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Comparative assessment of immunological tolerance in fish with natural immunodeficiency

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ABSTRACT

Natural occurrences of immunodeficiency by definition should lead to compromised immune function. The major histocompatibility complexes (MHC) are key components of the vertebrate adaptive immune system, charged with mediating allorecognition and antigen presentation functions. To this end, the genomic loss of the MHC II pathway in *Syngnathus* pipefishes raises questions regarding their immunological vigilance and allorecognition capabilities. Utilising allograft and autograft fin-transplants, we compared the allorecognition immune responses of two pipefish species, with (*Nerophis ophidion*) and without (*Syngnathus typhle*) a functional MHC II. Transcriptome-wide assessments explored the immunological tolerance and potential compensatory measures occupying the role of the absent MHC II. Visual observations suggested a more acute rejection response in *N. ophidion* allografts compared with *S. typhle* allografts. Differentially expressed genes involved in innate immunity, angiogenesis and tissue recovery were identified among transplantees. The intriguing upregulation of the cytotoxic T-cell implicated *gzma* in *S. typhle* allografts, suggests a prominent MHC I related response, which may compensate for the MHC II and CD4 loss. MHC I related downregulation in *N. ophidion* autografts hints at an immunological tolerance related reaction. These findings may indicate alternative measures evolved to cope with the MHC II genomic loss enabling the maintenance of appropriate tolerance levels. This study provides intriguing insights into the immune and tissue recovery mechanisms associated with syngnathid transplantation, and can be a useful reference for future studies focusing on transplantation transcriptomics in non-model systems.

1. Introduction

The multifaceted vertebrate immune system comprises a primordial evolutionary conserved innate branch coupled with the adaptive immune system representing one of the hallmarks of vertebrate evolution (Cooper and Alder, 2006; Flajnik and Kasahara, 2010). Understanding the evolution of the immune system is important as it holds the key to adaptation and co-evolution in host-parasite interactions, which in turn is integral for advancing effective medical treatments (Stearns et al., 2010). The adaptive immune system promotes specific pathogen responses, contributing to immunological memory which can be used to evoke a stronger response upon re-infection (Cooper and Alder, 2006; Farber et al., 2016). Among the key components of the vertebrate adaptive immune repertoire are the major histocompatibility complexes (MHC), which shape specific immune responses to a vast diversity of pathogens (Benacerraf, 1981; Neefjes et al., 2011). The evolution of the spectacularly diverse assortment of MHC alleles is postulated to be influenced by factors such as MHC driven mate choice, pathogenic selection and host-pathogen co-evolution (Apanius et al., 1997; Borghans et al., 2004; Penn and Potts, 1999; Takahata and Nei, 1990; Trachtenberg et al., 2003). MHC genes are key mediators for determining self from non-self, stimulating T- and B-cell activation and providing the link between antigen presentation and pathogen eradication (Shiina et al., 2009).

The discovery of MHC proteins in teleost fish arrived many years after their identification in mammals (Hashimoto et al., 1990), with ancestral genome duplications and rearrangements in early teleosts giving rise to a vast diversity of MHC structures (Grimholt, 2016; Wilson, 2017). For years MHC II was believed to be synonymous among teleosts and mammals, however, the discovered genomic loss of MHC II in Gadiformes challenged this idea (Star et al., 2011). Since then other MHC II genomic absences in anglerfish (Dubin et al., 2019; Star et al., 2011), *Syngnathus* pipefish, as well as functional pathway loss in *Hippocampus* have also come to light (Haase et al., 2013; Roth et al., 2020).

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These findings highlighted the plasticity of the teleost adaptive immune system and brought into question the immunological capacity of syngnathid fishes with particular concerns over their ability to determine self from non-self.

Allorecognition can be assessed using allogeneic transplants, with historical research carried out on mammals highlighting the importance of MHC in mediating allograft rejection and determining histocompatibility (Dausset, 1981; Snell, 1981; Snell and Higgins, 1951). Fish related transplantation experiments have also provided sufficient grounding for evaluating allorecognition, however, none have investigated fish with natural MHC II deficiency as well as their implied natural immunodeficiency, and the majority have been unable to utilise next generation sequencing advances to substantiate physical phenotypic observations (Cardwell et al., 2001; Hildemann, 1972; Hildemann and Haas, 1960; Kallman, 1970; Kallman and Gordon, 1957; McKinney et al., 1981). Owing to the vast array of effector mechanisms adopted by the immune system to eliminate invading pathogens, it is crucial that the immune system avoids promoting an immune response against self, a process known as self-tolerance (Goodnow et al., 2005). Recognition process complications often contribute to the development of autoimmune diseases (Fernando et al., 2008; Nepom and Erlich, 1991). As MHC are important mediators of self-tolerance, it stands to reason that the evolutionary loss of MHC II in *S. typhle* may have impacted its ability to determine self from non-self and in turn facilitated an increased level of immunological tolerance to non-self tissue.

This investigation utilized allograft and autograft fin-transplant experiments to assess the perceived immunological deficiency of the MHC II devoid broadnosed pipefish, as well as its pipefish relative *Nerophis ophidion*. *N. ophidion* was chosen due to it possessing a functional MHC II pathway, providing an important reference for assessing the allorecognition and immune defence pathways in *S. typhle*. Allografts and autografts were compared along with two control groups, in an attempt to distinguish foreign tissue immune response elements from those evoked through alternative effectors. Following two weeks of monitoring for visual signs of inflammation and tissue rejection, transcriptome-wide gene expression assessments were carried out, to understand the molecular underpinnings that characterise transplant immune responses. Focus was also given to potential compensatory mechanisms in *S. typhle* that may have evolved following MHC II, as well as attempting to highlight other immune, physiological and tissue recovery processes that are activated upon transplantation. In light of the documented immunological rearrangements in syngnathid fishes, from a visual standpoint, it was hypothesised that **(i)** allograft transplants in this study would elicit stronger signs of rejection in *N. ophidion* than in the MHC II devoid *S. typhle* due to the perceived increased immunological tolerance in *S. typhle*. Leading on from this, the second hypothesis of this study predicted through utilising transcriptomic data, that **(ii)** *N. ophidion* would express a higher degree of allograft rejection compared with *S. typhle*. **(iii)** It was also predicted that MHC pathways would exhibit a greater downregulation in *N. ophidion* autografts compared with *S. typhle*. Lastly, based on the fundamental histocompatibility differences between autograft and allograft transplants it was hypothesised that **(iv)** within species, signs of rejection would be greater in allografts compared with autografts.

2. Materials & methods

2.1. Ethics statement

All aquaria maintenance methods and experimental procedures used in this investigation meet the guidelines issued by the German Animal Welfare Association (Permit no. V242 – 35167/2018) and are in accordance with German Law. No wild-caught individuals and no endangered species were used in this investigation.

2.2. Animals

Lab bred, *S. typhle* and *N. ophidion* native to the south Baltic were reared in the aquaria facilities at GEOMAR in Kiel. All stock fish were maintained under the standard conditions used by (Beemelmanns and Roth, 2016) in species-specific tanks (100 L). *S. typhle* were fed frozen and live mysids twice a day while live and frozen *Artemia salina* were fed to *N. ophidion*.

2.3. Fin transplants

Caudal fins were transplanted between genetically distinct individuals (allograft; **ALLO**) or within the same individual (autograft; **AUTO**). Surgeries were carried out on females for each species with grafts exchanged between same sex subjects. In addition, a "surgical control" (**SC**) was implemented, consisting of fish that underwent the surgical procedure and incisions but were without the attached transplant. A second control (**C**) was included which were fish that had not undergone surgery (surgical control), but were kept in the same post-surgery tank conditions as the transplantees.

