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Immunological tolerance in the evolution of male pregnancy

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Abstract

The unique male pregnancy in pipefishes and seahorses ranges from basic attachment (pouch-less species: *Nerophinae*) of maternal eggs to specialized internal gestation in pouched species (e.g. *Syngnathus* and *Hippocampus*) with many transitions in between. Due to this diversity, male pregnancy offers a unique platform for assessing physiological and molecular adaptations in pregnancy evolution. These insights will contribute to answering long-standing questions of why and how pregnancy evolved convergently in so many vertebrate systems. To understand the molecular congruencies and disparities in male pregnancy evolution, we compared transcriptome-wide differentially expressed genes in four syngnathid species, at four pregnancy stages (nonpregnant, early, late and parturition). Across all species and pregnancy forms, metabolic processes and immune dynamics defined pregnancy stages, especially pouched species shared expression features akin to female pregnancy. The observed downregulation of adaptive immune genes in early-stage pregnancy and its reversed upregulation during late/parturition in pouched species, most notably in *Hippocampus*, combined with directionless expression in the pouch-less species, suggests immune modulation to be restricted to pouched species that evolved placenta-like systems. We propose that increased foeto-paternal intimacy in pouched syngnathids commands immune suppression processes in early gestation, and that the elevated immune response during parturition coincides with pouch opening and reduced progeny reliance. Immune response regulation in pouched species supports the recently described functional MHC II pathway loss as critical in male pregnancy evolution. The independent co-option of similar genes and pathways both in male and female pregnancy highlights immune modulation as crucial for the evolutionary establishment of pregnancy.

KEYWORDS
evolution, immunity, immunological tolerance, male pregnancy, syngnathidae, transcriptomics

1 | INTRODUCTION

Pregnancy encompasses zygote implantation, embryonic retention, growth, and ends with the release of offspring at parturition. Viviparity has evolved independently more than 150 times across vertebrate species, with phylogenetic studies supporting the notion that gestation holds oviparous roots (Blackburn, 2015; Dudley et al., 2021). A culmination of morphological and physiological innovations that provide progeny with nutrient support, gas exchange and osmoregulation have evolved to shape this

complex reproductive strategy (Bainbridge, 2014; Gittleman, 1981; Wourms, 1981). Viviparity therefore enables species to provide protection from environmental threats and facilitates the production of larger offspring with higher survival rates compared to oviparous species.

One of pregnancy's evolutionary quandaries concerns the conflict between avoiding embryonic rejection through immunological tolerance, whilst also maintaining the parent's immunological vigilance towards invading pathogens (La Rocca et al., 2014). In vertebrates, the capacity to determine self from nonself can be partly attributed to the diverse set of major histocompatibility complex (MHC) genes (Edwards & Hedrick, 1998; Ljunggren & Kärre, 1990). The implantation of the semi-allogeneic foetus into the maternal uterine wall without rejection in mammals seemingly contradicts the laws of transplantation proposed by Medawar (1953). However, mammals and reptiles have evolved a number of mechanisms to circumvent embryonic rejection. MHC II and its coactivator (CIITA) expression are absent in invading embryonic trophoblast cells in mammals (Von Boehmer & Kisielow, 1990; Murphy & Tomasi, 1998), while MHC I is downregulated and nonclassical MHC I upregulated to help modulate the first immune interactions between foetus and mother (Hiby et al., 2004; Murphy et al., 2009). Mammalian regulatory T cells and natural killer cells control immune responses across the foetoplacental bridge maintaining self-tolerance (Ernerudh et al., 2011; La Rocca et al., 2014; Svensson-Arvelund et al., 2015), while early inflammation facilitates implantation and reduced inflammation thereafter assists with pregnancy maintenance (Chavan et al., 2017; Mor et al., 2011).

Another key adaptation in the evolution of advanced pregnancy is the maternal placental link to the embryo's metabolism, which allows for nutritional provisioning of the offspring by the mother (Bell & Ehrhardt, 2002; Garnica & Chan, 1996). Maternal metabolic processes that fluctuate during pregnancy support the stage-specific growth demands imposed by the embryo and help maintain maternal homeostasis (Alvarez et al., 1996; Denne et al.,1991; King, 2000; Naismith & Morgan, 1976). Metabolic rate fluctuations during pregnancy have been reported in lizards (Beuchat & Vleck, 1990; DeMarco, 1993), mammals (Lain & Catalano, 2007; Reynolds et al., 1986; Zeng et al., 2017) and fish (Masonjones, 2001). In humans, a maternal shift from an anabolic to catabolic state towards the end of gestation helps support the physiological demands of the offspring (Herrera, 2002; Lain & Catalano, 2007).

The majority of pregnancy research focuses on human and mouse systems; however, as pregnancy is a synapomorphy in mammals, gaining insights into the evolutionary mechanisms that shaped phenotypic differentiation is challenging. To this end, comparative studies of phylogenetic alternatives outside of the mammalian lineage are required to develop a better understanding of how and why pregnancy evolves, and to compare the genetic intricacies and adaptations that helped shape this reproductive process as a whole.

Conceivably, the most enigmatic pregnancy form can be attributed to syngnathids (seahorses, pipefishes, pipehorses and seadragons; family Syngnathidae), which have evolved unique male pregnancy (Dawson, 1985; Herald, 1959). Pregnant males carry the fertilized eggs for the duration of embryonic development, utilizing specialized skin patches located on either their trunk or tail, which can feature intricate skin extensions or pouches. Consequently, brooding type and brood pouch morphology are highly diverse within the lineage. Among these brooding forms is *Hippocampus's* marsupium-like pouch, possessing muscle operated opening and closing capabilities, and the less derived *Syngnathus*' pouch, which varies in prominence and is formed of muscular skin flaps that cover the fertilized eggs (Carcupino et al., 2002; Ripley et al., 2010; Wilson et al., 2001). Nerophinae, however, develop integument tissue, which swells in order to help hold and partially immerse the eggs (Carcupino et al., 2002) (Figure 1).

Syngnathid research has highlighted genetic and morphological congruencies found between male and eutherian mammal reproductive systems (Roth et al., 2020; Small et al., 2013; Stölting & Wilson, 2007; Whittington et al., 2015), with special interest being funnelled into immunological and nutrient transfer processes (Beemelmanns et al., 2019; Beemelmanns & Roth, 2017; Ripley & Foran, 2009; Roth et al., 2012, 2020; Skalkos et al., 2020). Similarly,

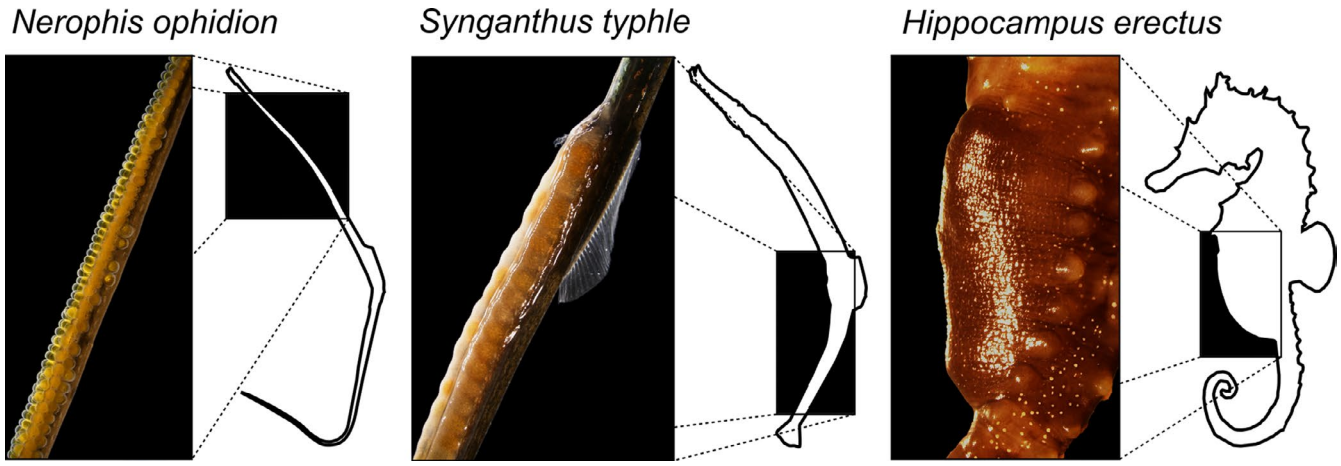

FIGURE 1 Syngnathid brooding types: *Nerophis ophidion* (external egg-gluing/pregnancy), *Syngnathus typhle* (inverted brood pouch) and *Hippocampus erectus* (sealed brood pouch)

several studies have characterised brood pouch tissue gene expression at multiple pregnancy stages in *Hippocampus abdominalis* (Lin et al., 2017; Whittington et al., 2015) and more recently two *Syngnathus* species (Keller & Roth, 2020; Roth et al., 2020; Small et al., 2013), showing transcriptional changes in pathway processes such as tissue remodelling, nutrient transport and immunity. The functional absence of MHC II pathway components in some pouch bearing syngnathids (*Syngnathus* and *Hippocampus* (Haase et al., 2013; Luo et al., 2016; Roth et al., 2020) has stimulated discussions about the loss's potential immune modulatory role in the evolution of advanced paternal pregnancy. The close phylogenetic relationships of syngnathid fishes renders them conducive to as yet lacking comparative multispecies molecular studies. In particular, this group provides an excellent platform to investigate pregnancy related evolutionary traits, both morphological and molecular, as it possesses both basal (*Nerophinae*) and highly specialised brooding forms (*Hippocampinae*) with several transitions (*Syngnathus*).

This study aimed to build on previous single species/brooding type transcriptome analyses of syngnathids, by extending gene expression profile analyses of brooding tissue to four syngnathids on a positive investment gradient: *Nerophis ophidion* (external egg gluing), *Syngnathus rostellatus* and *Syngnathus typhle* (inverted brood pouch) and *Hippocampus erectus* (sealed brood pouch). Gene expression profiles were assessed throughout the gestation period, by subdividing the term into nonpregnant, early, late and parturition stages. We expected specific pathways and genes to be activated in the corresponding pregnancy stages in each species and thus hypothesized that (i) replicates will cluster into their respective stages, within species, based on their gene expression profiles. Second, (ii) we expected similar pathways and genes to be differentially expressed in the corresponding pregnancy stage across species. However, as immunological tolerance is supposed to be of main relevance in the pouched species with intimate contact from fathers to embryos, we proposed that (iii) the immunological influence during pregnancy could be documented in pouched syngnathids but remained absent in the less intimate external brooders. Specifically, following inferences regarding immune modulation during pregnancy, we hypothesized that (iv) syngnathids exhibit pronounced immune suppression/modulatory activity in brooding tissue during early gestation that are distinct from late gestation. Lastly, we proposed that (v) homologous female pregnancy-related genes and pathways known from mammals were expressed in syngnathid paternal brooding tissue, while alternative pathways specific to male pregnancy can be observed additionally.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Work was carried out in accordance with German animal welfare law and with the ethical approval given by the Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Ditgitalisierung (MELUND) Schleswig-Holstein (permit no. V242-57983/2018). No wild endangered species were used in this investigation.

