II pathway's proposed loss of function in the seahorse (Roth et al., 2020). These points considered, *mhc II* expression could be redundant or share an alternative unknown function that is independent of CD74. Extracellular non-classical MHC II functions have been proposed previously (Hauschildt et al., 1993; Mourad et al., 1990; Spertini et al., 1992), which in principle would require cellular transport assistance provided by the previously mentioned pathway components. Intracellular MHC II non-classical function has also been proposed by way of promoting TLR-related innate immune responses through interactions with the tyrosine kinase Btk and CD40 (Liu et al., 2011). CD40

gene upregulation was observed in *TV* along with MHC II when compared with *NV*, which could indicate a possible non-classical function of MHC II, as an adapter for the promotion of TLR-triggered responses, relating to innate immunity in seahorses. This theory relies on the assumption that as an intracellular process, assisted transport via CD74 may not be as essential for surface-related functions. The absence of non-classical class II DM molecules in teleost fishes, components tasked with dissociating bound CD74 from classical MHC II in mammals, suggests unique MHC II peptide loading methods, potentially independent of CD74, exist among teleosts and engage in alternative functions (Dijkstra et al., 2013). If the classical MHC II pathway function is absent or has a non-classical function in seahorses, it raises questions regarding the immunological measures that operate in its place. The MHC I pathway, which is mediated via $CD8^{+}$ cytotoxic T-cell allorecognition, has been a suggested compensatory mechanism to cope with the loss of MHC II in pipefish and codfish (Parker et al., 2022; Parker and Roth, 2022; Roth et al., 2020; Star et al., 2011). Higher MHC I diversity in seahorses compared with other non-brooding syngnathids has also been stipulated, further supporting a potential increased dependence on the MHC I pathway (Parker et al., 2023b; Roth et al., 2020). If classical MHC II function is in fact missing in *H. erectus*, MHC I upregulated expression in *TT* replicates compared with *NT* found here could also support this proposition, as well as the fourth hypothesis of this investigation **(iv)**.

4.5. Angiogenesis

Gills are highly vascularized tissues that allow for optimal respiratory capacity through regular circulation of oxygenated blood; however, this, along with the semi-permeability of the tissue, also renders it susceptible to microbial influx (Bjørgen and Koppang, 2021; Evans et al., 2005; Koppang et al., 2015). To counter this, dense vascular networks and a rich blood supply establish a conduit for circulating immune cells in the gills, while specialized structures known as the gill-associated lymphoid tissue (GIALT) provide a mucosal hub for immune cell populations (Salinas, 2015). This investigation exhibited signs of angiogenic activity, through GO enrichment and upregulated vascularization genes, in double-exposed replicates compared with single-exposed individuals. In turn, angiogenic processes could be an adaptive response to secondary challenges, which potentially assist with immune cell recruitment. This is supported by previous findings indicating that vascular endothelial cells are influenced by immune cell derived soluble factors and have adhesion molecules capable of dictating the extravasation of immune cell types (Mora and Von Andrian, 2006; Young, 2012). Integrins are crucial for such cell-adhesion processes, facilitating vascular immune cell trafficking, tissue migration, phagocytosis, and the formation of immune synapses, rendering them an integral component in the immune system in mammals (Luo et al., 2007; Pribila et al., 2004). Universal upregulation of integrin genes in *TT* replicates, as well as *itgb7* in *TT* and *VT,* propound a similar importance in seahorse gill tissue. Further functional studies should identify if ITGB7, known for its immune homing role in the gut in humans (Arthos et al., 2008), plays a similar migratory role in syngnathid gills. The absence of these immune reaction traits in *VV* gives further support that an increased naiveness of the *H. erectus* immune system exists against *T. maritimum*. These vascular-related gene expression findings accentuate the growing disparities between *V. aestuarianus* and *T. maritimum* gene expression profiles. Blood vessel formation in seahorse gills appears to be an important process following secondary exposure and could be linked to processes involved in syngnathid immunity. This is in line with previous research in mammals which associates infection with increased angiogenetic activity (Noonan et al., 2008; Ribatti and Crivellato, 2009; Takeda and Akira, 2004), as well as in pipefish when in response to allogenic tissue (Parker and Roth, 2022). Genes involved in BMP signalling are also linked to angiogenesis and vascular development processes in humans (Cai et al., 2012; de Vinuesa et al., 2016). The upregulated expression of BMP-related genes in *TT* compared with *NT* also matches the upregulated angiogenic trend exhibited in the same example, while evidence from previous studies in humans supports a potential role in immune modulation (Chen and Ten Dijke, 2016; Sconocchia and Sconocchia, 2021; Yoshioka et al., 2012). However, little is known about immune-related BMP signalling in teleosts, and would likely require further research to substantiate the inferences addressed here.

