Evolution of male pregnancy associated with remodeling of canonical vertebrate immunity in seahorses and pipefishes

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A fundamental problem for the evolution of pregnancy, the most specialized form of parental investment among vertebrates, is the rejection of the nonself-embryo. Mammals achieve immunological tolerance by down-regulating both major histocompatibility complex pathways (MHC I and II). Although pregnancy has evolved multiple times independently among vertebrates, knowledge of associated immune system adjustments is restricted to mammals. All of them (except monotremata) display full internal pregnancy, making evolutionary reconstructions within the class mammalia meaningless. Here, we study the seahorse and pipefish family (syngnathids) that have evolved male pregnancy across a gradient from external oviparity to internal gestation. We assess how immunological tolerance is achieved by reconstruction of the immune gene repertoire in a comprehensive sample of 12 seahorse and pipefish genomes along the “male pregnancy” gradient together with expression patterns of key immune and pregnancy genes in reproductive tissues. We found that the evolution of pregnancy coincided with a modification of the adaptive immune system. Divergent genomic rearrangements of the MHC II pathway among fully pregnant species were identified in both genera of the syngnathids: The pipefishes (Syngnathus) displayed loss of several genes of the MHC II pathway while seahorses (Hippocampus) featured a highly divergent invariant chain (CD74). Our findings suggest that a trade-off between immunological tolerance and embryo rejection accompanied the evolution of unique male pregnancy. That pipefishes survive in an ocean of microbes without one arm of the adaptive immune defense suggests a high degree of immunological flexibility among vertebrates, which may advance our understanding of immune-deficiency diseases.

immunological tolerance | major histocompatibility complex | male pregnancy | seahorse | comparative genomics

Pregnancy is the most dramatic form of parental investment, protecting embryos from extreme temperatures, anoxia, osmotic stress, and predation at the cost of fewer young, less effective dispersal, and high-energy demand (1, 2). Although this trait requires multiple anatomical and physiological changes (3) involving development, morphology, osmoregulation, endocrinology, and immunology (4, 5), it has evolved independently in more than 150 vertebrate lineages. A fundamental problem for pregnancy to evolve is the rejection of the embryo that is recognized as foreign tissue by the vertebrate’s adaptive immune system, as it displays alleles also from the other parent. Modulation of the immune system to tolerate foreign protein signatures of the embryonic tissue, in turn, is conflicting with the maintenance of immunological vigilance toward pathogens (6).

Being mammals themselves, researchers have almost exclusively focused on mammalian pregnancy to assess the key adaptations for pregnancy evolution. In vertebrates, the unique diversity of the classic major histocompatibility complex (MHC) class I and II genes (7–9) plays a key role for self/nonself-recognition. While in mammals an initial inflammation seems crucial for embryo implantation (10), during pregnancy mammals prevent an immunological rejection of the embryo with tissue layers of specialized fetal cells, the trophoblasts (11–13). Trophoblasts do not express MHC II (14–16) and thus prevent antigen presentation to maternal T-helper (Th) cells (17), which otherwise would trigger an immune response against nonself. Additionally, expression of classic MHC I genes (HLA-A, -B, and -D) is down-regulated (18). These immunological adaptations are mediated by a cross-talk between the placental trophoblasts and uterine immune cells, in particular natural killer cells and regulatory T cells (Tregs) (19, 20). Tregs maintain self-tolerance by suppressing inflammatory Th1 immune responses to foreign tissue by the vertebrate’s adaptive immune system. We show that the unique evolution of male pregnancy in pipefishes and seahorses coincided with a genomic modification of one arm of the adaptive immune system. Our findings indicate a trade-off between immunological tolerance and embryo rejection to accompanying the emergence of male pregnancy. That syngnathids survive in an ocean of microbes despite their drastically modified immune defense suggests an unexpected immunological flexibility. Our results may improve the understanding of immunodeficiency diseases and call for a reassessment of vertebrate immunity.