Fish were starved for 24 h prior to surgery then anaesthetized with dissolved MS-222 (Tricaine, 100 mg L^{-1}; Sigma-Aldrich, Munich, Germany) until a surgical level of sedation was reached at which time individuals were withdrawn. Sedation assessments were in accordance with those recommended guidelines (Neiffer and Stamper, 2009). Anaesthesia was maintained during surgery by pipetting aerated anaesthetic seawater over the gills and mouth.

Allograft transplantations were conducted on two fish simultaneously in order to minimise the time under anaesthesia. For *S. typhle*, caudal fins were severed at the base to ensure connective tissue retention and then inserted into an anterior-posterior incision made below the anus (Fig. 1). For *N. ophidion*, small tissue clips were removed from the end of the tail and relocated to the same anatomical location as *S. typhle* transplantees. Following surgery, subjects were returned to smaller individual tanks in a reduced light environment and assessed to ensure the resumption of normal breathing and swimming activity. Slowly propelling the fish forward through the water was found to assist with breathing recovery and regaining of consciousness. Normal activity resumed between 5 and 15 min post-surgery.

2.4. Tissue sampling

Fish were monitored daily for 14 days post-surgery documenting changes in behaviour and movement. At day 15, all fish were euthanized with an overdose of MS-222 (Tricaine, 500 mg L^{-1}; Sigma-Aldrich, Munich, Germany) and the surgical area tissue was removed, without the transplant, as well as the gills, before being immersed in RNAlater. Samples were then immediately incubated at 4 °C for one week to allow for percolation, before relocating them to −20 °C in preparation for RNA extraction. Tissues were thawed and homogenised using a tissue shredder (Qiagen) prior to total RNA extraction utilising the RNeasy Tissue Mini Kit (Qiagen, Hilden Germany).

2.5. Transcriptome sequencing and de novo assembly

Extracted RNA quality was assessed using the NanoDrop-1000 spectrophotometer (NanoDrop) and Fragment Analyzer (Agilent Technologies) before library preparation. Libraries were prepared using an Illumina TruSeq stranded mRNA kit, before being paired-end sequenced (Illumina, NovaSeq 6000, 2 × 150bp reads) at the Competence Centre for Genomic Analysis (CCGA) in Kiel. Adapters were trimmed from all resultant raw reads using Trim galore! (v0.6.5) (Krueger, 2015) prior to FastQC (v0.11.9) and MultiQC quality checks (v1.9)(Andrews, 2010; Ewels et al., 2016).

Processing raw reads for transcriptome assemblies were carried out as follows: Rcorrector package (v1.04) with standard settings was used

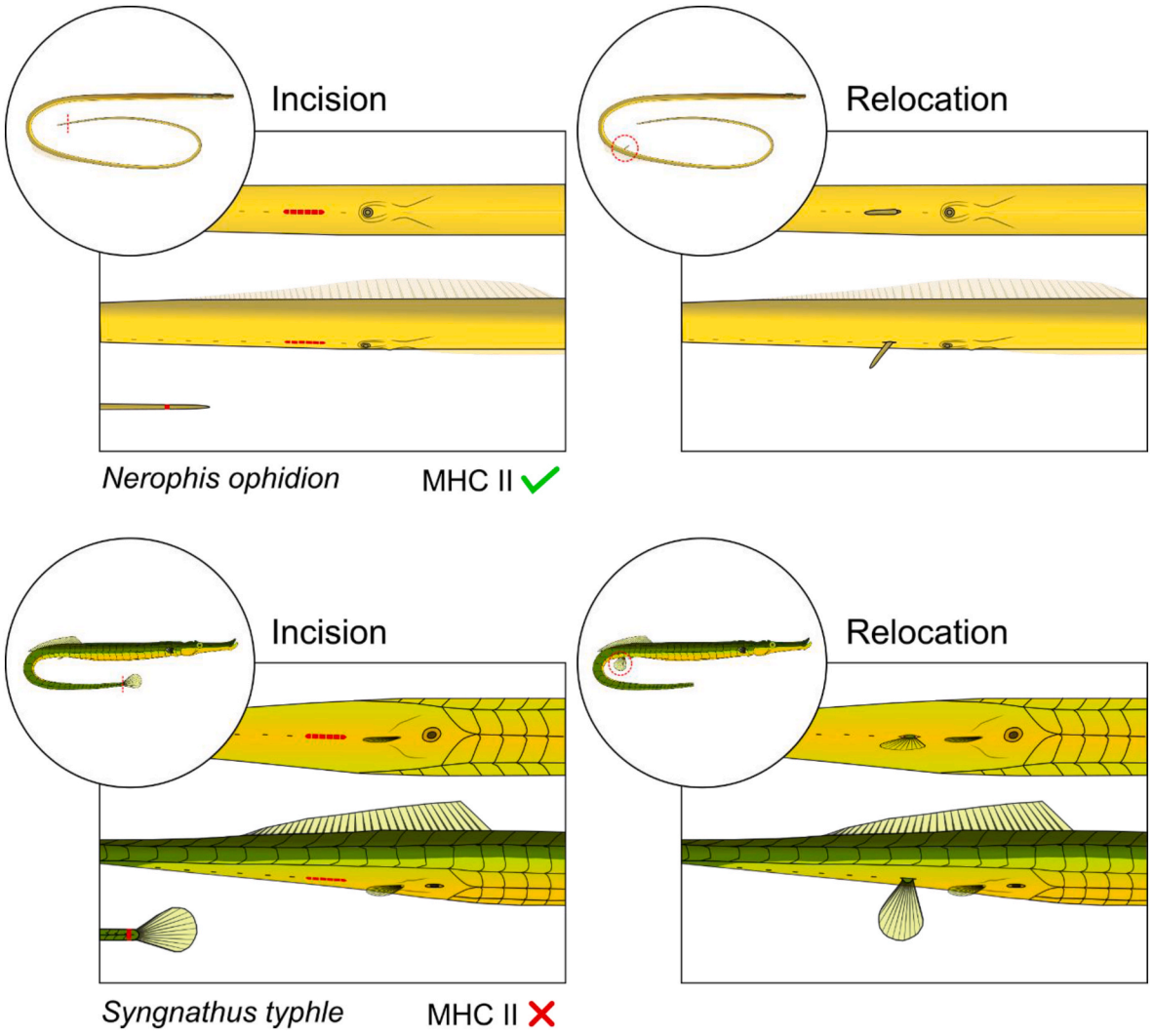

Fig. 1. Fin transplant incisions and relocations for both syngnathid species, *N. ophidion* and *S. typhle*. Green MHC II indicates presence of MHC II and red indicates loss of gene and function. Red dotted lines at day 1 show the incision location and dotted red circles indicate the relocation area.

to correct for errors (Song and Florea, 2015). Quality and adapter trimming were carried out on corrected reads using the fastp program (v0.20.0) (default setting) (Chen et al., 2018). Owing to the large number of reads, Bignorm software was utilized for normalization, using the recommended settings (Wedemeyer et al., 2017). Using default settings and disabling the normalization option, the normalized reads were assembled to the transcriptome using the Trinity package (v2.8.5) (Haas et al., 2013). For annotation, contigs were analysed with the Trinity package associated tools, TransDecoder (v5.5.0) and Trinotate (v2.0.0), under the author's recommendation (Bryant et al., 2017). The TransDecoder prediction step was conducted using the options '—retain_pfam_hits' and '–retain_blastp_hits'. The required databases (Pfam-A, Rfam and Uniprot) for these tools were downloaded in November 2019.