4.6. Concluding remarks

Deciphering the intricacies of immune activities using transcriptome data can be a challenging prospect, with time of dissection, tissue type, age, species, and environment all potentially influencing the subtle gene expression changes. In particular, immune responses in fish have been shown to be influenced by temperature, environmental factors, antigen characteristics, exposure dose, and time in previous studies (Anderson et al., 1982; Lillehaug et al., 1993; Mota et al., 2019; Rijkers et al., 1980). Gene expression findings that concern the *NN* replicates in this experiment compared with *TV* and *VV* replicates were unexpected, based on the unusual downregulation of immune-related genes in *VV* and *TV*. Considering that stringent measures were taken to avoid the influence of confounding variables during these experiments, no clear explanation for these unexpected results could be found. Immune defence strategies come at a metabolic cost, therefore, expending excessive energy when dealing with bacterial exposure may not always be the optimal course of action (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996; Viney et al., 2005). The general immunological sedateness characterising *V. aestuarianus* replicates in this investigation could in part be explained by this energy balancing process; whereby the costs of raising an immune response in response to *V. aestuarianus* are greater than the benefits.

In this investigation, reactions to bacterial exposure were discussed under the umbrella terms of innate and adaptive immunity, despite the knowledge that both branches are intimately linked and constituents often contribute to both processes (Iwasaki and Medzhitov, 2015; Lanier and Sun, 2009; Vivier et al., 2011). However, segregating the terms for the purposes of disentangling the differences between primary and secondary exposures is a robust approach adopted previously (Keller and Roth, 2020). This approach held further importance for interpreting MHC II pathway functionality and potential compensatory gene activations. Similarly, using different heat-killed bacteria for this experiment provided the opportunity to decipher strain-specific characteristics when it comes to immune responses. In turn, disparities were found between the reactions induced by both heat-killed bacteria with *T. maritimum* treatments, especially *TT*, exhibiting the most pronounced adaptive defences compared with any other treatment. As mentioned previously, this could largely be due to fish in this investigation being more accustomed to *V. aestuarianus* infection, resulting in a less intensive immunological presence, compared with the unknown *T. maritimum*. The differences in vascular-related growth processes were also an interesting contrast between the two strain effects; future functional studies should be arranged to investigate angiogenesis' significance within the context of syngnathid immunity. Overall, bacterial induction of the immune system in both these Gram-negative bacterial strains, by and large, optimises the same pathways and defence mechanisms upon infection, but secondary exposure appears to promote differences between the two, which supports the fifth hypothesis of this study **(v)**.

Gene expression differences found between *V. aestuarianus* and *T. maritimum* replicates in this investigation highlighted the presence of bacterial-specific immune responses in seahorses, with unfamiliar strains tending to evoke a stronger immune reaction. Adaptive immune response characteristics were observed prominently in replicates twice exposed to unfamiliar bacteria supporting the capacity of adaptive immune memory in seahorses. Additionally, the first potential indicators of trained immunity in seahorses were observed, which could supplement

or compensate for adaptive immune functional losses that may have occurred in this genus. In turn, trying to understand the weight and relevance of innate immunity in syngnathids should be prioritized as it could play a role in offsetting the functional repercussions of adaptive immune gene loss. While the upregulated expression of MHC II requires further clarification as to whether its function relates to antigen-presentation or an alternative non-classical function. Lastly, this study raised the potential significance of angiogenic and vascular processes in mounting syngnathid gill immune responses and should encourage future research tasked with understanding their functional role.

Data accessibility

All raw sequencing data are available at NCBI SRA under BioProject PRJNA1019256. Genome data are available at NCBI SRA under BioProject PRJNA613176.

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CRediT authorship contribution statement

Jamie Parker: Formal analysis, Writing – original draft, Writing – review & editing. **Silke-Mareike Marten:** Methodology. **Tadhg C. Ó Corcora:** Methodology. **Jelena Rajkov:** Methodology. **Arseny Dubin:** Data curation. **Olivia Roth:** Conceptualization, Funding acquisition, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declarations of competing interest

None.

Data availability

Raw read data has been uploaded to NCBI and will be made open for access immediately after the paper is finalized and published. Details can be found at the end of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2024.105136.

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