2.2 | Fish

Captive-bred *H. erectus* were acquired through aquarium breeders (Seepferdchen24 Meeresaquaristik GmbH, Ottersberg) and bred in our laboratories for several generations prior to the experiment, laboratory-bred pipefish (*S. typhle*, *S. rostellatus and N. ophidion*) were reared in the aquaria facilities at GEOMAR in Kiel until they were reproductively active. Seahorses were kept at 25°C matching conditions in Qin et al. (2020) while pipefish were kept at 18°C replicating conditions used by Beemelmanns and Roth (2016). All fish were kept in species-specific breeding groups in 100 L tanks and fed live and frozen mysids twice a day except *N. ophidion*, which was fed live *Artemia salina*. Careful reproduction assessments were carried out in order to separate pregnant individuals into single-sex tanks to avoid additional mating of polygamous species.

2.3 | Tissue sampling

Four pregnancy stages were targeted in this investigation: Nonpregnant, early and late pregnancy, and parturition (Figure 2). Using in-laboratory embryo staging tables, embryos were classified as early if eye pigmentation was not yet visible, which aligns roughly to <6 (*H. erectus*), <8 days (*Syngnathus*), <12 days (*N. ophidion*), following fertilization. Conversely, late staged individuals were classified as those with defined, fully pigmented eyes, corresponding to the latter half of development. Pregnant individuals were euthanized with an overdose of MS-222 (500 mg/l; Sigma-Aldrich). Six individuals per pregnancy stage for four syngnathid species of different brooding type were sampled: *N. ophidion* (external), *S. typhle* and *S. rostellatus* (inverted brood pouch) and *H. erectus* (sealed brood pouch). Only inner pouch-lining tissue (*Hippocampinae*), internal flap and pouch tissue (*Syngnathus*) and adhesive skin tissue (*Nerophinae*) were dissected to minimise the influence of unwanted cell types. Optimal pouch fleshiness for dissection was represented in parturition pouch tissue compared to the nonpregnant stage tissue, especially in the case of *N. ophidion*. Due to this imbalance, pouch tissue of parturition replicates was used as the baseline gene expression control in this study. Tissue was preserved immediately in RNAlater following dissection and kept at 4°C for one week, before transferring all samples to −20°C for long-term storage. Further details on tissue sampling methodology can be accessed here (Supporting Information S1).

2.4 | RNA extraction, library synthesis, sequencing, de novo assembly and transcript abundance

Following RNA extraction and quality checks, library preparation was conducted using Illumina TruSeq stranded kit. Paired-end

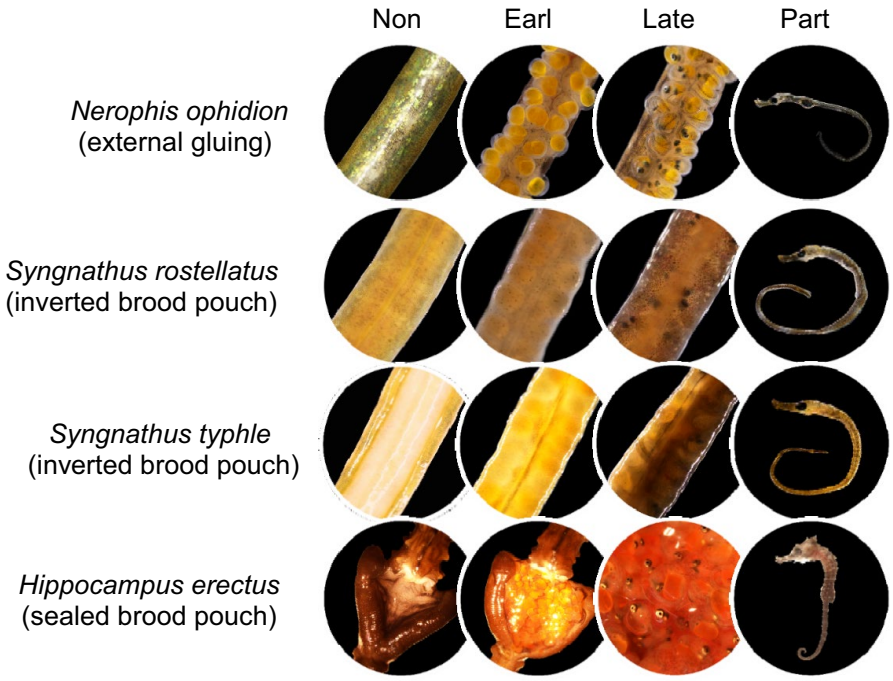

FIGURE 2 Visualisation of the different syngnathid brooding types used in this investigation and pregnancy stages at which brooding tissue was extracted. Gestation lengths: *Nerophis ophidion* (24–28 days), *Syngnathus* (26–30 days) and *Hippocampus erectus* (14–16 days)

sequencing (Illumina, HiSeq-4000, 150 bp reads) was carried out at Norwegian Sequencing Centre (NSC; https://www.sequencing.uio.no), University of Oslo, Norway. Resultant reads were quality checked and trimmed prior to analyses (Andrews, 2010; Krueger, 2015).

The Trinity package (v2.8.1) (Bryant et al., 2017; Haas et al., 2013) was used for transcriptome assembly, following read correction (Song & Florea, 2015) and normalization (Wedemeyer et al., 2017). Transcript abundance was determined for each species by aligning reads to their respective transcriptomes using RSEM (v1.3.3) (Langmead & Salzberg, 2012; Li & Dewey, 2011). Abundance estimates were transferred then (tximport (v1.18.0) (Soneson et al., 2015) for gene expression analyses. Detailed extraction, de novo assembly and raw read processing information is accessible here (Supporting Information S1).

2.5 | Multispecies orthologue comparisons

Transcriptome assemblies underwent gene orthology analysis (OrthoFinder; v2.4.0) (Emms & Kelly, 2015, 2019), utilizing 11 fish species as references (including the four used in this study) (Table S1). Multiple sequence alignment was achieved using DIAMOND (v0.9.21) (Buchfink et al., 2015) and MAFFT (v7.475) (Katoh & Standley, 2013), and subsequent tree inference using FastTree (v2.1.10) (Price et al.,2010). Trinity gene identifiers and their associated gene and GO annotations were matched to each of the corresponding orthologues. To compare expression of each orthologue between stages and across species, transcripts per million (TPM) values for each orthologue were extracted, standardized to account for small differences in replicate TPM sum totals and log(n+1) transformed. Multiple copies of the same orthologue were tallied prior to standardization and transformation.

2.6 | Orthologue comparison: Statistical analysis

Principal component analysis (PCA) was carried out first on orthologues from within species to help determine if expression values were conducive to stage-specific clustering. Multivariate analysis of variance (MANOVA) was carried out on individual species principal component scores (PCs; used: PC1-PC8) with stage as a predictor variable to identify which PCs in particular reflected stage differences. Analysis of variance (ANOVA) was carried out on the scores of the most influential PCs according to MANOVA analyses, followed by post-hoc Tukey's tests to highlight exact significant stage differences in R (v4) (R Development Core Team, 2013). PCs selected for visual representation exhibited the greatest overall stage difference significance (MANOVA; $p < .05$).

For the combined species comparison, gene-wise linear models were produced to account for species as the first dominant clustering factor and accordingly only resulting residuals were utilised for PCA, to facilitate stage-specific separation of replicates. Subsequent MANOVA of residuals of all stage replicates, with stage as a predictor variable was conducted on all species replicates combined. Then as with the single species approach, ANOVA was used on the most influential PCs and post-hoc Tukey's tests were carried out to pinpoint precise stage difference significances. Lastly, the pouched species comparison (*S. typhle*, *S. rostellatus* and *H. erectus*) was performed using the same procedure as the all species data set, except all *N. ophidion* replicates were removed prior to PCA.

2.7 | Orthologue comparison: Functional group enrichment analysis

Gene ontology (GO) functional group annotation analyses were carried out on the top 675 most influential orthologues, using DAVID (v6.8) (Sherman & Lempicki, 2009) with *D. rerio* and human as a background. GO_FAT biological process functional groups were established by collating genes possessing similar functional roles. DAVID's high stringency setting was used, with each functional group given an enrichment score and FDR-corrected value to account for multiple hypothesis testing. The most influential orthologues for *N. ophidion* were also subjected to GO functional enrichment analysis for comparative purposes only.

2.8 | Species pairwise differential gene expression analysis

Mean variance stabilizing transformation (VST) was carried out on all remaining counts for each species individually, proceeded by principal component analysis (PCAs) and uniform manifold approximation projection UMAP (McInnes et al., 2018) to help expose potential outliers. Gene expression was analysed with DESeq2 (v.1.22.2) (Love et al., 2014) in R (v4) (R Development Core Team, 2013). Differential gene expression analysis was carried out using the following pregnancy stage pairwise comparisons: Nonpregnant versus parturition, early vs. parturition, and late vs. parturition. Resulting *p*-values were corrected for multiple testing using the Benjamini and Hochberg method (Benjamini & Hochberg, 1995). Only genes with an adjusted *p*-value < .05 and an absolute log-2fold change expression of >1 were considered for downstream analyses.

2.9 | Smooth clustering (mFuzz)

Smooth clustering was carried out on each species individually utilising the count data set as its basis for analysis. Mean expression levels per pregnancy stage were clustered using the mFuzz soft-clustering algorithm (v3.12) (Futschik & Carlisle, 2005). Gene expression values were z-score normalized (mean = 0; standard deviation = 1) and the mFuzz "mestimate" function was utilized to provide optimal fuzziness values for each species: (2.56) *H. erectus*, (2.72) *S. rostellatus*, (2.51) *N. ophidion* and (3.75) *S. typhle*. Genes were grouped into clusters based on expression similarities and assigned a cluster membership value between 0 (low) and 1 (high), corresponding to their cluster membership credibility. The optimum number of clusters was determined in a way that reduced the chance of two clusters sharing similar expression profiles and helping to ensure the most distinct clusters possible for each gene set. Genes with membership values >0.4 were used to characterize each cluster and carried forward for enrichment and GO analysis.

2.10 | Gene ontology and functional group enrichment analysis (mFuzz)

Clustered gene groups were subjected to two different GO characterisations to help elucidate general and more distinct functional groups. Firstly, all annotated genes were run through the GO Slim Term Mapper (Accessed May 2020) (Boyle et al., 2004; Mungal, 2003) and binned into broad functional groups and percentage representations for each group were reported. Secondly, cluster assigned genes were fed into the database for annotation, visualization and integrated discovery database (DAVID; v6.8) (Sherman & Lempicki, 2009) for GO_FAT functional annotation. Clusters with >25 genes were used to improve the robustness of the analysis. Due to gene numbers, human annotation background was utilised because of its superior gene recognition success rate, allowing for sufficient gene carryover for meaningful analysis. GO functional clustering was set to "high" stringency and each group was assigned a functional category, enrichment score, and FDR value.

3 | RESULTS

3.1 | Orthologue species comparisons

RNA-Seq produced a total of 140–190 M paired end reads per pregnancy stage with an average of 25.8 M per sample. An initial 7256 orthologue groups were recognised across all four syngnathid species. Scaled transcripts per million (TPM) values were used for the orthologues expressed during all pregnancy stages across the species used in this study. Only orthologues that were expressed in at least three replicates in all species were retained, resulting in 6751 orthologues for downstream analyses. One nonpregnant *S. rostellatus* individual was excluded from all across-species analyses, due to tissue type inconsistencies (Figure S1).