2.6. Transcript abundance and pairwise differential gene expression analysis

Deduction of transcript abundance was conducted by utilising RSEM v1.3.3 (Li and Dewey, 2011) and bowtie2 v2.4.2 (Langmead and Salzberg, 2012) to align reads to each respective transcriptome assembly. Tximport (v1.18.0) (Soneson et al., 2015) was used to transfer abundance estimates prior to downstream differential gene expression analysis. Data filtering was carried out, excluding genes with an expression value of <5 and present in <3 replicates. All remaining counts were transformed using mean variance stabilizing transformation (VST), before principle component analysis (PCAs) and uniform manifold approximation projection UMAP (McInnes et al., 2018) were adopted to reveal unusual outliers. The DESeq2 package (v.1.22.2) was used for all differential gene expression analysis (Love et al., 2014) in R (v4) (R Development Core Team, 2013). Three pairwise comparisons were carried out between the following pairs incorporating the non-surgical controls: **allograft vs control (ALLO/C)**, **autograft vs control (AUTO/C)** and **surgical control vs control (SC/C)**. In an attempt to remove the influence of surgical trauma associated immune activity, two more pairwise comparisons were carried out; **allograft vs surgical control (ALLO/SC)** and **autograft vs surgical control (AUTO/SC).** Lastly, a pairwise comparison between **allograft vs autograft (ALLO/AUTO)** was conducted in an attempt to highlight genes strongly associated with the two transplant types. Multiple testing corrections were implemented using the Benjamini and Hochberg adjustment (Benjamini and Hochberg, 1995). Genes with a log-2fold change expression of >1 and adjusted p-value < 0.05 were used for downstream analysis. The same process was carried out on the sequenced RNA extracted from gill tissue as an immunological reference to assist with downstream analyses. The gill was chosen based on its richness of blood and therefore presence of circulating immune cells.

2.7. Functional annotation of genes

Following transcript mapping and differential gene expression analysis a table of all differentially expressed transcripts was established. A Trinity annotation record was created based on all transcripts in each syngnathid species, which were all BLAST aligned to determine putative gene identification. The closest BLAST matches were used to identify the most accurate gene and Uniprot ID, independent from species, for each differentially expressed transcript. Orthology checks were carried out to ensure that related orthologs could be found in related teleost species. Uniprot identifiers for each transcript were then fed into the latest Uniprot database (Consortium, 2021) in order to extract gene information including: functional descriptions, tissue information, key words, GO IDs and GO terms. Word and phrase searches were carried out on the data table to extract information concerning gene functionality and potential pathway involvements, while external literature searches were carried out on every gene to extract further relevant information. Broadly speaking, words utilized for searches were related to immunity, tissue remodelling and wound healing. A comprehensive list of all words and phrases that were used in this search can be found in the supplementary material (supplementary; Table S1).

3. Results

3.1. Visual assessments

Each fish was assessed visually throughout the post-surgery period and all pre-mature fish mortalities were documented. A number of

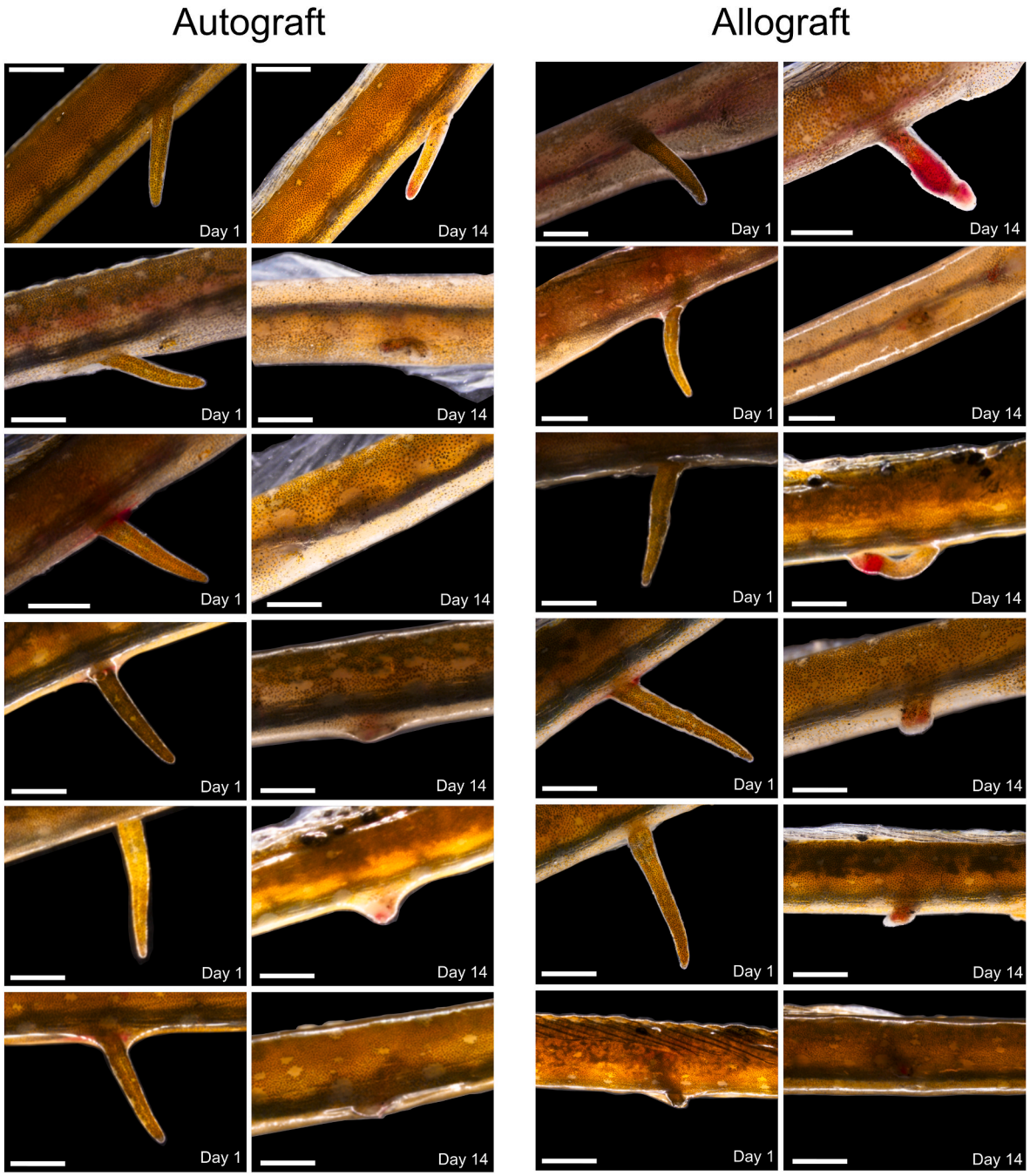

Fig. 2. Surgical transplant images of autograft and allografts in *Nerophis ophidion* directly after surgery (day 1) and prior to dissection (Day 14). Scale bars represent 1 mm.

fatalities were observed in allograft (*N. ophidion*: 9, *S. typhle*: 2) and autografts (*N. ophidion*: 6, *S. typhle*: 3). Fin 'sloughing', described as the shedding or loss of tissue from transplant, was observed at varying degrees in both species which is congruent with previous transplant studies on Xiphophorin fishes (Kallman and Gordon, 1957). The complete loss of fin transplant was also observed in some instances likely owing to ineffective tissue connection (supplementary; Fig. S1). In an initial trial carried out on *S. typhle* individuals, two transplantees were shown to maintain allograft transplants following 30 days after surgery (data not shown). Prior to evaluations of gene expression changes, transplants were observed for visual signs of innate and adaptive immune responses, potentially leading to localised inflammation. Visual indicators and behaviours that were used to determine whether such immune processes were active, included: transplant colour change/colour loss, swelling of the transplant and/or host, tissue sloughing, change in swimming and feeding activity. These points considered, initial observations following the 14 days period, suggested a more acute immune reaction in *N. ophidion* (Fig. 2) compared with *S. typhle* (Fig. 3). Prominent tissue swelling and increased reddening of the transplant tissue in *N. ophidion* allografts were the driving forces for this diagnostic distinction, appearing to be more severe than *S. typhle* allografts and *N. ophidion* and *S. typhle* autografts. Within species, visual transplant responses in *S. typhle* autografts and allografts appeared to be less distinct from each other than in *N. ophidion*. Additionally, evidence of possible revascularisation can be observed in both species suggesting the transfer of host blood to the donor tissue and establishment of a histological connection.