Significance

Among vertebrates, pregnancy has evolved more than 150 times independently. A fundamental problem for pregnancy to evolve is inadvertent rejection of the embryo when being recognized as foreign tissue by the vertebrate’s adaptive immune system. We show that the unique evolution of male pregnancy in pipefishes and seahorses coincided with a genomic modification of one arm of the adaptive immune system. Our findings indicate a trade-off between immunological tolerance and embryo rejection to accompanying the emergence of male pregnancy. That syngnathids survive in an ocean of microbes despite their drastically modified immune defense suggests an unexpected immunological flexibility. Our results may improve the understanding of immunodeficiency diseases and call for a reassessment of vertebrate immunity.

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Data deposition: The sequences reported in this paper have been deposited in the European Nucleotide Archive, https://www.ebi.ac.uk/ena (accession no. PRJEB32126). Gene alignments for positive selection analyses can be found at Figshare: doi:10.6084/m9.figshare.11499360.

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Here we present comprehensive genome data on 12 representative species of the Syngnathiformes covering a broad range of parental reproductive investment. By assessing their immune gene repertoire, we reconstructed the evolutionary acquisition of immunological tolerance within this unique lineage. As a non-mutually exclusive explanation, we also assessed whether immune gene regulation contributes to immunological tolerance in syngnathids, similar to mammals, and measured differential gene expression during male pregnancy in Syngnathus typhle using comparative transcriptomics to assess whether immunological tolerance can also be achieved by gene regulation in syngnathids, similar to mammals.

**Genome Size Evolution in Syngnathiformes**

We selected one species, *S. typhle*, to obtain a high coverage and contiguous genome assembled to a high-quality draft stage sufficient to achieve gene repertoire completeness using a combination of paired-end and mate-pair libraries. The genomes of 11 additional species were assembled to draft stage (*SI Appendix, Table S2*). Based on the whole-genome datasets, including the already published genomes of *Syngnathus scovelli* and *Hippocampus comes* (29, 30), Bayesian phylogenetic analyses (*SI Appendix, Table S3*) places the origin of the Syngnathiformes clade at 80 Mya (*SI Appendix, Fig. S1*). Surprisingly, the Syngnathiformes lineage contains species with very divergent genome sizes, spanning from 347 Mbp (*Syngnathus rostellatus*) to 1.8 Gbp (*Entelurus aequoreus*) (Table 1). Syngnathiformes species lacking male pregnancy—namely *Fistularia tabacaria*, *Mullus surmuletus*, *Dactylopterus volitans*, *Aeoliscus strigatus*, and *Macroramphorus scolopax*—displayed larger genomes than both genera with full male pregnancy (i.e., all *Hippocampus* and *Syngnathus* species). In contrast, the *Nerophinae* pipefishes with external male pregnancy, specifically *Nerophis ophidion* and *E. aequoreus*, have significantly larger genomes (Table 1). Concordantly, during 50 million years of evolution, transposable elements have expanded in...

![Fig. 1. Morphology of brood pouches of subfamily Nerophinae (A), and of the genera Syngnathus (B), and Hippocampus (C) and display of the placenta-like structures in syngnathids (only *Syngnathus* and *Hippocampus*). The placenta-like structure with lumen, apical pore, CRM (cells rich in mitochondria), ions, epithelial cells, capillaries, and the egg are drawn after figure 1 of ref. 28.](image_url)
Nerophinae, most likely explaining the large genome sizes within this subfamily (SI Appendix, Fig. S2 and Table S4).

Modification of MHC II Pathways in Syngnathus and Hippocampus

In order to correlate the modification of adaptive immunity with the degree of male pregnancy, a set of key genes involved in adaptive immunity was analyzed from the assembled genomes presented here along with two previously published syngnathid genomes (29, 30) (SI Appendix, Table S2). MHC I and MHC II are essential for the recognition process of nonself-peptides by presenting them to CD8+ and CD4+ T cells, respectively. In line with our hypothesis, all fully pregnant species (i.e., genera Syngnathus and Hippocampus) underwent considerable modifications of their adaptive immune system characterized by losses or changes of key genes of the MHC II pathway (Fig. 2; details on ortholog search and analyses can be found in the SI Appendix, sections 5.1. and 5.5.11).