3.2 | Principal component analyses

Within-species principal component analyses (PCA) demonstrated stage-specific sample clustering (Figure 3). Multivariate analyses of variance (MANOVA) confirmed a significant stage (PCs; used: PC1–PC8) effect for each syngnathid species (Table S2), with *S. typhle* and *N. ophidion* displaying the strongest stage significance. Analysis of variance (ANOVA) and post-hoc Tukey's tests highlight significant stage-specific differences within each PC, for each species (Tables S3, S4). The axes displayed in the species-specific PCA plots were selected based on the ANOVA results.

In *H. erectus* (PC2), parturition replicates were significantly clustered from nonpregnant and early pregnant replicates, while nonpregnant and late pregnant replicates differences were also shown to represent distinct clusters (Figure 3a). Early pregnancy stage replicates were significantly different from both nongravid

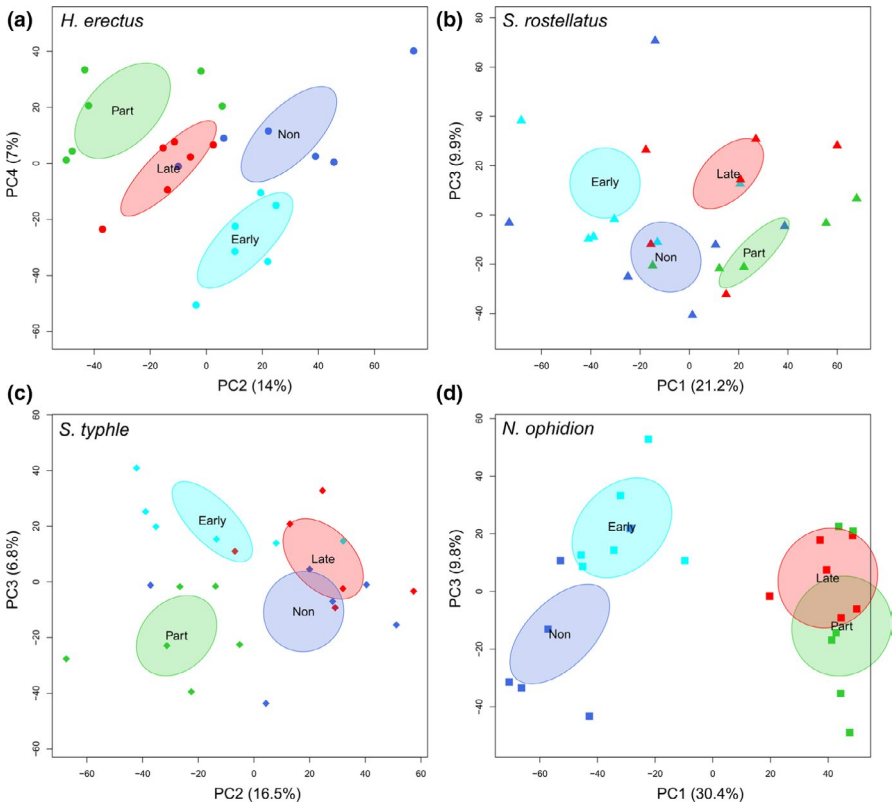

FIGURE 3 Principal component analysis for (a) *Hippocampus erectus*, (b) *Syngnathus rostellatus*, (c) *Syngnathus typhle* and (d) *Nerophis ophidion* replicates based on orthologue loadings. Replicates pregnancy stage identification: nonpregnant (blue), early (cyan), late (red) and parturition (green). Ellipses represent 70% confidence. PCs represent the two most significant PCs for each species, for other comparisons see Supporting Information

stages when considering PC4. For *S. rostellatus*, PC1 exhibited the most significant stage differences, distinguishing early-stage replicates from both late and parturition stage replicates (Figure 3b). The parturition cluster of *S. typhle* was significantly distinct from both gravid stages (early and late) along the PC3 axis, while PC2 separated late and early stage replicates. Parturition stage represented a distinct cluster from late and nonpregnant individuals (Figure 3c). Lastly in *N. ophidion* (PC1), differentiation of samples belonging to the nonpregnant replicates from those associated with early stage was observed, while PC3 exhibited significant differences between early and the two nongravid stages (nonpregnant and parturition) (Figure 3d). Other PCA plot comparisons for each species were also conducted as a reference (Figures S2–S5).

MANOVA on the residuals data set (species effect removed) identified significant stage differences within the first eight principal components (Table S5). PCA plots of the residuals suggested stage-specific separation of replicates (Figure 4a–c). Three PCA comparisons were used to distinguish differences between combined replicate groups; however, it was not possible to convincingly discriminate each pregnancy stage independently. Instead, PC2 (~12.9% variation) and PC4 (~4.6% variation) accounted for the starkest contrast found between the "initial" (nonpregnant and early) and "concluding" (late and parturition) pregnancy stages. This contrast was confirmed to be statistically significant and represented the most distinct stage difference recorded in this study following ANOVA and post-hoc Tukey's testing (Tables S6, S7). PC7 (~2.3% variation), appeared to account for the separation of nongravid (nonpregnant and parturition) and gravid (early and late) replicates. ANOVA and post-hoc Tukey's tests of PC7 yielded supporting significance of this separation (Tables S6, S7).

As with the all species data set, MANOVA of the pouched species data set confirmed significant differences found between stages (PC1:PC8). PC3 and PC4 explained ~6.6% and ~5.5% (Figure 4d) of the variation, respectively, with PC3 separating nonpregnant/early replicates from late/parturition, while PC4 divided gravid (early/late) from nongravid (nonpregnant/parturition) replicates. This trend was also evident along PC5 (~3.7% variation) and PC7 axes (~2.5% variation). ANOVA of each of the most influential principal components positively supported each respective separation trend after post-hoc Tukey's testing. All PC score data used in this investigation can be accessed in Supporting Information S1.

The top 675 highest-ranking (10%) orthologues were either assigned a positive or negative loading value (Table S8). These values can be used to determine how close the expression of an orthologue is associated to a particular gestation stage. Influential orthologues of interest driving the separation of gestation stages were highlighted for their potential roles during pregnancy in other viviparous species (Figure 5). These highlights were confined to the two most significant PCs explaining the highest degree of variation: PC2 and PC4 (all species), and pouched species (PC3 and PC4). All orthologue gene references for this section can be found in Supporting Information (Tables S9–S12).

The "all species" comparison highlighted a number of genes related to various immunological and physiological pathways (Figure 5). Most notably, genes associated with MHC I antigen presentation, angiogenesis and catabolism functions aligned with the concluding stages of gestation. Upregulated pathway components

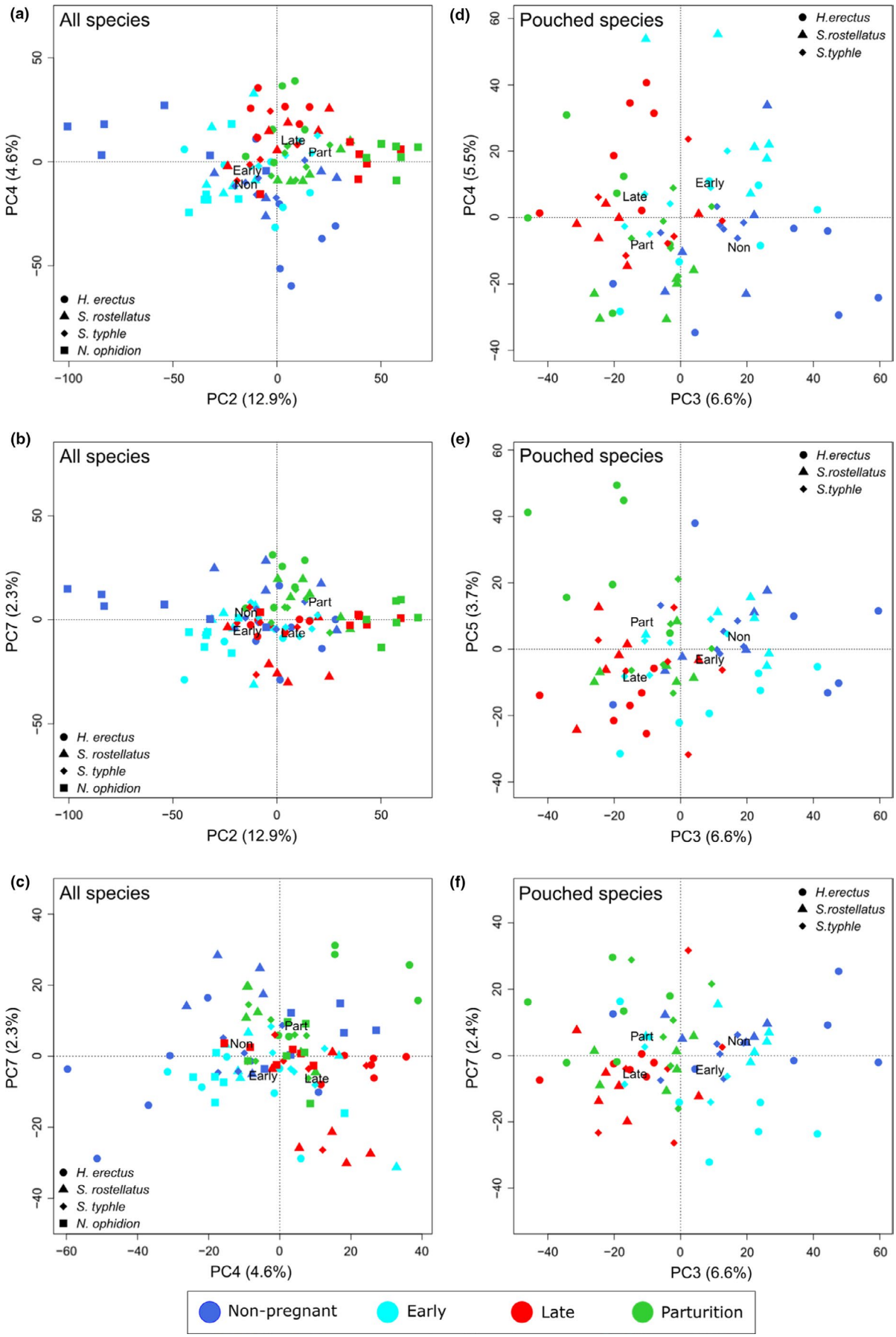

FIGURE 4 PCA component visualizations for all species combined (a–c) and pouched species only (d–f). Stage text labels represent stage means