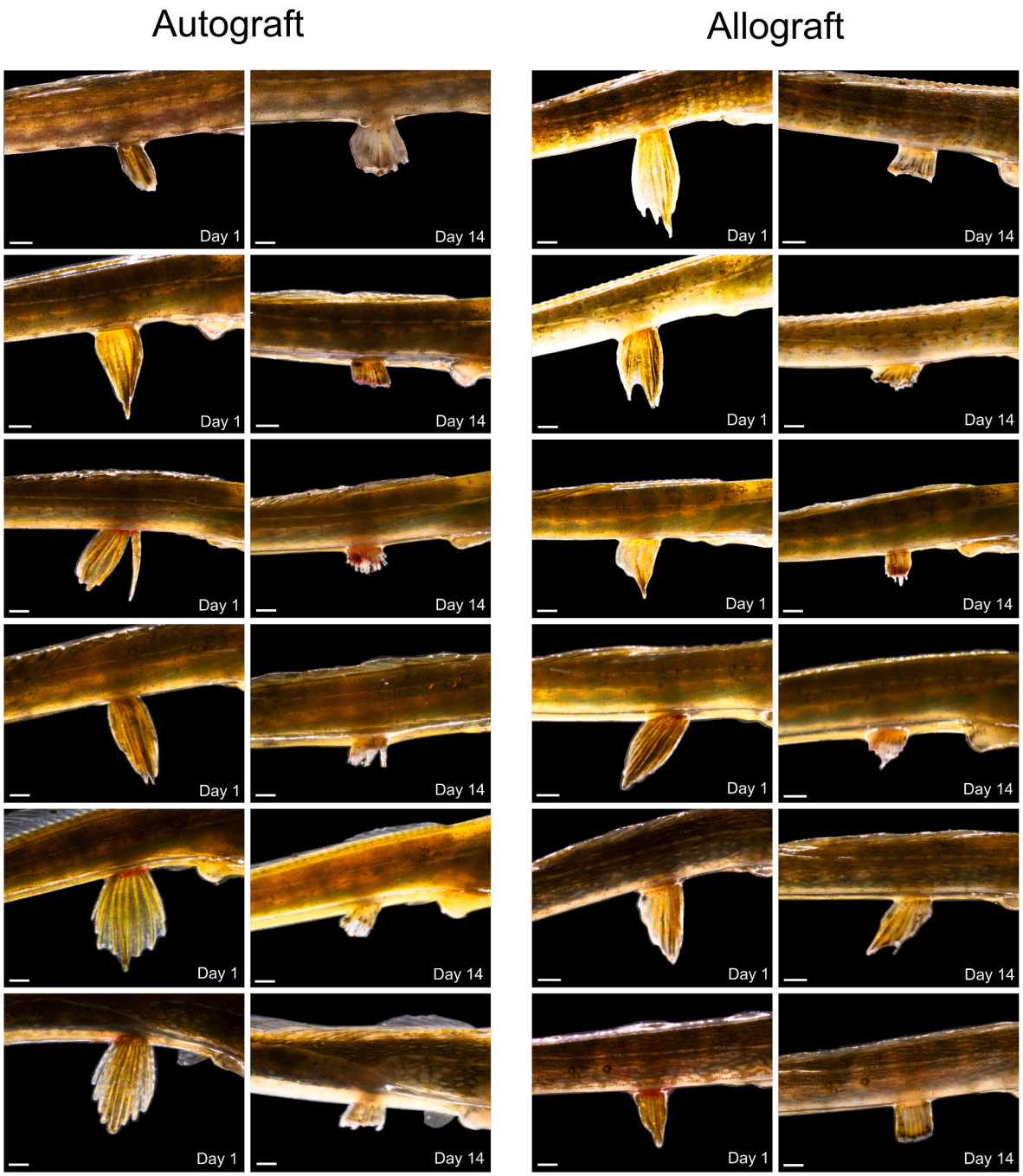

Fig. 3. Surgical transplant images of autograft and allografts in *Syngnathus typhle* directly after surgery (day 1) and prior to dissection (Day 14). Scale bars represent 1 mm.

3.2. Differential gene expression of surgical area tissue

Differential gene expression analysis was carried out on the extracted surgical area tissue from two syngnathid pipefish species, *S. typhle and N. ophidion*. Four surgical types; allograft **(ALLO)**, autograft **(AUTO)**, surgical control **(SC)**, control **(C)**, each with 6 replicates were carried forward for gene expression assessments. Following RNA sequencing, the total read ranges for all replicates of each surgical type was 222–349 million paired-end reads, with an individual sample average of 23.5 million reads. A total of 2550 and 1233 annotated genes were differentially expressed across all replicate pairwise comparisons in *S. typhle* and *N. ophidion* surgical tissue, respectively (supplementary; Fig. S2). Genes of interest were selected based on their relevance to processes such as immune system function, tissue remodelling, wound healing, and regeneration. The six pairwise comparisons used in this investigation were as follows; allograft vs control **(ALLO/C)**, autograft vs control **(AUTO/C)**, surgical control vs control **(SC/C),** allograft vs surgical control **(ALLO/SC),** autograft vs surgical control **(AUTO/SC)** and allograft vs autograft **(ALLO/AUTO).**

In both species the majority of annotated differentially expressed genes are shared between allograft (ALLO/C) and autograft (AUTO/C) comparisons within species, when using the non-surgical control (C) replicates for pairwise comparisons (Fig. 4) (supplementary; Tables S2–3, Figs. S3 and S4). Similarly, a number of genes were shared between the *S. typhle* transplant types and their equivalences in *N. ophidion*. Shared upregulated allograft genes in the ALLO/C comparison, included *ctsk* (inflammation), *tnnl1* (muscle function), *mmp13* (collagen degradation) and *gas2* (apoptosis), with the latter three also showing upregulation in autograft replicates in both species. Downregulated genes shared few similarities among allograft subjects, with one notable gene (*vwa7*) which is present within the MHC III region and thought to control lung tumour susceptibility (Snoek et al., 2000). Further notable upregulated genes specific to autograft, when compared with control replicates in both species included, *tlr1* (innate immunity), *cmlkr1* (anti-inflammation and pro-inflammation), *adam12* (muscle regeneration). Two additional muscle related genes called *ahnak* (smooth muscle migration), *myh10* (muscle development), as well as *slc4a1* (erythrocyte structure) were among the few genes that exhibited