The invariant chain of MHC II (CD74), preventing premature peptide binding of MHC II, displayed a divergent exon 3 in Syngnathus and Hippocampus compared to both mammals and other teleosts (Fig. 3). Additionally, Hippocampus had a sequence substitution of exon 6b, while Syngnathus displayed a divergent exon compared to other fish and human. Both exons 3 and 6b are located in the protein region protruding into the endosomal lumen. Several lines of evidence suggest that these losses are impairing functions of CD74. In human, exon 3 of CD74 covers the region associating with MHC II (CLIP) [amino acids 108 to 124 in SI Appendix, Dataset 1: 10CD74_clean (31)]. Exon 6b is annotated as Thyroglobulin type I repeats, which are proposed cysteine protease inhibitors (this exon consists of six conserved cysteine residues) and are implicated in delaying the degradation of internalized antigens subsequently preserving epitopes for antigen presentation (32, 33).

As the most drastic change in gene repertoire, all Syngnathus species have lost the genes encoding the classic MHC II α- and β-chains, implying that the presentation of antigens to the T cell receptor on CD4+ T lymphocytes is disabled (Fig. 2). This is supported by a loss of CD4, mediating successful receptor binding and activation of CD4+ T lymphocytes—AICDA, responsible for the unique receptor diversity of the antibodies and CIITA, the MHC II transactivator—which control the expression of MHC II genes in antigen-presenting cells. The only canonical gene of the MHC II pathway remaining in the Syngnathus genomes was the autoimmune regulator (35), driving negative selection on self-recognizing T cells (36). While leading and trailing exons of CIITA were well conserved among all investigated Syngnathiformes species compared to reference sequences from other fish families, several other exons diverged markedly or homologous sequences were not found [Fig. 3 and SI Appendix, Dataset 1: 13AIRE_exon_overview (31)]. In the genera Syngnathus and Hippocampus, exons 3, 4, 5, 6, and 12 of AIRE were lost or substituted with very divergent sequences that could not be aligned. Putative loss of MHC II-related function of the AIRE transcription factor is further emphasized by the lack of expression in various S. typhle tissues, which could result in insufficient negative selection of T cells in the thymus (36). Overall, our findings suggest that the MHC class II pathway was lost in Syngnathus.

The situation in Hippocampus was more complex. Similar modifications as in Syngnathus for the CD74 gene were observed in terms of a divergent exon 3, and in a substitution of exon 6b. Importantly, no loss of the MHC II genes as in all three Syngnathus species was observed. However, in Hippocampus, the MHC II gene sequences, in particular of the β-copy, were highly distinct from other functional MHC II genes found in species with functional MHC class II such as zebrafish, seabass, salmon, and guppy [SI Appendix, Dataset 1: 29MHCII_beta_complete (31)]. In parallel, small sections of some CIITA exons displayed substitutions compared to both other teleosts and mammals. This is in line with findings for AIRE in both Hippocampus and Syngnathus, where several exons were either lost or diverged markedly compared to other teleosts, indicating most likely an alternative function not related to MHC II (Fig. 3). Moreover, the tertiary structure of MHC II genes of Hippocampus was predicted to lack two critical cysteine bridges that are essential to form the peptide-binding pocket of the MHC II molecule [SI Appendix, Dataset 1: 29MHCII_beta_complete (31)]. In line with these findings, in Hippocampus we identified positive selection in sequences of genes that were lost in Syngnathus (AIRE, CD4, and CIITA), which may suggest neo- or subfunctionalization (SI Appendix, Table S11).

A closer examination of the invariant chain encoding gene CD74 also suggests that the evolution of adaptive immunity has taken distinct routes in the two sister genera Syngnathus and Hippocampus. A shared relaxed selection on CD74 in the common ancestor of syngnathids resulted in a loss-of-function by either sequence substitution in the Hippocampus or divergence