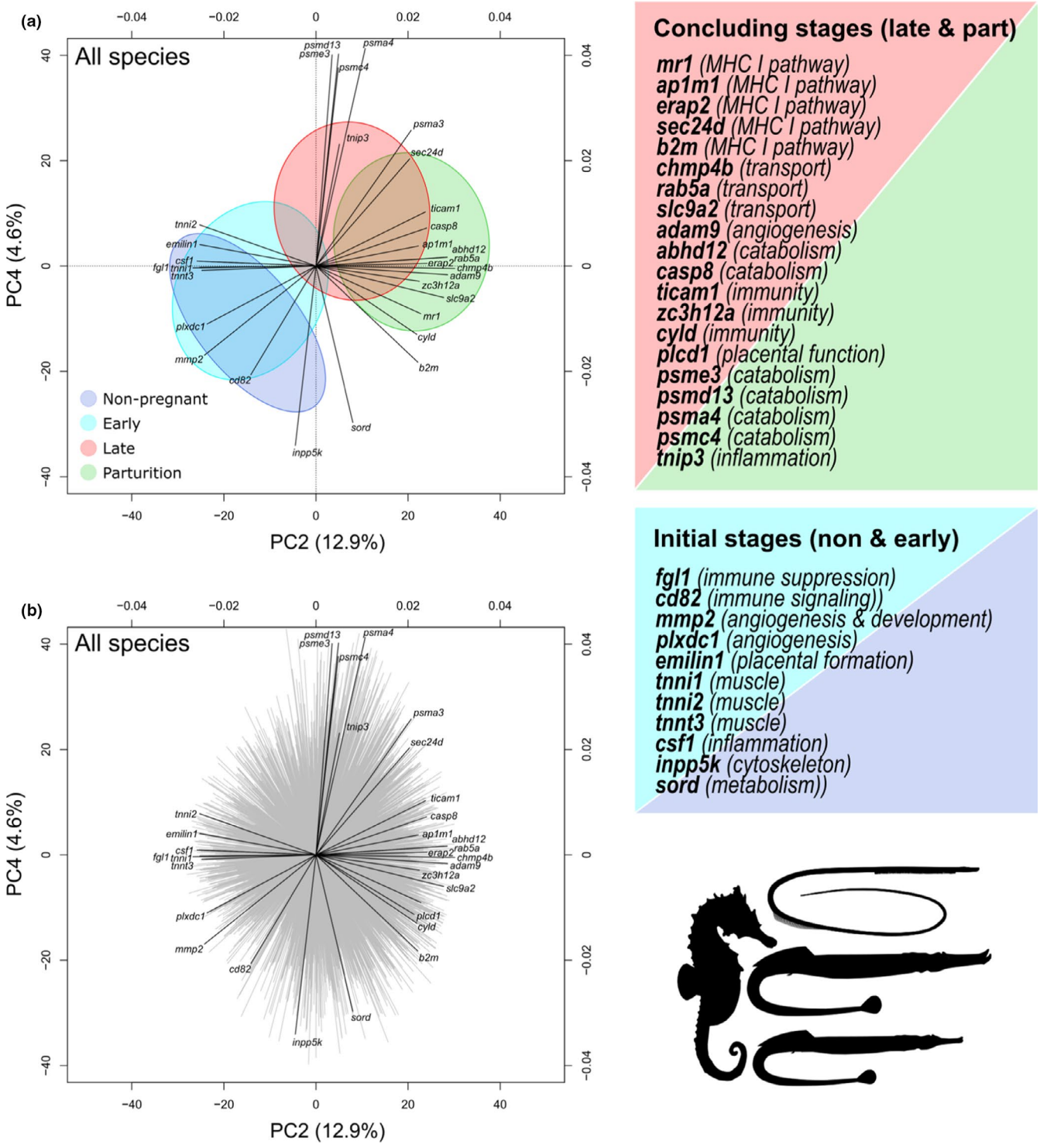

FIGURE 5 (a) PCA component plots for all species data set with genes of interest within the top 675 most influential orthologues and pregnancy stage ellipses. (b) PCA component plots with genes of interest and all other orthologue loadings used in analyses (grey). Ellipses represent 70% confidence

characterising the beginning stages included angiogenesis, immune suppression, and inflammation. The "pouched species" data set similarly expressed a number of MHC related genes in addition to those with alternative immunological function (Figure 6). As with the "all species" data set, pouched species featured upregulated gene expression contributions from angiogenic and inflammatory mediators during the initial gestation stages. Additional orthologue and pathway information highlighted here concerning influential orthologues can be accessed in the supplements (Supporting Information S1).

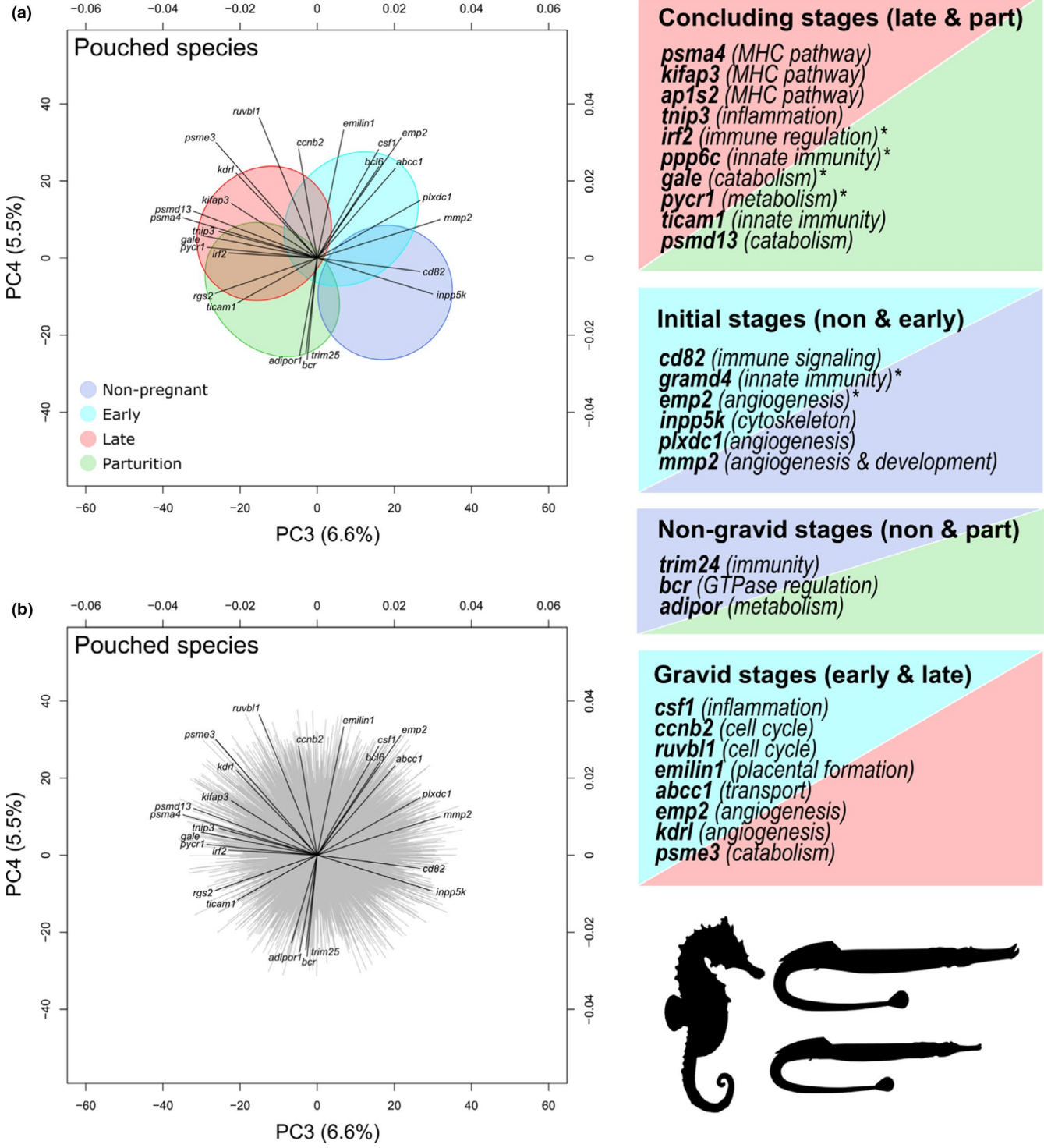

FIGURE 6 (a) PCA component plots for pouched species data set with genes of interest within the top 675 most influential orthologues and pregnancy stage ellipses. (b) PCA component plots with genes of interest and all other orthologue loadings used in analyses (grey). Ellipses represents 70% confidence

3.3 | GO functional annotation

GO functional enrichment analysis of the most influential orthologues highlighted a number of upregulated physiological pathways. When considering the "all species" data set (Tables S13–S16), metabolic processes were shown to retain significance following FDR correction during the initial, nonpregnant/early stages. Additionally, the concluding, late/parturition stages were characterised by enriched catabolic and transport processes. Indications from the "pouched species" data set also shows significant catabolic process enrichment towards the end of gestation, while gravid stages (early/late) are associated with cell division activity (Tables S17–S20).

Human background GO functional enrichment, uncovered similar pathway enrichment findings, but with a higher degree of significance (Tables S21–S28). This background was used to provide a perspective on the orthologue roles within another pregnancy system. The all species analyses highlighted viral and antigen processing activity, as well as catabolic processes during the concluding pregnancy stages, while the initial stages were characterized by muscle related mechanisms. The late/parturition stages in pouched species exhibited a more accentuated enrichment of antigen processing and presentation processes than in the all species comparison in addition to immune regulation processes, whilst also maintaining the catabolic process consistencies found during the pregnancy's culmination. Additionally, the initial stages in pouched species exhibited enrichment of hypoxia related functions. Lastly, cell division processes, as with the all species data set, dominated expression in the gravid pregnancy stages. Influential orthologues characterising the two most significant stage defining PCs (PC1 and PC3) for *Nerophis ophidion* did not highlight any upregulated antigen processing and presentation processes (Tables S29–S32).

3.4 | Differential gene expression of syngnathid brood-pouch tissue

The total number of annotated pairwise-differentially expressed genes for all stage comparisons varied between the species, ranging from 11,914 genes in *N. ophidion*, 2088 genes in *H. erectus*, 3,196 genes in *S. rostellatus* and 1158 genes in *S. typhle* (Figure 7). All gene reference sources referred to in this section can be found in the Supporting Information (Tables S33–S36). Only genes with a log2fold change >1 were considered for discussion. We focused on differentially expressed genes with potential or confirmed roles in pregnancy, immune system function, and reproduction, as well as those will high/low expression levels. These were collated for each species, with prominent components of inflammatory, tissue remodelling and adaptive immune pathway being represented (Figure 8; Supporting Information S1).

All species differentially expressed a number of antigen processing and presentation related genes. An overall downregulation of these genes was exhibited in early pregnancy in pouched fish (Table S37). One exception to this rule was found in *H. erectus and S. typhle*, where *h2-k1* expression was induced. Due to the confirmed loss of MHC II in both *Syngnathus* species (Roth et al., 2020), the upregulated MHC II associated genes (*cd209*, *racgap1*) found here probably represent an alternative function. *N. ophidion* expressed a larger array of MHC I and MHC II related genes in nonpregnant and early pregnancy than the pouched species, and, unlike the pouched species, no clear expression direction was identified. In some cases, multiple histocompatibility antigens were identified, with some exhibiting contradictory expression directions.