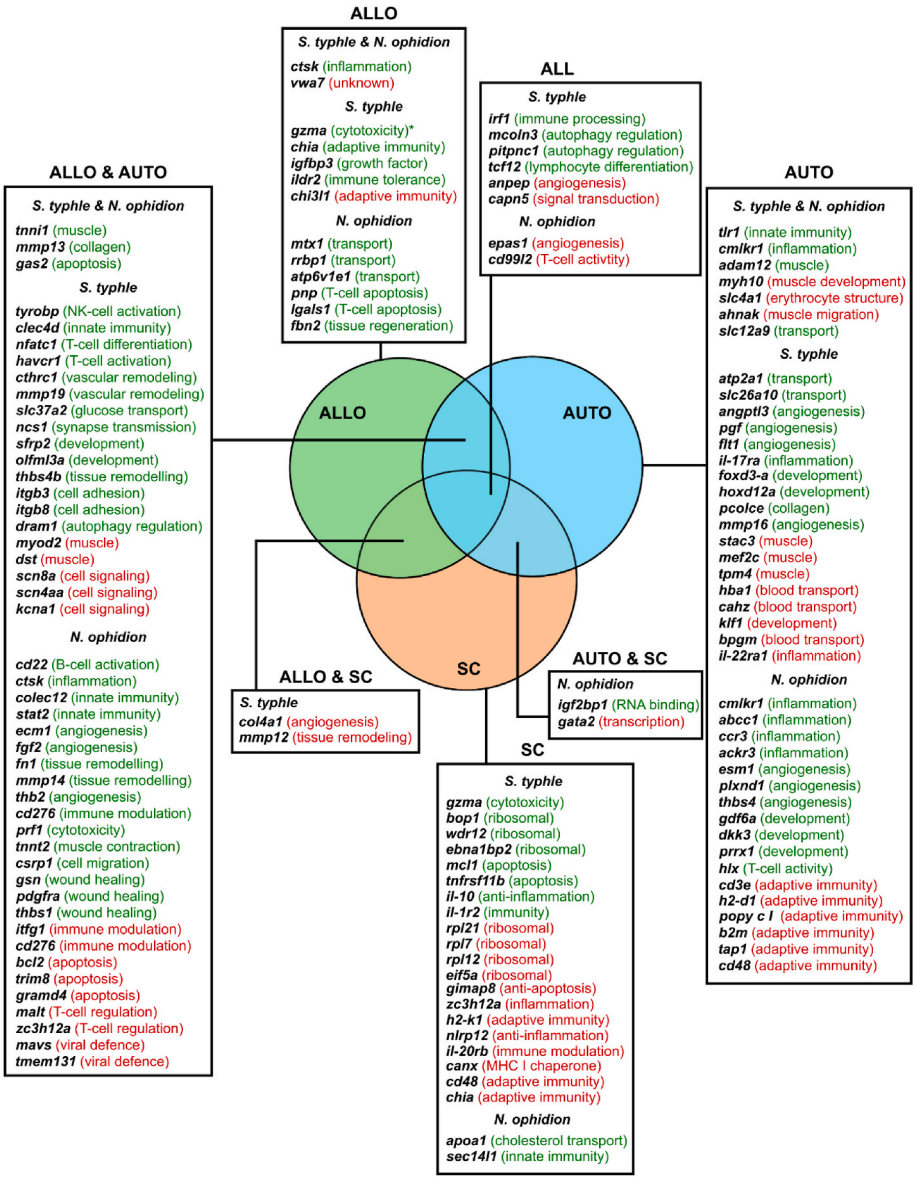

Fig. 4. Schematic diagram showing differentially expressed genes of interest in allograft (ALLO), autograft (AUTO) and surgical control (SC) replicates. Upregulated (green) and downregulated (red) genes are in relation to control replicates. Genes shown possess roles in immunity and/or have high/low expression levels. Upregulation in ALLO-AUTO comparison indicated by *.

a shared downregulation in autografts across species. Surgical control (SC) replicates in *S. typhle* and *N. ophidion*, when compared to C, did not share any differential expression similarities (see Fig. 5).

3.3. *Syngnathus typhle* - species-specific gene expression

3.3.1. Shared gene expression among transplant types

Species-specific gene expression observations for *S. typhle* uncovered a number of shared similarities between ALLO/C, AUTO/C and SC/C pairwise comparisons with control replicates (Fig. 4). The transcriptional activator, *irf1*, which is implicated in innate, adaptive immune processing and apoptosis, was one of the most upregulated genes in all three transplant types. Other shared genes exhibiting upregulated expression included *mcoln3, pitpnc1* (autophagy regulation), and the transcription factor *tcf12* (lymphocyte differentiation regulation). Notable genes with immune function that were upregulated in ALLO and AUTO tissue compared with C include *tyrobp, clec4d* (innate immunity) and *havcr1* (T-cell activation). In addition, genes involved in vascular (*cthrc1*, *mmp19*), tissue (*thbs4b*) and bone (*nfatc1*) remodelling, glucose transport (*slc37a2*), synapse transmission (*ncs1*), development (*sfrp2*, *olfml3a*), cell adhesion (*itgb3*, *itgb8*) and autophagy regulation (*dram1*), while *gzma* was upregulated in both ALLO and SC replicates but not differentially expressed in AUTO.

When compared with C, two protease genes, *anpep* (angiogenesis) and *capn5* (signal transduction) showed downregulated expression in ALLO, AUTO and SC, while *col4a1* (angiogenesis) and *mmp12* (tissue injury and remodelling) exhibited downregulated expression in both ALLO and SC. Shared gene downregulations were exhibited in ALLO and AUTO when compared with C, these included genes with functions in muscle differentiation (*myod2*, *dst*) and cell signalling (*scn8a*, *scn4aa*, *kcna1*). No genes with a prominent immune function were identified.

ALLO/SC and AUTO/SC comparisons, using SC as the comparative control produced a large number of differentially expressed genes in *S. typhle*. Ribosomal processing and translation (*eef1a*, *eef-2*, *rpl3*), NK-cell activation (*tyrobp)*, inflammation (*il-17ra, nlrp12, fstl1*) and tissue remodelling (*mmp2*, *mmp14, tnfrsf11a*) genes, were all upregulated in ALLO and AUTO when compared with SC. Conversely, *polr3e* (innate immunity), *nfil3* (T-cell activity) and *tcim* (apoptosis) were jointly downregulated in ALLO and AUTO samples.

3.3.2. Allografts

A number of upregulated genes with immune function were identified in *S. typhle* ALLO replicates when compared with C including *igfbp3* (growth factor modulator), *ildr2* (T-cell regulation) and the chitinase coding *chia* (adaptive immunity). Incidentally, *chia* was also shown to be upregulated in allografts when compared with the surgical control.