### Table 1. Summary of species, estimated genome sizes, and assembly statistics

| Species Name | Estimated genome size (Mbp) | Assembly size (Mbp) | N50 scaffold (Mbp) | N50 contig (%) | BUSCO complete (%) |
|--------------|-----------------------------|---------------------|-------------------|----------------|-------------------|
| *Gulf pipefish* | NA                          | 307                 | 12400.1           | 27.8           | 85.8              |
| *Tiger tail seahorse* | NA                          | 494                 | 2034.5            | 39.6           | 89.4              |
| *Yellow seahorse* | 478                         | 445                 | 31.2              | 10.3           | 83.9              |
| *White’s seahorse* | 461                         | 433                 | 40.8              | 10.3           | 86.0              |
| *Straightnose pipefish* | 1581                        | 976                 | 6.8               | 5.2            | 33.6              |
| *Tiger tail seahorse* | 494                         | 2034.5              | 39.6              | 89.4           |                   |
| *Yellow seahorse* | 478                         | 445                 | 31.2              | 10.3           | 83.9              |
| *White’s seahorse* | 461                         | 433                 | 40.8              | 10.3           | 86.0              |
| *Straightnose pipefish* | 1581                        | 976                 | 6.8               | 5.2            | 33.6              |
| *Tiger tail seahorse* | 494                         | 2034.5              | 39.6              | 89.4           |                   |
| *Yellow seahorse* | 478                         | 445                 | 31.2              | 10.3           | 83.9              |
| *White’s seahorse* | 461                         | 433                 | 40.8              | 10.3           | 86.0              |
| *Straightnose pipefish* | 1581                        | 976                 | 6.8               | 5.2            | 33.6              |
| *Tiger tail seahorse* | 494                         | 2034.5              | 39.6              | 89.4           |                   |
| *Yellow seahorse* | 478                         | 445                 | 31.2              | 10.3           | 83.9              |
| *White’s seahorse* | 461                         | 433                 | 40.8              | 10.3           | 86.0              |

NA, not applicable. *Already published genomes.*
in the *Syngnathus* for exon 6b, accompanied with a divergent exon 3 in both lineages. While subsequently several core genes of the MHC II pathway were lost in *Syngnathus*, genes of the MHC II pathway were under positive selection in *Hippocampus* [Fig. 3 and SI Appendix, Table S11 and Dataset 1, gene alignments (31)] and showed clear sequence divergence compared to other teleosts and humans (Fig. 3). Different scenarios may explain the observed pattern in the MHC II pathway of *Hippocampus*.

First, the sequence divergence of the MHC II core genes in contrast to other teleosts and the signs of positive selection could indicate that in *Hippocampus* the MHC II genes were taking over alternative or novel functions. CD74 is pivotal for a functional MHC II pathway, as supported by an impaired assembly and surface expression of MHC II and a defective antigen presentation in invariant chain knockout mice (37). While generally the CLIP of CD74 (exon 3) associates with MHC II, the remaining exons of CD74 act as chaperone, transporting MHC II to the loading compartment. The loss of exon 6b in *Hippocampus* could indicate a compromised loading process. Accordingly, the MHC II system in *Hippocampus* is likely to be less efficient in contrast to other vertebrates, which may suffice to permit the evolution of full male pregnancy.

Second, the MHC II pathway may not be compromised in its function despite the lost and diverged exons of CD74 over a functional rearrangement of the immune system. However, this is less likely, as mice with transgenic expression of a truncated CD74 protein lacking the CLIP region (the part of the gene that diverges from other teleosts in *Hippocampus*) could not pursue MHC II trafficking (38) (Fig. 3 and SI Appendix, Table S11).