3.5 | Pregnancy stage soft-clustering (mFuzz)

Species-specific soft-clustering was carried out on all differentially expressed transcripts from within-species over four stages of male pregnancy of *H. erectus* (1990 genes), *S. rostellatus* (481 genes), *S. typhle* (80 genes) and *N. ophidion* (10,500 genes). Clustering divided

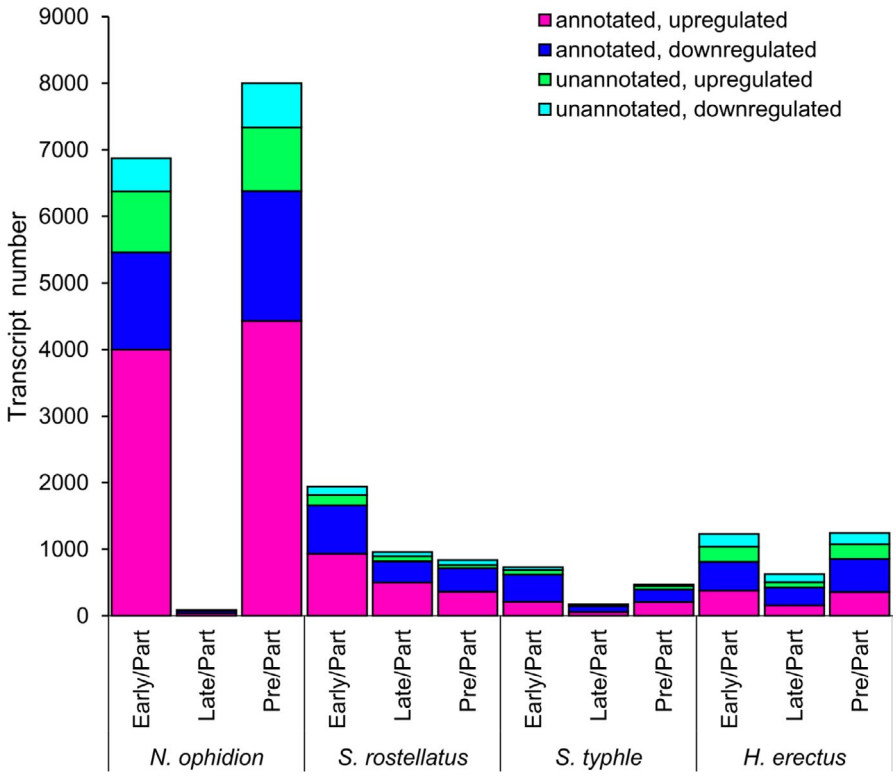

FIGURE 7 Differentially expressed transcript numbers for pregnancy stage pairwise comparisons (up-/downregulated)

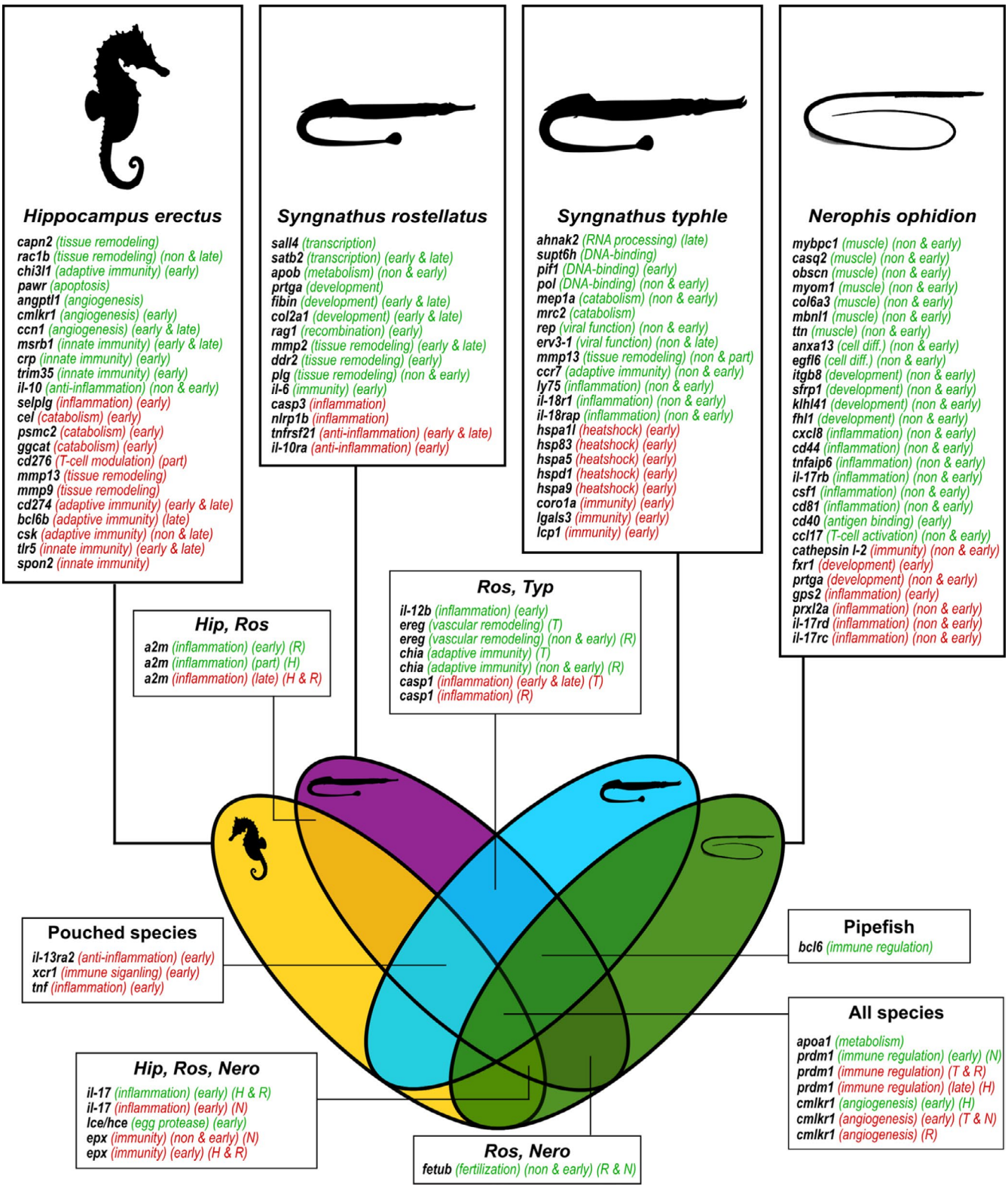

FIGURE 8 Schematic diagram depicting differentially expressed genes of interest in *Hippocampus erectus*, *Syngnathus rostellatus*, *Syngnathus typhle* and *Nerophis ophidion*. Genes highlighted are selected based on high and low expression and/or their roles in immunity and pregnancy. Upregulated genes are represented in green, downregulated genes are represented in red. Genes not labelled with any specific stages are expressed in all stages and letters represent species; *H. erectus* (H), *S. rostellatus* (R), *S. typhle* (T) and *N. ophidion* (N)

genes into groups with similar expression profiles culminating in gestation stage clusters for *H. erectus* (6), *S. rostellatus* (5), *S. typhle* (4) and *N. ophidion* (5) (Figures 9–12) (Table S38). Owing to the differences in total number of expressed genes used, the number of genes per cluster varied among species. Not all species produced a representative cluster for every stage. All analysed species showed a distinct early and nonpregnant stage upregulation cluster, while exclusive late stage clusters were only observed in *H. erectus* and *S. rostellatus*. Some genes formed clusters representative of more than one stage, such as the late/parturition upregulation clusters in *H. erectus* and *N. ophidion*. In addition, while attempting to avoid indistinguishable clusters within species some groupings exhibited striking similarities; clusters in *H. erectus* (3–4) and *N. ophidion* (2–3). No combined early-late cluster was found in any species.

3.6 | GO functional annotation (mFuzz)

Broad GO slim term mapper annotations produced a number of patterns when comparing functions between pregnancy stages (Supporting Information S1 and Table S39).

Functional gene annotations also unearthed a number of pathways specific for each cluster (Tables S40–S42), with only clusters possessing >25 annotated genes being used. The most enriched pathways and their associated genes are reported here, while individual genes with putative functions in pregnancy are also highlighted. The upregulated nonpregnant expression cluster was observed in all species. *S. rostellatus* and *S. typhle* accrued a reduced number of genes compared with the other two species, but notable genes with high membership values included *paqr9* (development; *S. rostellatus*) and *ccdc3* (metabolic regulation; *S. typhle*). *H. erectus* and *N. ophidion* clusters were characterised by genes involved in cell motility regulation (*dpp4*, *ddr2*, *evl*) and MAPK signalling (*pik3cb*, *dusp5*). Similarly, a representative early pregnancy stage cluster was found in all species. *Lgr6* (signalling) and *lrrc17* (development) both possess high membership scores for *S. rostellatus*, while *S. typhle* also highlighted *lgr6* as a prominently expressed gene. However, as with the nonpregnant clusters, a lack of genes prevented enrichment analysis for both species. The upregulated genes shaping the early stage cluster in *H. erectus* encompassed blood circulation (*slc8a2*, *edn2*), epidermal development (*tgm1*, *tgm3*) and muscle function (*cacna1d*), while *serpinb1* (inflammation regulation) and *rhcg* (ammonium transport) possessed some of the highest membership scores in the cluster. For *N. ophidion*, transport (*xpo4*, *slc9a4*, *mfsd10*) was the most prominent process during early pregnancy.

During late pregnancy, seahorse-pouch expression was characterised by cytoskeletal organisation and protein assembly processes (*tpm1*, *arpc5*, *pak3*, *pfn2*), while *tgfb3* (growth factor), *lep* (metabolism) and *cd58* were among the highest-ranking members within the seahorse late pregnancy cluster. *S. rostellatus* produced a late stage cluster, with *fosl2*