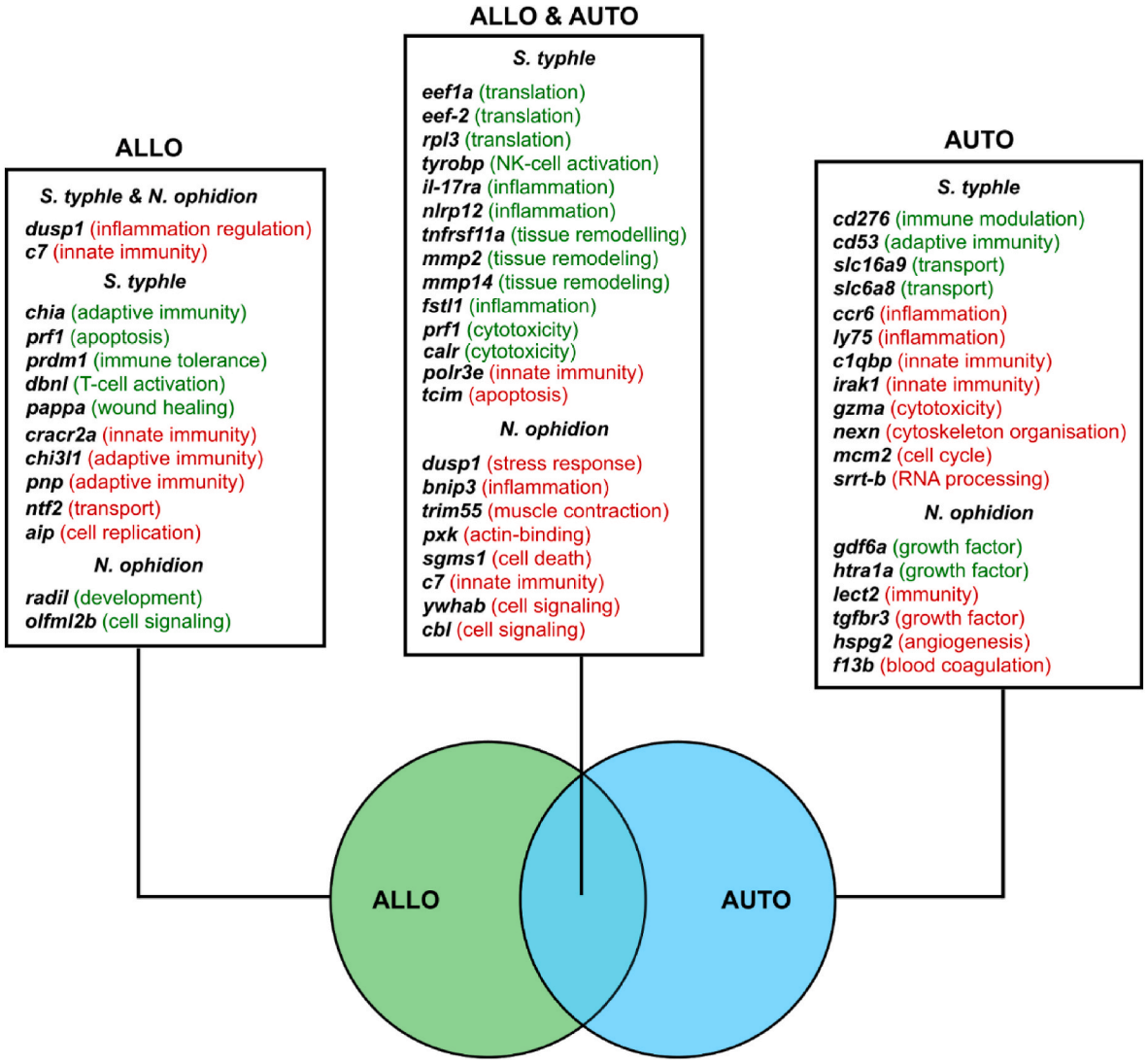

Fig. 5. Schematic diagram showing differentially expressed genes of interest in allograft (ALLO) and autograft (AUTO) replicates. Upregulated (green) and downregulated (red) genes are in relation to surgical control replicates. Genes shown possess roles in immunity and/or have high/low expression levels.

Conversely, a different chitinase (*chi3l1*) with adaptive immune function was shown to be strongly downregulated in ALLO in both C and SC comparisons. Additional immune genes found to be upregulated in ALLO when compared with SC included; *prf1, calr* (cytotoxicity), *prdm1* (immune and tolerance function), *dbnl* (T-cell activation) and *pappa* (wound healing). While allografts exhibited downregulated expression of *cracr2a*, *c7* (innate immunity) and *samsn1* (adaptive immunity regulator), when compared with the surgical control. Among the most downregulated genes in the aforementioned comparison were *dusp1* (inflammation regulation), *ntf2* (transport) and *aip* (cell replication).

Only three annotated genes were found to be differentially expressed in the ALLO/AUTO pairwise comparison, with a single protease (*gzma*) showing an upregulation in ALLO. The other two genes exhibiting downregulated expression in ALLO compared with AUTO include *chi3l1* (adaptive immunity) and the motor protein, *dnah11*.

3.3.3. Autografts

In autografts, *il-17ra* and *il-22ra1* (inflammation) were shown to be upregulated and downregulated, respectively. Functions for other upregulated genes among autograft replicates included transportation (*slc12a9, atp2a1*, *slc26a10*), angiogenesis (*angptl3, pgf, flt1, mmp16*), development (*foxd3-a*, *hoxd12a*) and collagen interaction (*pcolce*). A number of downregulated genes with muscular (*stac3*, *mef2c*, *tpm4*), and erythrocyte/developmental function (*hba1*, *cahz*, *klf1*, *bpgm*), were identified specifically in AUTO compared with SC. The AUTO/SC pairwise comparison highlighted number of immune genes, namely the immune regulator *cd276* and surface protein *cd53* (adaptive immunity), which were both upregulated in AUTO, while inflammation implicated (*ccr6, ly75*), innate immunity (*c1qbp, irak1*) and cytotoxic (*gzma*) related genes, showed the opposite expression direction. The cytolytic *prf1*, which was similarly upregulated in allografts in the ALLO/SC comparison, also exhibited upregulated expression in autografts in AUTO/SC. Highly downregulated expression of *nexn* (cytoskeleton organisation), *mcm2* (cell cycle) and *srrt-b* (RNA processing) was found in AUTO compared with SC, while upregulated expression was exhibited by the solute-carriers *slc16a9* and *slc6a8*.

3.3.4. Surgical controls

The amount of differentially expressed genes in the SC/C pairwise comparison exceeded those in both ALLO/C and AUTO/C. Notably, a number of genes involved in ribosomal processing were shown to be upregulated (*bop1*, *wdr12*, and *ebna1bp2*) and downregulated (*rpl21*, *rpl7*, *rpl12*, *eif5a*) in surgical control tissue compared with control replicates, while a couple of apoptotic genes (*mcl1*, *tnfrsf11b*) also showed positive upregulation. As with allograft and autograft tissue there was an upregulation of immune genes such as *il10* (anti-inflammation) and *il1r2* (innate immunity). Equally, other immune genes, which were predominately related to innate immune defences, were shown to be downregulated in SC replicates compared with C, including; *gimap8* (anti-apoptosis), *zc3h12a* (inflammation), *il20rb* (immune modulation) and *nlrp12* (anti-inflammation). The only antigen processing and presentation related genes to be differentially expressed in *S. typhle* were *h2-k1* (MHC I), *canx* (MHC I chaperone) and *cd48* (adaptive immunity), which were all downregulated in SC in comparison with C. In contrast to ALLO, the chitinase *chia* (adaptive immunity) was downregulated in SC when compared with C.

3.4. Nerophis ophidion - species-specific gene expression

3.4.1. Shared gene expression among transplant types

The T-cell extravasation implicated *cd99l2* and angiogenic *epas1* exhibited downregulated expression in ALLO, AUTO and SC when compared with C. Between ALLO/C and AUTO/C comparisons in *N. ophidion* a large number of differentially expressed genes shared similar expression patterns. Upregulated in ALLO and AUTO replicates compared to C, were genes with immune functions such as B-cell activation (*cd22*), innate immunity (*ctsk*, *colec12*, *stat2*), angiogenesis (*ecm1*, *fgf2*), anti-angiogenesis (*thb2*) and *prf1* (cytotoxicity), tissue remodelling (*fn1, mmp14*). Conversely, a number of immune genes including *itfg1* (immune modulation), *malt* (T-cell regulation), *zc3h12a* (inflammation), *mavs*, *tmem131* (viral defence), *bcl2*, *trim8 and gramd4* (apoptosis) and were downregulated in ALLO and AUTO with C tissue. Two orthologs of the immune modulator *cd276* were shown to be differentially expressed in *N. ophidion*. One was upregulated in both ALLO and AUTO compared with C, while the other was downregulated in ALLO and AUTO compared with C. In the same comparison, a number of wound healing (*gsn*, *pdgfra*, *thbs1*) related genes were upregulated in ALLO and AUTO tissue, as well as two muscle tissue related genes *tnnt2* (muscle contraction) and *csrp1* (cell migration). Notable genes that shared similar expression in AUTO and SC replicates include *igf2bp1* (RNA binding), which was upregulated compared to C replicates, while the multifunctional *gata2* was downregulated.