**Modifications of the MHC I Pathway under Pregnancy**

In Gadiformes (cod-like fishes) an independent loss of the MHC class II pathway was recently reported, and the observed diversification of MHC I genes was hypothesized to compensate for the loss of a functional MHC II pathway (39, 40). Accordingly, we assessed MHC I copy number in syngnathids using the most conserved exon 4 of the MHC I gene and found it to be higher in all species displaying male pregnancy (the *Nerophinae* with external male pregnancy [27 to 42 copies], *Hippocampus* [20 to 36 copies], and *Syngnathus* [24 to 44 copies] with full male pregnancy) compared to species without pregnancy (5 to 10 copies) (Fig. 2). While all identified MHC I sequences in Syngnathiformes are part of the U lineage (41), the distinct cluster of syngnathid MHC I sequences supports a potential coevolution of MHC I with male pregnancy (*SI Appendix, Figs. S9 and S10*). These lineage-specific MHC I variants likely increase the ligand repertoire and suggest a possible function within the cross-presentation pathway, in contrast to Atlantic cod, where cross-presentation could be hindered due to loss of the entire CD74, a gene with crucial function in the MHC I cross-presentation pathway (42). Moreover, key genes of the MHC I pathway, such as β2-Microglobulin (*B2M*, important for the availability of MHC I light-chain proteins) and CD8 (responsible for activation of CD8+ T lymphocytes), were under positive selection in syngnathids, similar to RAG1 that facilitates V(D)J recombination and TAP1/TAP2 that function as heterodimers in the transport of antigens (*SI Appendix, Table S11*). This supports a shift from the MHC II to MHC I cross-presentation pathway as all of the latter genes (*CD8, RAG1*, and *TAP1/TAP2*) also have important functions in the MHC I cross-presentation pathway.
identification of marked positive-selection signals support an interpretation of the MHC I pathway to coevolve with male pregnancy. The expanded MHC I repertoire is likely to be linked to the simultaneous loss/rearrangement of the MHC II pathway and may compensate its function/deficiency over the MHC I cross-presentation pathway.

Pregnancy requires special physiological adaptations to assure the oxygen supply to the growing embryo. In line with these expectations, the repertoire of hemoglobin genes encoding oxygen transport show signs of coevolution with male pregnancy. All synagnostids have lost the hemoglobin gene alpha 6, while those genera with full male pregnancy, *Syngnathus* and *Hippocampus*, have also lost the alpha 5 gene. Conversely, fully pregnant species have gained alpha 1 and alpha 2 hemoglobin genes (*SI Appendix*, Figs. S14–S16). It is tempting to speculate that the shift in hemoglobin gene repertoire indicates selection for more effective oxygen transfer from father to offspring in male pregnancy evolution.

**Modulation of Gene Expression during Pregnancy**

Next, we assessed whether or not the evolution of immunological tolerance required the cooption of similar genes and physiological processes in female and male pregnancy. To do so, we analyzed global gene-expression patterns using RNA sequencing in our model species *S. typhle* in brood pouch tissues during pouch development and pregnancy. At the same time, this approach assessed whether the evolution of immunological tolerance in male pregnancy was also achieved by immune gene regulation as in mammals, in addition to the identified changes in gene repertoire. We examined the gene-expression profiles of male undeveloped brood pouch tissues (control) against developed pouch tissue from mature and receptive males (43); pouch tissue at early- and at late-pregnancy genes with a false-discovery rate-corrected P value of <0.05, as determined by the cuffdiff algorithm, were categorized as differentially expressed (44). All differentially expressed genes were searched for potential functions via homology, using reported functions in female pregnancy of mammals, in the squamate reptile *Chalcides ocellatus* (45) and in male pregnancy of *S. scovelli* (29) and *Hippocampus abdominalis* (46). A total of 141 genes were significantly up- or down-regulated during male pregnancy in *S. typhle* and *S. scovelli* (29). The direction of expression in differentially expressed genes correlated between *S. typhle* and *S. scovelli* (29). The expression of hemoglobin genes shows signs of coevolution with male pregnancy. All fully male-pregnant species have also lost the alpha 5 gene. Conversely, fully pregnant species have gained alpha 1 and alpha 2 hemoglobin genes (*SI Appendix*, Figs. S14–S16). It is tempting to speculate that the shift in hemoglobin gene repertoire indicates selection for more effective oxygen transfer from father to offspring in male pregnancy evolution.
Immune Gene Expression and Male Pregnancy

Next, we focused on immune gene-expression changes that accompany the modification of the MHC II pathway and the MHC I gene repertoire expansion. We analyzed the differential expression of immune genes that are either known to have a function in female pregnancy in mammals or known to also show expression changes in pregnancy of reptile, of seahorse, or of another pipefish species [in bold italics below; in the parentheses \(M\) indicates a gene known to have a function in mammals, \(R\) in the reptile \(C.\) ocellatus (45), \(S\) in the seahorse \(H.\) abdominalis (46), and \(P\) in the pipefish \(S.\) scovelli (29)] (SI Appendix, Table S12) or those with a fold-change \(> 2\) (220 genes in total, including 30 immune genes) (only in italics) (Fig. 5 and SI Appendix, Table S13).