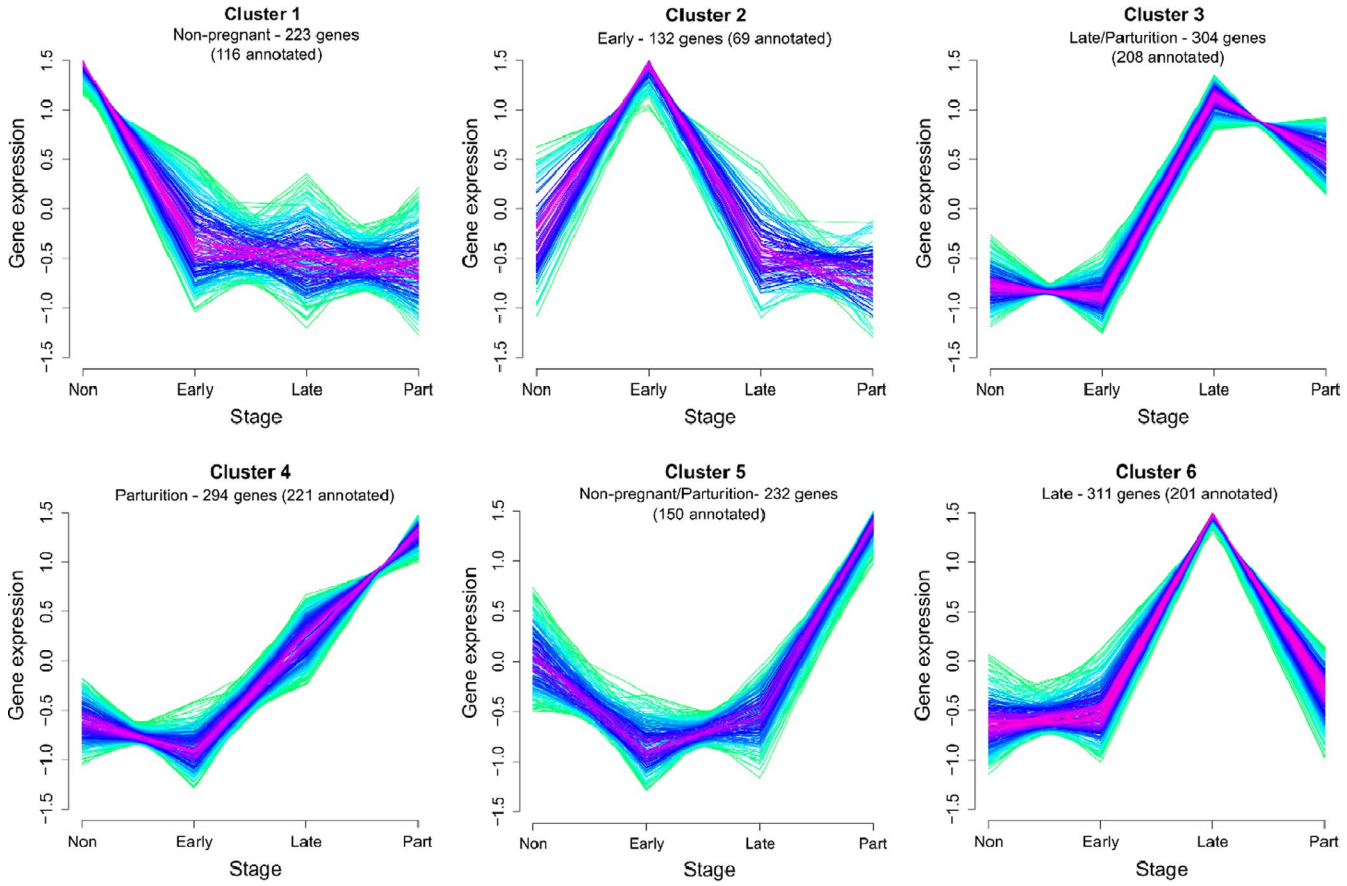

FIGURE 9 Soft-clustering of differentially expressed genes, characterising specific male pregnancy stages in *H. erectus*

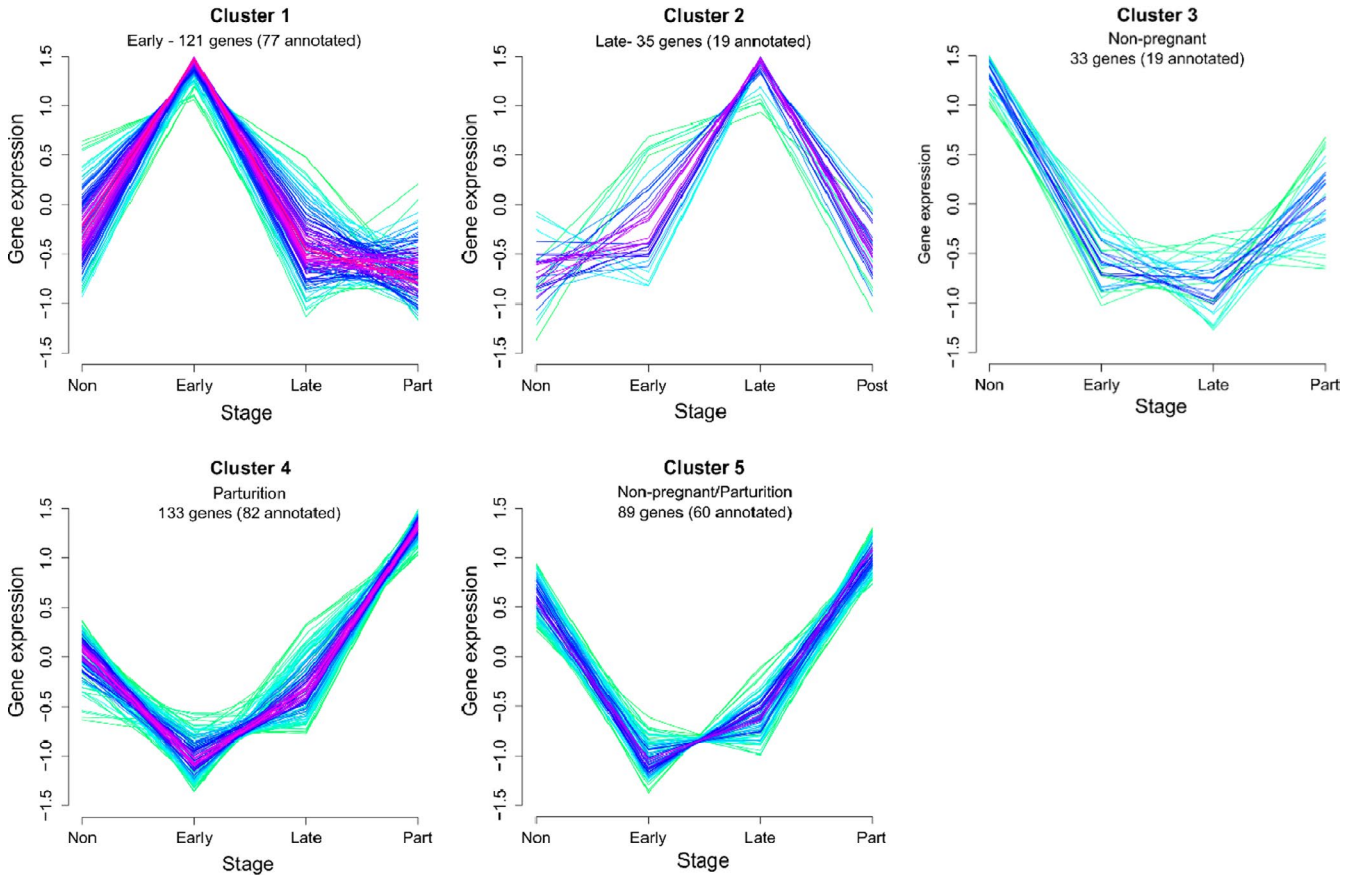

FIGURE 10 Soft-clustering of differentially expressed genes, characterising specific male pregnancy stages in *S. rostellatus*

(transcription), *baiap2l1* (actin-binding) among the genes with the highest membership values.

The parturition cluster identified a strong catabolic process presence in *H. erectus* (*plaa*, *psmd6*, *psma1*, *ubqln4*) as well as a number of immune related pathways in *H. erectus* only (*adam8*, *psmd3*, *psmd1*). The *S. rostellatus* parturition tissue is characterised by membrane remodelling and protein disassembly processes (*ist1*, *chmp1b*, *vps4b*), while *cuzd1* (uterine function) was also shown to have elevated expression during this time. Gene highlights for *S. typhle* following juvenile expulsion include *plpp3* (metabolism) and *tgfb3* (growth).

Key findings regarding dual-stage profiles can be observed in the combined late-part clusters (cluster 3 and 4) for *H. erectus*, where functional groups involved in antigen processing and presentation (*dync1li2*, *canx*, *psma5*, *ap1s2*) and metabolism/catabolism (*psmb3*, *psmc3*, *skp1*, *odc1*) were elevated. The two nonpregnant-early clusters found in *N. ophidion* (cluster 2 and 3), were characterised by parasitic/viral associated (*itga2*, *klc1*, *eif4g1*) and cell migration regulation processes (*ddr2*), while in *S. rostellatus*, the nonpregnant/parturition cluster (cluster 5) highlighted a number of genes involved in metabolic processes (*sgpp2*, *galc*).

4 | DISCUSSION

Female gestation stages are defined by a plethora of intricate physiological and morphological changes that are often shared across the independent events of pregnancy evolution (Bauersachs & Wolf, 2012; Brandley et al., 2012; Soncin et al., 2018). These changes can be assessed by profiling stage-specific gene expression, something that has been carried out in mammals and reptiles previously (Griffith et al., 2013; Helguera et al., 2009; Kim et al., 2015). Comparing the diverse reproductive forms of the Syngnathiformes, which range from oviparity to advanced male pregnancy, can provide insights into the key adaptations of male pregnancy evolution. Moreover, investigating the male pregnancy stage specifics of these fishes can reveal shared or disparate evolutionary traits that also manifested through female pregnancy evolution.

Principal component and time-series soft-clustering analyses highlighted stage-specific clustering in all four syngnathid species assessed giving support for both previous data (Roth et al., 2020; Small et al., 2013; Whittington et al., 2015) and for our first hypothesis (i). The presence of an early soft cluster in all species, is in line with data from mammalian pregnancy, implying that both female and male early pregnancy stimulate unique physiological and morphological changes in the pregnant parent and represent an important yet delicate stage in an embryo's development following fertilization (Weissgerber & Wolfe, 2006). The lack of an upregulated early-late combined cluster suggests that very few genes share a sustained upregulated expression profile throughout the entire pregnancy period. This supports an existing distinction between early and late pregnancy on a transcriptome level and confirms that male gestation is highly dynamic (Roth et al., 2020; Whittington et al., 2015).

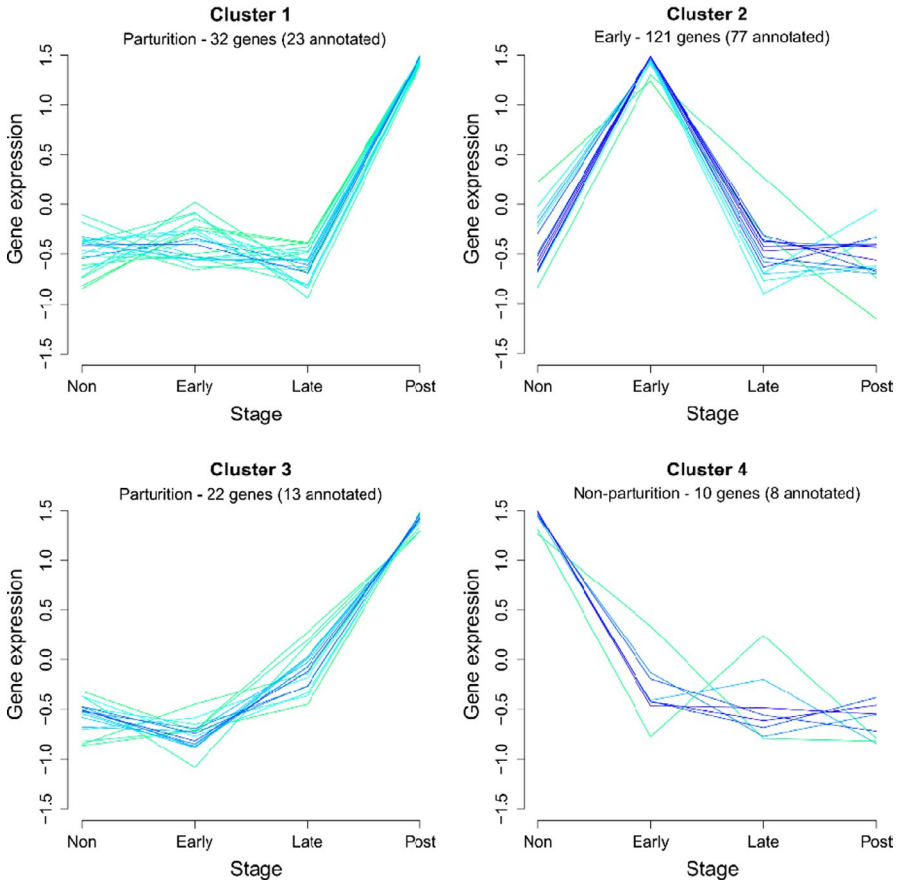

FIGURE 11 Soft-clustering of differentially expressed genes, characterising specific male pregnancy stages in *S. typhle*

In contrast, the unique male pregnancy evolved with a diversity of shared stage-specific expression changes as indicated by combined gene expression profiles of all species. In the combined species analysis the differentiation of initial (nonpregnant/early) versus concluding (late/parturition) pregnancy stages is of particular strength in syngnathid fish. This brings to light shared orthologues and metabolic pathway fluctuations that occur during male gestation, supporting our second hypothesis (ii). Parental metabolism dynamics in mammals during pregnancy shift from a predominantly anabolic state, where nutrient stores are sequestered for the concluding stages, to a catabolic state where nutrient stores are released to facilitate the embryo's steep growth phase (Lain & Catalano, 2007). Shifting metabolic rates in syngnathids have previously been recognised during pregnancy (Whittington et al., 2015; Zhang et al., 2016).