Shared genes found to be differentially expressed in the ALLO/SC and AUTO/SC pairwise comparisons utilising SC as a control included *nfil3* (T-cell activity) and *dusp1* (inflammation regulation). Both matched the expression direction of the same genes in *S. typhle*. The gene encoding for the apoptosis-inducing BNIP3, was also shown to be upregulated in both ALLO and AUTO replicates when compared with SC.

3.4.2. Allografts

Genes with immunological function highlighted in ALLO replicates included two involved in T-cell apoptosis (*pnp* and *lgals1*) and one believed to be involved in graft related tissue regeneration (*fbn2*) (Gilpin et al., 2017), both of which were upregulated compared with C tissue. Conversely, in the ALLO/SC comparison a component of the complement innate immune system (c7) was downregulated. Prominent differentially expressed genes with alternative function in the ALLO/SC comparison included *radil* (development) and *olfml2b* (signaling), which were both upregulated in ALLO, while *trim55* (muscle contraction), *pxk* (actin-binding), *sgms1* (apoptosis) *ywhab* and *cbl* (signaling) were conversely downregulated.

3.4.3. Autografts

Overall, in the AUTO/C comparison *N. ophidion* autograft replicates showed a general downregulation of antigen processing and presentation related genes including, *h2-d1, popy class I histocompatibility antigen, b2m*, *tap1*, *cd48* and *cd3e* which incidentally were not downregulated in allograft replicates. Despite these downregulations, *N. ophidion* AUTO replicates also upregulated, compared with C, inflammation (*cmklr1*, *abcc1*, *ccr3*, *ackr3*) and T cell activity (*hlx*) genes. The AUTO/SC pairwise comparison unearthed a few differentially expressed immune genes tasked with roles in neutrophil chemotaxis (*lect2*) and blood coagulation (*f13b*), both of which were upregulated in autografts. Genes with alternative functions such as angiogenesis and endothelial cell migration (*esm1*, *plxnd1*, *thbs4*), and development (*gdf6a*, *dkk3*, *prrx1*) were also upregulated in AUTO tissue compared with C, while the growth factors *gdf6a* and *htra1a* were upregulated in AUTO tissue compared with SC.

3.4.4. Surgical controls

Among the genes exclusively expressed in SC replicates were *apoa1* (cholesterol transport) and *sec14l1* (innate immunity), were standouts concerning upregulated genes with immunological function when compared with the standard control.

4. Discussion

This investigation was the first transcriptome-wide assessment of the immunological capacity of a naturally immunodeficient fish species; utilising allograft and autograft fin-transplants to understand the mechanisms that govern allorecognition, rejection and tissue recovery. Investigating the immunodeficient *S. typhle* provides a fascinating opportunity to understand the impact MHC II loss on self non-self

recognition, as well as the potential to shed new light on compensatory measures involved in pipefish allorecognition. Comparing *S. typhle* with *N. ophidion*, a pipefish with a fully functional MHC II pathway, can provide some crucial evolutionary context for the adaptive immune system rearrangements.

Historically, allogeneic transplantation studies were used to assess histocompatibility and led to the discoveries of the MHC molecules, with experiments carried out on mice (Auchincloss Jr and Winn, 2004; Snell, 1948; Snell and Higgins, 1951), lizards (Cuellar and Smart, 1977; Manríquez-Morán and Méndez-de la Cruz, 2008) and fish (Cardwell et al., 2001) varying in degrees of success. MHC I and MHC II are both recognised mediators of transplant rejection (Ayala García et al., 2012; Snell and Higgins, 1951), however, the comparative importance of MHC I from MHC II in this process is difficult to differentiate. Allograft rejection has been reported previously in fish (Nardi, 1935; Sauter, 1934; Šećerov, 1912), however, a detailed inspection of the underlying molecular pathways in play have not been explored. Visual signs of rejection induced by fin transplants were also documented here, with *N. ophidion* allografts appearing to evoke a more acute rejection response than *S. typhle* (Fig. 2), supporting the first hypothesis of this study **(i)**. The increased mortality among *N. ophidion* allografts compared with *S. typhle* also provides support for this rejection effect.

Following a molecular examination, the role of MHCs in transplant rejection in both species was not as prominent as would have first been thought. Appropriately, MHC II related genes were not detected in *S. typhle* in this study, however, perhaps more surprising was the lack of MHC II expression in *N. ophidion*. MHC II is exclusively expressed on antigen presenting cells such as dendritic cells and mononuclear phagocytes (Stastny et al., 1986). The lack of MHC II expression in *N. ophidion* could be due to a low number of these associated immune cells in the examined tissue. To see if any MHC II expression could be observed elsewhere, *N. ophidion* gill tissue was also examined. Gills are likely to harbour a greater number of antigen-presenting cells, based on their role as an immunologically active mucosal interface between the vascular system and environment. However, the absence of MHC II related gene expression also in gills remains difficult to interpret. As of yet, it is not possible to exclude that the *N. ophidion* MHC II pathway, despite its genomic presence (Roth et al., 2020), might have lost its function. The functional loss of MHC II was recently suggested for seahorses for which sequences of the MHC II pathway remained largely unaffected but a lost exon of the MHC II invariant chain CD74 was observed (Roth et al., 2020). This previous study emphasised the flexibility of the vertebrate immune system and challenged the preconceptions of what immune system requirements are needed to survive. With future work focusing on comparative single-cell expression approaches (Parker et al., 2022b) across syngnathids combined with morphological assessments of immune cells the aim is to shed light on these open questions.

Although a role of MHC II in transplant rejection could not be identified, a number of key indicators shed light on the mechanisms that are active in its place. One such finding is the upregulated expression of *gzma* in *S. typhle* allografts when compared with autografts. *Gzma* codes for the protease granzyme a, an abundant constituent of natural killer cell (NK-cell) and cytotoxic T lymphocyte (CTL) granules (Hayes et al., 1989; Peters et al., 1991; Salcedo et al., 1993), and has been recorded in *S. typhle* immune cells previously (Parker et al., 2022b). Both CTLs and NK-cells to lesser extent are present in acutely rejecting mammalian allografts (Hanson et al., 1988; Trentin et al., 1992), while the expression of *gzma* by tissue graft infiltrating CTLs is associated with, and used as a marker for acute rejection in human kidney transplants (Kummer et al., 1995; Van Ham et al., 2010). The putative activity of $CD8^+$CTLs in allograft tissue could be an indication that allorecognition in *S. typhle* is in part controlled by MHC I, with which CTL non-self recognition is associated. This highlights the importance of MHC I mediated allorecognition in *S. typhle* and perhaps hints towards immunological compensation in the wake of MHC II pathway loss. Further research is certainly required to confirm this postulated role. While upregulated in allografts and autografts, compared with the surgical control, the identification of apoptotic perforin-1 (*prf1*) and its chaperone calreticulin (*clr*) further supports the activity of cytotoxic T-cells in *S. typhle* (*Andrin* et al., *1998*). As with granzyme a, perfornin-1 is a recurring factor highlighted in allograft transplant research (Choy, 2010; Clement et al., 1991; Griffiths et al., 1991) and shown to be highly expressed in mice $CD8^+$ CTLs within pancreatic islet transplants two weeks post-surgery (Baas et al., 2016). Possessing a complete MHC repertoire compared with *S. typhle*, *N. ophidion* was expected to exhibit a more elevated allograft rejection response following transcriptome assessment. This rejection trend in allografts specifically, was not distinct between the species based on the transcripts examined, and therefore support could not be given to the second hypothesis of this study **(ii)**.