Collectively, the observed gene-expression changes during male pregnancy contribute to immunological tolerance during pregnancy already apparent from the gene repertoire. In particular, we identified expression changes of proinflammatory Th1 and antiinflammatory Th2 responses and a simultaneous down-regulation of the MHC I pathway during pregnancy, which resemble the expression changes during mammalian pregnancy. An inflammation response was suggested to be important for successful implantation in mammalian pregnancy (10). The key genes mediating this specific inflammation at implantation in mammals—IL6R (M/P), TNF (M), and PTGS2 (M) (10, 47)—were up-regulated during pouch development in pipefish. Here, other inflammation responses dropped [down-regulation of proinflammatory interleukins IL1B (M) and IL2RG (M), and S100A13 (interleukin secretion gene); the proinflammatory cytokine MIF; FHL2 involved in inflammation response; ADSS1L1C involved in antimicrobial peptide synthesis; the antimicrobial peptide PLE3; JUND involved in LPS response; and up-regulation of GSN (M/R) that binds to LPS].

Simultaneously, lymphocyte maturation and proliferation were suppressed through the down-regulation of CHIA and MEF2C (M/S) (maturation of B cells and important in mammalian embryo development), the up-regulation of GIMAP4 that enhances lymphocyte apoptosis, and the up-regulation of the transcriptional repressor PRDM1 (M/P) that initiates in mammals a lineage-restricted progenitor cell population contributing to placental growth and morphogenesis (48). Consistent with a shift from Th1 to Th2 responses during mammalian pregnancy, CEBPB (M/S/P), which represses Th1 but facilitates Th2 immune response, was up-regulated during pipefish male pregnancy. This coincided with expression dynamics of EPX (M) mediating eosinophil activity and promoting mammalian placental development (49). Lymphocyte maturation and proliferation remained consistently repressed during pregnancy as indicated by down-regulations of RPL18A (R) (activation of T cell proliferation [Th1]), of FCRL5 (enhancing B cell development), of the proinflammatory interleukin IL2RG (R/S), and of the interleukin secretion genes S100A13 and IL20R (R/S).

During late pregnancy only, GPR97 and MFNG (both responsible for B cell differentiation) were down-regulated along with the genes NEATC4 and HAVCR1, which are involved in T cell maturation. Few genes involved in Th1 immune response were up-regulated during pregnancy [TNF (M), CLCF1 (M), KLF4 (M/S), and TNFRSF21 (M)]. In female pregnancy, those genes were shown to have additional functions: TNF (M) mediates placental development and implantation (10, 50), CLCF1 (M) is responsible for the onset of labors at term [a process resembling inflammation (51)], and KLF4 (M/S) is key for the maintenance of gestation (52). The two inflammation genes, PLA2G4A (P) and IL17REL, were up-regulated during pouch development but not during pregnancy. In summary, inflammation responses during male pregnancy could be overlapping with previously identified expression patterns of homologous genes responsible for the regulation of inflammation during egg implantation and female pregnancy.

Analogous to human pregnancy where CASP3 (M/S) modifies the MHC class I pathway (53, 54) to support immunological tolerance (55), CASP3 (M/S) was up-regulated during pipefish pregnancy. Throughout early mammalian pregnancy, TAP1 (M) is increasingly expressed on placenta-specific trophoblasts and plays an important role in preventing maternal immune attacks toward the embryos (56). Such up-regulation of TAP1 (M) was
S. typhle pregnancy is key for maintaining the acceptance of the semi-male. Syngnathus suggests a change of function for antigen recognition over the MHC I pathway could also be (M). Recognition, presentation, and processing were down-regulated during pipefish male pregnancy. Homologs of genes marked with (M) possess known immune genes during pouch development (DEV), early pregnancy (EP), and late pregnancy (LP). In pipefish, the complete loss of functional flexibility than previously assumed, in line with recent findings in the Gadiformes lineage (34, 39). The complete loss of classic MHC II pathway loss is still elusive, we provide evidence among Syngnathiformes that modification and loss of adaptive immune genes and pathways is associated with the evolution of male pregnancy, which potentially selected for immunological tolerance. As Syngnathids and Gadiformes are only distantly related, losses and divergence of key genes of the MHC II pathway in each of those groups represent independent evolutionary events, likely driven by different selection factors.