Male pregnancy has evolved on a gradient from oviparity to viviparity surrounding several intermediate forms. As such, syngnathid species have evolved a number of brooding methods, which vary in tissue structure, morphological location and degree of investment (Carcupino et al., 2002; Stölting & Wilson, 2007; Wilson et al., 2001). Nutrient provisioning from father to offspring has been suggested in pouched syngnathid species (Kvarnemo et al., 2011; Ripley & Foran, 2009; Skalkos et al., 2020), while evidence of its presence in the more basal pregnancy forms of the *Nerophinae* are more tenuous (Berglund et al., 1986). Catabolic process upregulation in the concluding stages and the converse downregulation during its initiation periods suggest similar metabolic process fluctuations exist in male pregnancy as in mammals indicating that metabolic processes are a key requirement for the evolution of pregnancy. Catabolic processes were retained in pouched species and in parturition/late stage soft cluster of *H. erectus*. This suggests that metabolic shifts during pregnancy support embryonic growth with nutrients via a placenta-like system, and that this pattern has convergently evolved in diverse forms of male and female pregnancy. Internal pouch tissue expulsion and swift consecutive brooding found in syngnathids (Vincent, 1990; Watanabe et al., 1999) also supports a required upregulation of these processes. Similarly, the retained upregulation of catabolic processes in the basal pregnancy of *N. ophidion* probably aligns with this cyclic process rather than a link to embryonic growth.

Vascularisation and tissue remodelling are crucial prior to and throughout pregnancy in mammals (Read et al., 2007) and pouched syngnathids (Carcupino et al., 1997; Dudley et al., 2021; Laksanawimol et al., 2006), in order to facilitate embryonic growth. This is supported here with the upregulation in pouched species of *emp2* (early) and *kdrl* (late), which have roles in placental vascularisation and parturition processes, respectively, in mice (Williams et al., 2017). Furthermore, *ereg*, which was upregulated during pregnancy in the pouched pipefish species, is attributed with roles during early pregnancy in mice and tissue remodelling, inflammation and angiogenesis regulation in humans (Riese & Cullum, 2014), and could also therefore be important for successful male pregnancy.

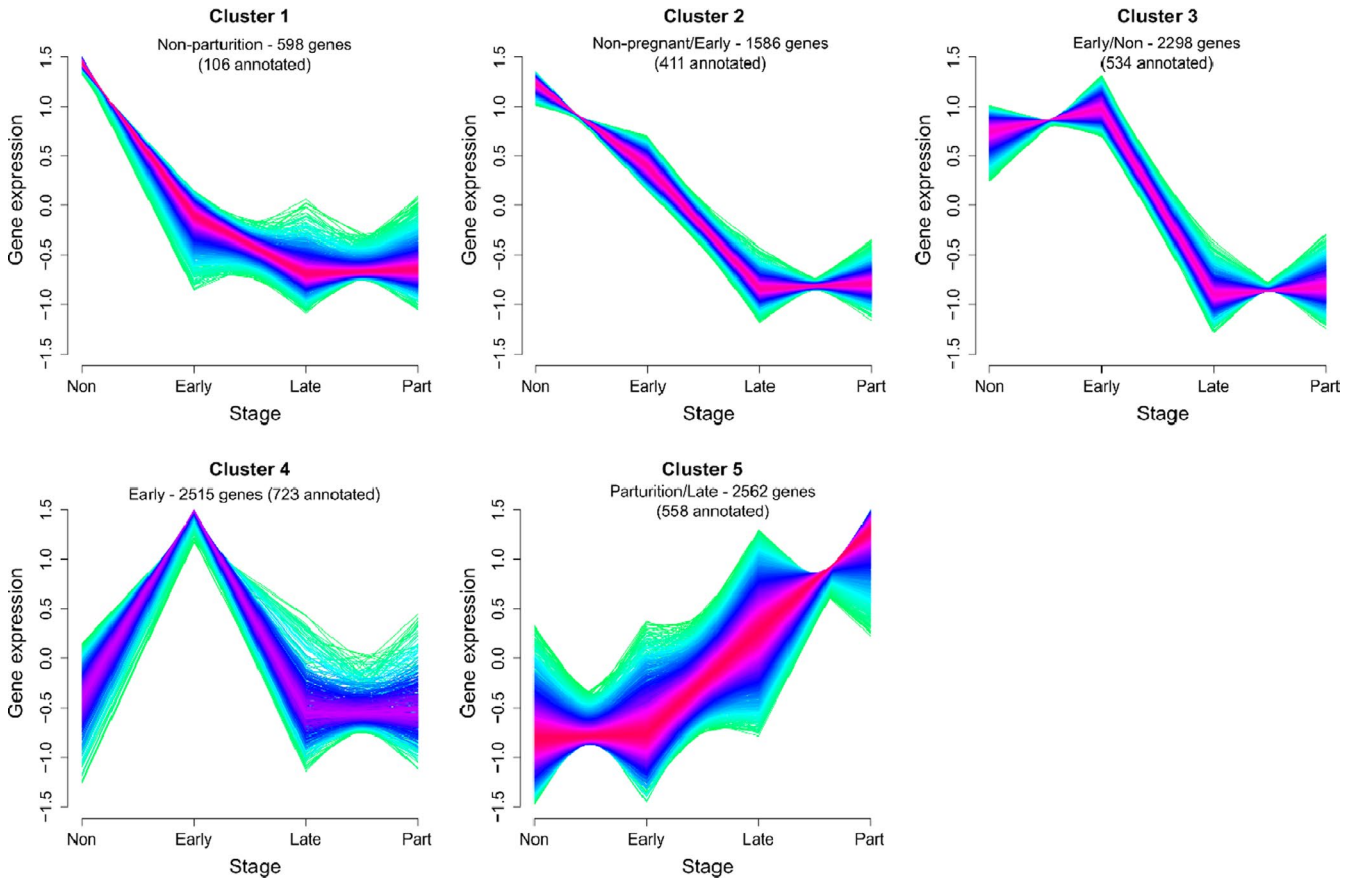

FIGURE 12 Soft-clustering of differentially expressed genes, characterising specific male pregnancy stages in *N. ophidion*

The enrichment of antigen processing and presentation towards the end of male gestation suggests the necessity of a decreased activity of the adaptive immune system during early pregnancy in contrast to the final phase of pregnancy. We could not identify pregnancy-dependent changes in the expression of MHC pathway related genes in nonpouched species (*N. ophidion*), but found a pronounced enrichment of the respective genes in the pouched species. In addition, antigen processing and presentation genes were present in the upregulated pathway of the *H. erectus* late/parturition soft cluster. We thus suggest that downregulation of the immune system during pregnancy and its upregulation towards the end of pregnancy must be specifically linked to the necessary adaptations in the evolution of a male brood pouch. Taken with the downregulated expression of MHC related genes during early pregnancy, and the elevated immune response towards pregnancy's conclusion, could indicate that the intimate patrotrophic dependencies of the progeny are reduced at these later stages, compared with early gestation, and are no longer in direct contact with the paternal immune system.

Reasons for this immune gene upregulation could be in response to the influx of pathogen containing, environmental water which enters the pouch during this time. Bacterial activity and growth is thought to be facilitated in the enclosed brood pouch, in particular during late pregnancy (Wang et al., 2019; Whittington & Friesen, 2020), supported by active pouch defence mechanisms in seahorse pregnancy (Wu et al., 2021; Zhang et al., 2019). Syngnathid larvae are most vulnerable and dependent on paternal support during early pregnancy, while more developed, hatched juveniles are less at risk from environmental seawater (Linton & Soloff, 1964). Observed opening of the seahorse pouch leading up to parturition, could assist the progeny's gradual acclimation to external seawater, encourage initial microbial colonization (Beemelmanns et al., 2019), and explain the immune process upregulation. This process may be less distinct in *Syngnathus* as the pouch is not completely sealed (Wilson et al., 2003). Osmotic stability is a key advantage of pregnancy (Carcupino et al., 2002; Watanabe, 1999), the above suggested opening of the sealed seahorse pouch may thus induce osmotic stress for the developing embryos. The enrichment of genes associated with "cellular nitrogen compound metabolism" in the seahorse during these periods may support pouch osmoregulation, however, this requires further experimental corroboration.

Nerophis ophidion is the only species in this study to retain a fully functional set of MHC I and II pathway components (Roth et al., 2020). Unlike in pouched pipefish species (Haresign & Shumway, 1981; Kvarnemo et al., 2011; Ripley & Foran, 2009), there is little evidence suggesting that syngnathids with external pregnancy are physiologically connected to the progeny during gestation (Berglund et al., 1986; Kronester-Frei, 1975). Accordingly, the reduced degree of foeto-paternal intimacy may render immune suppressive measures that are present in pouch bearing syngnathids, immunologically redundant in *N. ophidion*. While in pouched species suppression

of MHC pathway related genes was dominant during early pregnancy, this pattern could not be identified in *N. ophidion*. This lack of expression direction could indicate that the tissue of *N. ophidion* may not be operating with immunological tolerance function that accommodates the progeny, as deduced in pouched individuals. Moreover, the similar expression of many of these MHC genes with nonpregnant *N. ophidion* replicates, also infers modulatory mechanisms are not in play. As a result, the third hypothesis of this study which predicted immunological distinctions between pouched and nonpouched species, was supported (iii), while other physiological indications found here also suggest disparities do exist between the pouch-brooding forms. To confirm these immunological differences the next step would be to carry out specific functional experimentation to further clarify if nonpouched syngnathids implement immune modulation measures during pregnancy.