In addition to their role in antigen presentation MHC are charged with maintaining immunological tolerance (Von Boehmer and Kisielow, 1990). Brood pouch related immune modulation has been exhibited in syngnathids previously through the downregulation of MHC I (Parker et al., 2022a; Roth et al., 2020). Expression findings here in autograft replicates in both species also appear to have adopted similar mechanisms with regards to the suppression of the MHC I pathway. The downregulation of the aforementioned *gzma* in autografts compared with surgical control (*S. typhle*) contrasts its upregulated role in allogenic transplant rejection. While the downregulation of a number of MHC I related genes (*h2-d1*, *popy c l*, *tap1*) in *N. ophidion* autografts could confirm a transplant and host tissue cell recognition of self, resulting in a downregulation to avoid an autoimmune response. This immune modulation provides support to the third hypothesis of this investigation **(iii)**. Moreover, this MHC downregulation and the upregulated involvement of *gzma* in *S. typhle* allograft provides additional support to the fourth hypothesis here, as they both indicate expression disparities between autograft and allografts within species **(iv)**. Disentangling the specific drivers and importance of the MHC in immune modulation and transplant rejection in pipefish is an intriguing challenge. Based on these results it appears that MHC I does participate in these immunological functions, however, additional observations suggest that allogenic rejection may be controlled in part by other distinct immune mediators.

The innate immune system and inflammatory processes are recognised influences involved in transplant rejection (Braza et al., 2016; Mori et al., 2014). These associations were supported by a number of gene expression trends identified in this study. Chitinases in humans are expressed prominently in innate phagocytes (macrophages and neutrophils) at sites of infection, inflammation and tissue repair (van Eijk et al., 2005, 2007). Specifically, chitinases have been associated with M2 macrophages (Hartl et al., 2009), a subtype identified in *S. typhle* previously (Parker et al., 2022b). M2 macrophages possess an anti-inflammatory role involved in wound healing and tissue remodelling (Gordon, 2003; Murray and Wynn, 2011; Xiao et al., 2008). Similar processes may be occurring in *S. typhle*, with the upregulation of *chia* in allografts, however, the converse downregulation of *chi3l1* suggest that these chitinases may simultaneously perform different immunological and tissue regenerative roles upon allograft transplantation (Parker et al., 2022a; Roth et al., 2020; Small et al., 2013). Further in-depth functional analyses should be encouraged to help elucidate the specific function of chitinases within the Syngnathiformes order.

Irf1 has a multi-scale role in the immune system lending assistance to both innate and adaptive branches (Kröger et al., 2002; Wang et al., 2020). The involvement of *Irf1* in apoptotic processes around the incision site (Gao et al., 2010; Kim et al., 2004) was supported by a similar upregulation of *dram1* found here, which is involved in autophagy regulation and defences (van der Vaart et al., 2014; Zhang et al., 2013). *Irf1* is also known to control $CD8^+$ lymphocyte development in mammals (Penninger et al., 1997; Yang et al., 2018), a similar function to that of *tcf12* which is crucial in the differentiation regulation of maturing B-, T- and invariant natural killer T (iNKTs) cells (D'cruz et al., 2010; Emmanuel et al., 2018; In et al., 2017). The importance of apoptosis in

wound healing (Wu and Chen, 2014) explains why *irf1* upregulation in this study is only found in fish which underwent surgery.

Another crucial process involved in wound healing is angiogenesis, which stimulates blood vessel formation providing oxygenated blood for the healing tissue (Li et al., 2013; Rajnoch and Viklický, 2004; Tonnesen et al., 2000). Angiogenesis is also an immune inflammation characteristic with significance in pathologic and transplant related reactions (Ferrara, 2000; Folkman, 1995; Lemstrom et al., 2002). Vascular formation processes appear to be upregulated in both autograft and allograft transplants in both pipefish species, based on the upregulation of genes such as *mmp19*, *angptl3* and *flt1*. In addition, the upregulation of a number of metallopeptidases in this study, known to exhibit elevated expression following tissue injury, are also implicated in muscle and fin regeneration processes (Bai et al., 2005; Bellayr et al., 2009; Lei et al., 2013; Mohammadabadi et al., 2021), supporting the active occurrence of tissue healing in these fishes.

Syngnathids are defined by their unique evolution of male pregnancy (Dawson, 1985; Herald, 1959). Initial proposals suggest that MHC II loss in *S. typhle* may have helped facilitate the evolution of advanced male pregnancy, as a proxy for immunological tolerance (Parker et al., 2022a; Roth et al., 2020). From a conceptual point of view, semi-allogeneic embryos are similar to allograft transplants in that they both possess genetic material from a different individual and should in principle evoke an immune response. This study, however, suggests that immunological tolerance measures evoked during pregnancy and transplantation are fundamentally different and must thus be considered different phenomena. Moreover, the structure and physiology of the two tissue types (surgical area and brooding tissue) are likely to react differently to foreign tissue, therefore could require alternative immune mediation. It is conceivable that male pregnancy evolved as a result of the interaction of functional MHC II loss with additional adaptations that act exclusively during pregnancy or locally within brood pouch and placenta-like system. This is supported by immune response downregulation during early pregnancy in pouched syngnathids (Parker et al., 2022a). Findings here reinforce the complexities encompassing immunological tolerance, how it has evolved in syngnathids and the challenges that accompany it when attempting to assess these modulatory processes.

This investigation utilized novel transplantation techniques in order to explore the workings of complex immunological processes. Establishing viable methodologies in transplantology is incredible challenging, even when considering humans of which applied research holds most focus. These difficulties are exceeded in syngnathids due to the peculiar morphologies, small size and limited understanding of the fish group. Measures were taken here to reduce the effect of external pathogens and additional controls were incorporated to account for inevitable surgical trauma induced immune activity. To reduce harmful bacteria counts protein skimmers, bio-filters and UV filters were all installed in the recovery tank system, while regular water changes were also implemented. To reduce the chances of infection from overfeeding, uneaten food was swiftly removed from each tank to prevent bacterial colonization. It is conceivable that external effectors may in part mask the immune responses induced by the fin transplantation. Therefore, a careful analysis of gene expression patterns and in particular a comparison with the controls was important, before a measured deduction of the immune associated activity was put forward.

The immune processes that facilitate transplant rejection and destruction are highly dynamic. Immune cells that drive rejection impart their influence at various stages during the immune response making the timing of each response difficult to interpret. Skin grafts in mice highlighted the CTL accumulation in the surrounding area and allograft tissue 10 days following surgery (Celli et al., 2011). Despite the sampling taking place in this investigation 4 days later, the congruencies concerning CTL function at this progressive stage of rejection is promising. T-cell infection response and transplant rejection times in fishes have been shown to range from 5 to 20 days (Covacu et al., 2016; Kaastrup et al., 1989; Kallman and Gordon, 1957, 1958; Nakanishi, 1987). These studies give further support to the adopted 14 days sampling time used here, however, it cannot be ruled out that there were immunological factors involved in transplantation that were not detected due to sampling time. Elucidating the temporal dynamics of the rejection process in these fishes would be an interesting proposition, as it could shed additional light on the specific agents and sequence of events that shape an adaptive immune response.

Results from his study could imply a potential role of MHC I in transplant rejection and immunological tolerance in pipefish species, with and without MHC II. MHC I related CTL functions may even partially compensate for the loss of MHC II in *S. typhle*. Beyond the realms of MHC, details of physiological responses presented here involving angiogenesis, tissue regeneration and wound healing, impart compelling insight into how pipefish respond to transplantation and tissue trauma. Overall, this study expands on the growing knowledge concerning the complicated immunological workings and related evolution of the syngnathid lineage, while highlighting the subject of CTL activity as an interesting avenue to explore further in future experimental studies.

Data accessibility

Raw sequencing data can be accessed at NCBI SRA under project PRJNA787800. Transcriptome data available at Figshare at 10.6084/m9.figshare.15190101.

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Author contributions

OR and JP conceived the study. JP collected and processed samples at differing stages. JP and OR analysed the data. JP and OR wrote the manuscript.

Declaration of competing interest

None.

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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.2022.104393.

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