The loss of gut-associated lymphatic tissues (GALT), the spleen (62), and the immune genes (CD4, MHC II, AICDA, CIITA) in Syngnathus represent critical pathways that are attacked by the HIV (CD4+ T cells, GALT). As a natural “knockout” for the MHC class II pathway, Syngnathus may thus become instrumental in the future as a model for research on natural or disease-related immune deficiencies.

Materials and Methods
We have sequenced and assembled 12 Syngnathiformes genomes (SI Appendix, Table S2) and annotated the genome of S. typhle. We generated a time-calibrated phylogeny of Syngnathiformes (SI Appendix, section 3 and Fig. S1). To search for shifts in the optima of genome size in the different lineages, we applied the Ornstein-Uhlenbeck process using the Syngnathiformes phylogeny and the genome sizes of the species (SI Appendix, section 4.1 and Fig. S2). To investigate potential reasons for differences in genome size, a library of repeated elements was created (SI Appendix, section 4.2 and Table S4). For immune, pregnancy, and hemoglobin genes, translated query sequences, either as whole sequences and for MHC and hemoglobin also coincided with major alterations of the gene repertoire of both MHC pathways. While the identified rearrangement and loss of the core genes of the MHC II pathway is consistent with an adaptive explanation to modulate the immune system so as to prevent immunological rejection of the embryo, demonstrating causality would require future functional validation. While in Syngnathus, the genomic knockout of the MHC II pathway must have resulted in a loss-of-function, in Hippocampus the situation remains inconclusive.

One of the most unexpected findings was that even within a single fish family, the rearrangement of the MHC II pathway differed between the genera Hippocampus and Syngnathus. At the same time, this demonstrates both a strong selection for reduction of immunological vigilance displayed by the MHC class II pathway during pregnancy evolution and a remarkable flexibility of the vertebrate immune system in general. Because this unique fish family displays male pregnancy, any of the immunological adaptations are also not compounded by the sex per se (60) [i.e., the fact that the female sex through provisioning of eggs usually needs a more competent immune system under conventional sex roles, referred to as Bateman’s principle (61)].

The up-regulation of CD74 during pregnancy is puzzling as almost all other genes of the MHC II pathway are absent in S. typhle. As key exons of CD74 are diverged or substituted in Syngnathus, the up-regulation of CD74 during pregnancy rather suggests a change of function for CD74 in the evolution of male pregnancy. This suggests that consistent with female pregnancy, antigen recognition over the MHC I pathway could also be down-regulated during pipifish male pregnancy.

Discussion
Although pregnancy is widespread among the vertebrates, very little is known on the immunological modifications that are required to prevent embryo rejection other than within the class mammalia. Here, we present a major modification of the immune system associated with increasing investment into pregnancy in the fish family of pipefishes and seahorses that not only entailed gene-expression changes during pregnancy but also coincided with major alterations of the gene repertoire of both MHC pathways. While the identified rearrangement and loss of the core genes of the MHC II pathway is consistent with an adaptive explanation to modulate the immune system so as to prevent immunological rejection of the embryo, demonstrating causality would require future functional validation.
Upon gene alignments, gene trees were generated with RAxML (v8.2.10.) and a European Research Council Starting grant (malePREG) (to O.R.). Sequencing library creation and high-throughput sequencing was carried out at the Norwegian Sequencing Centre, University of Oslo (http://www.sequencing.uio.no), Norway and McGill University and Genome Quebec Innovation Centre, Canada. Computational work was performed on the Aberdeen Supercomputing Cluster (Norwegian Metacenter for High Performance Computing and the University of Oslo) operated by the Research Computing Services group at USIT, the University of Oslo IT-department (https://www.uio.no/it/jenester/it/forsknings/).

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More than just a...
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