The placental-uterine immunological environment during pregnancy is unique in as much as it deals with a conceptual balancing act of required alertness to pathogenic presence and tolerance of semi-allogeneic fetal antigens (La Rocca et al., 2014). Male immune capabilities are higher than in female syngnathids but have been shown to come at a cost during pregnancy (Lin et al., 2016; Roth et al., 2011). Immune system states of pouch bearing syngnathids fluctuate owing to the shifting foeto-paternal demands, and a consensus is growing that immune modulation is present and necessary (Keller & Roth, 2020; Li et al., 2020; Roth et al., 2020; Whittington et al., 2015). These observations are extended here in all pouched species, with many adaptive immune genes charged with tasks in antigen presentation and processing, showing a downregulation predominantly during early gestation (Table S37). (iv) These findings, taken with the upregulation of a few MHC isolates and enrichment of related antigen presentation components representing the late-parturition stages (cluster 5) in *H. erectus*, help support the fourth hypothesis of this study suggesting that generally immune suppression activity appears more pronounced in early pregnancy compared with late/parturition. This implies that while the suppression of allorecognition pathways is important to maintain foeto-paternal tolerance in general, immunological mechanisms during male pregnancy on a stage-specific level are probably more nuanced. In humans, the upregulation of nonclassical MHC I genes surrounding the foeto-maternal interface has been recognised (Rapacz-Leonard et al., 2014), with some being reported in peripheral blood of pregnant women and invading extravillous cytotrophoblasts (Hamai et al., 1999; Kovats et al., 1990). While previous *h2-k1* downregulation during early male pregnancy has been documented (Roth et al., 2020), it is conceivable that its upregulation here in *H. erectus*, may align with functions found in the aforementioned nonclassical MHC I orthologues in humans.

Findings here suggest a connection between brood pouch inflammatory processes and male pregnancy exists in a similar mode to that of mammals. Inflammatory fluctuations are recognised during mammalian pregnancy, driven by hormonal changes and the necessary embryonic demands to ensure successful implantation and parturition (Challis et al., 2009; Hilgers et al., 2021; Mor, 2007; Orsi & Tribe, 2008). Based on findings here, these mechanisms appear to hold true for male pregnancy also, indicating that convergent evolutionary adaptations were adopted to facilitate the key functional phases at pregnancies initiation and conclusion (Supporting Information S1).

The immune regulatory cytokine, IL-10, is known to be an integral mediator and anti-inflammation modulator during mammalian pregnancy (Wu et al., 2001) and has been reported at high levels during early pregnancy (Marzi et al., 1996). Findings here suggest IL-10's anti-inflammatory function may be similarly integral in the seahorse, especially during early pregnancy when inflammatory processes are dominant and require modulation. Incidentally, downregulation of the IL-10 binding *il-10ra* in *S. rostellatus* during early pregnancy could indicate it playing more of a role in advanced pregnancy forms. The converse downregulation of *il-10* in the seahorse, during parturition also supports the prominent proinflammatory response adopted in uterine tissue at that time of pregnancy in mammals (Hansen et al., 2017). Seahorse parturition is a more active process when compared with *Syngnathus* "birthing" and may be more reliant on proinflammatory processes during this time.

The proinflammatory orthologues, *ticam1* and *tnip3*, shared among all species were influential within the concluding pregnancy stage replicates and remained influential following the removal of *N. ophidion*. This may indicate that in pouched species proinflammatory processes help drive parturition.

Immunological tolerance during pregnancy is characterised by a shift from a T-helper type 1 (Th1) to a T-helper type 2 (Th2) cell dominant environment in mammals (Medawar, 1953; Raghupathy et al., 2000; Wegmann et al.,1993). Reports of syngnathid immunological tolerance have been propounded in previous work (Roth et al., 2020) and findings here indicate similar mechanisms. CCR7 plays a role in pregnancy establishment and tolerance (Förster et al., 2008; Teles & Zenclussen, 2013), while its negative inhibitory function of IL-12 production, a cytokine that promotes the activation of Th1 immune responses, could have implications by way of reducing the chances of preeclampsia (Sakai et al., 2002). *Ccr7* was only upregulated in one pouched species here (*S. typhle*), but its expression could be an indication that a Th2 dominant state is advantageous during pouched syngnathid gestation as it is in mammals. Tumour necrosis factor (*tnf*) characterises a Th1 environment, and its downregulation during pregnancy in all pouched species could also signal this shift. Chitin has a stimulatory function within both innate and adaptive immune system, exampling in the promotion of pro-Th2 mediators. In turn, chitinases, such as CHIA, are credited with immune modulatory function and associated with Th2 dominant immune environments in mammals (Cuesta et al., 2003; Komi et al., 2016; Lee et al., 2008, 2011). Roth et al. (2020) highlighted the downregulation of *chia* in developed nonpregnant *Syngnathus* pouch tissue, while Small et al. (2013) reported the chitinase to be significantly upregulated in pouch tissue during pregnancy. Here, prominent chitinase upregulation was also found in *S. typhle* (early & late), *S. rostellatus* (early) and *H. erectus* (early) in the form of *chia* (*Syngnathus*) and *chi3l1* (*Hippocampus*). This could further implicate the observed

shift toward Th2 conditions associated with female gestation. Interestingly, this strong upregulation was only observed in pouched syngnathid species, further implicating a role in syngnathid pouch function and male pregnancy. Importantly, this could be a defining factor when comparing less complex and advanced male pregnancy systems and their potential reliance on paternal immunological modulation. The immune modulator, *cd274*, has been shown to be highly expressed in the mammalian placenta during pregnancy with a role in maintaining immunological tolerance (Guleria et al., 2005). Interestingly, however, further studies have propounded oxygen as a potent modulator of CD274, with trophoblast exposure to high levels of oxygen having a positive effect on *cd274* expression (Holets et al., 2006). The exclusive strong downregulation of *cd274* throughout *H. erectus* pregnancy could indicate a different role in advanced syngnathid pregnancy. Alternatively, perhaps the sealed pouch oxygen levels are restricted, resulting in the downregulation of *cd274*. This raises further questions regarding the permeability and flexibility of the seahorse marsupium during pregnancy and its influence on the immune modulatory function.

The degree of foeto-paternal physiological connectivity in *N. ophidion* has come under question in this study, as when referring to genes with MHC pathway function patterns of expression do not match those found in pouched syngnathids. However, as with pouched species a number of genes with proinflammatory functions during early pregnancy were upregulated (cd44, cxcl8, tnfaip6). Interestingly, expressed genes with perceived roles in immune modulation were also recorded in this external brooding form. For example, *N. ophidion* was the only species to upregulate *cd276* and *prdm1* during pregnancy; the latter was even downregulated in the inverted and sealed syngnathid pouch. While these immune modulation mediators may be functional in the basal tissue integument and would require further functional analysis to confirm this, based on reduced intimacy deduced for this brooding form, and the inconsistent expression of MHC related genes, these regulators are probably not for promoting progeny protection. Excessive structural change in brooding tissue is not as pronounced in *N. ophidion* during pregnancy compared with pouched brooders, with the integument appearing consistent in form from egg deposition to egg hatching. Convincing evidence for the tissue's nutrient transfer and osmoregulation function, which characterises pouched syngnathid pregnancy (Kvarnemo et al., 2011; Ripley & Foran, 2009; Skalkos et al., 2020), is limited in nonpouched species (Berglund et al., 1986; Kronester-Frei, 1975). Considering the deductions made here regarding the lack of foeto-paternal immune connection, the scope for nutrient partitioning also appears implausible. In this case, it stands to reason that the *N. ophidion* brooding tissue is solely an egg-carrying mechanism that ensures protection from predators, and not a patrotrophic conduit that provides stage-specific assistance for the progeny.

Taking all the gene expression analyses into account, this study was able to identify a number of homologous genes that are expressed in both male and female pregnancy, and highlight potential physiological pathway congruencies found between the two gestation cycles. Consequently, (v) the fifth hypothesis of this study was supported, despite no clear male pregnancy specific genes being identified.

Within the syngnathid fish group, pouch/brooding integument tissue varies in structure and fleshiness and care needed to be taken to ensure that comparable tissue was utilised for transcriptome comparisons. Therefore, only the internal tissue of each of the pouched syngnathids and scrapings of the *N. ophidion* egg holding tissue were dissected. However, the differences between *H. erectus's* advanced marsupium-like pouch, the slightly less derived *Syngnathus*' pouch, and *N. ophidion's* swelling integument tissue (Carcupino et al., 2002; Ripley et al., 2010; Wilson et al., 2001) could be an influencing factor when it comes to the disproportionate amount of differentially expressed genes found in *N. ophidion*. Additionally, it is plausible that *N. ophidion* brooding integument tissue may have been over-proportionally sampled in pregnancy stages where tissue was more hypotrophic. These differing morphological features, which also change within each species depending on the pregnancy stage, are probably a driver of these expression disparities. Only the orthologue-based data set was utilised in the multispecies comparisons to ensure all discussed genes were present and the same in all species. The choice not to only use the same orthologues for the individual species analysis was to allow for the identification of genes specific to a particular species and it respective brooding tissue type.

As well as brooding tissue disparities, it should be acknowledged that differences exist between the offspring development staging in *N. ophidion* compared to pouched syngnathids. *N. ophidion offspring* develop fully inside the eggs until their release, whereas the progeny in *Syngnathus* and *Hippocampus* hatch earlier and continue their development within the pouch (Whittington & Friesen, 2020). This raised some issues when it came to determining the pregnancy stage dimensions for this investigation. To combat this issue it was decided that termination of "pregnancy" would be defined as the point in time at which paternal attachment/retainment ceased. This allowed for a cohesive comparison between species, with stages interpreted based on their relation to eggs being received and offspring being released entirely. Combining a transcriptome examination of brooding tissue with a defined embryo development staging assessment, in multiple syngnathid species, could be a fascinating topic for future research.

The encompassing period between conception and parturition is not of uniform function or process. Stages are adaptive, changing to provide for the progeny and maintain homeostasis between offspring and parent. In conclusion, here it has been shown to be possible to characterise the initial stages of pregnancy from those at its climax, based on inner brood pouch-tissue gene expression across multiple species. This propounds that male pregnancy shares some of the metabolic pathway constructs that define early and late stages in the female gestation, while also showing indications of proinflammation activity during early pregnancy, potentially to assist with egg engulfment. As with previous male pregnancy reports, a general downregulation of MHC pathway components was found,

especially during early pregnancy in pouched species. Contrastingly, findings here suggest that immune modulation processes during late/post-parturition in advanced male pregnancy are perhaps not as crucial to progeny survival as they are in mammals, potentially due to a physiological connection break between offspring and parent and exposure to the environmental seawater. With this in mind, further investigations are encouraged to help clarify this immunological shift and determine the role that MHC pathways have during the climax of male pregnancy.

Pinpointing shared expression similarities between male pregnancy brooding types, this study also highlights the potential distinctions in functionality between the brood pouch forms, especially when it comes to immunity, osmoregulation and nutritional supplementation. Comparative syngnathid studies allow for the subtle evolutionary changes that shaped male pregnancy to be observed and this study provides a supportive basis for future work. It also provides emphasis that more focus should be given to pregnancy strategies that transcend the human model, and that non-model organisms and multispecies comparisons can help annotate the genetic drivers of pregnancy evolution as a whole. Lastly, this study helps shed additional light on the complex evolutionary relationships found between pregnancy and the immune system; a fascinating yet contentious topic which when better understood could help support related medical practices.

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AUTHOR CONTRIBUTIONS

O.R., J.P. and S.J. conceived the study. J.P., O.R., R.S. and K.S.W. collected and processed samples at differing stages. J.P., A.B., R.S. and T.B. analysed the data. J.P. and O.R. wrote the manuscript with input from all coauthors.

DATA AVAILABILITY STATEMENT

Raw sequencing data has been made available in NCBI SRA under project PRJNA755822. Transcriptome data are openly available at Figshare at 10.6084/m9.figshare.15190101.

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