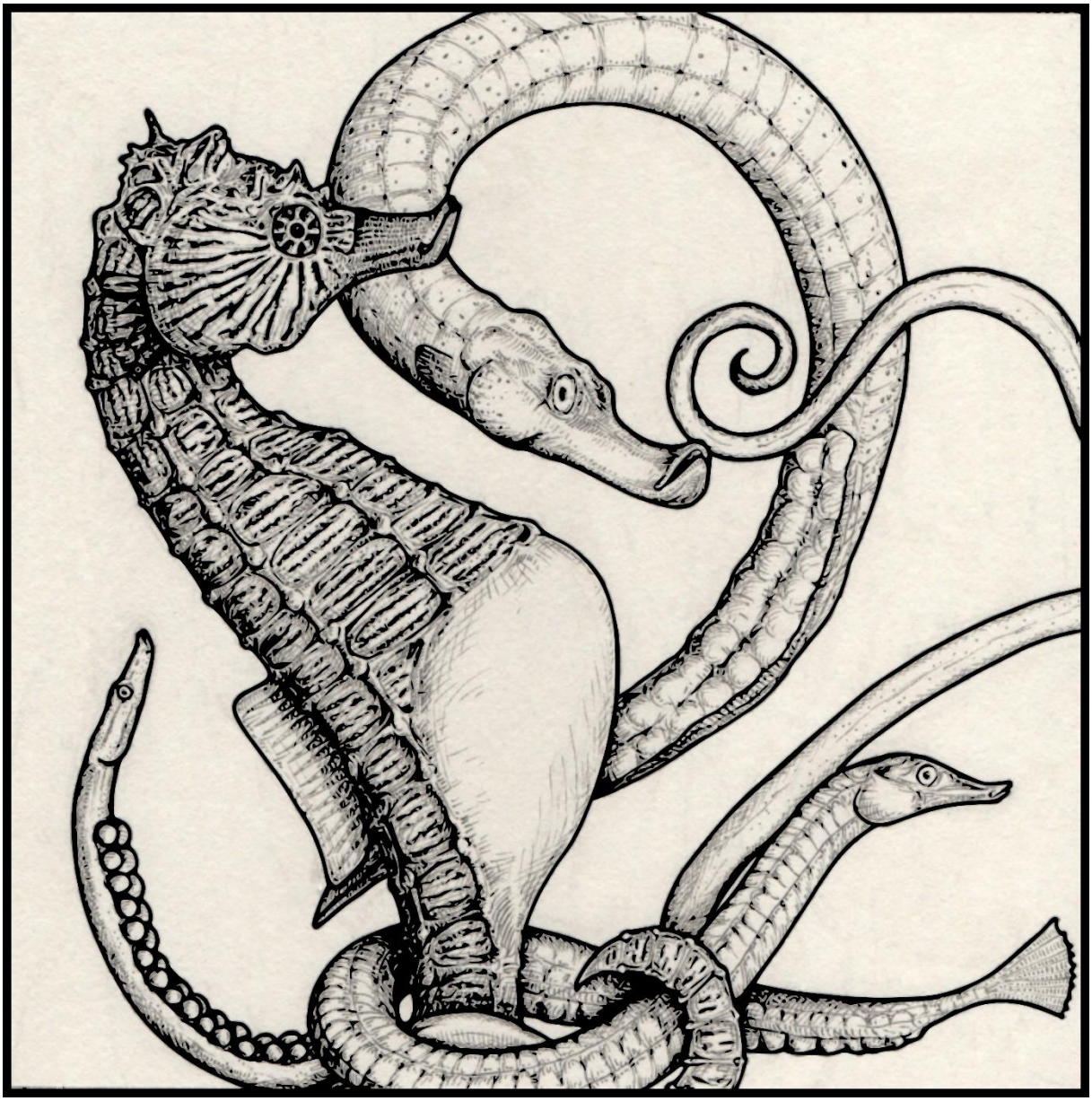
MALE PREGNANCY AND THE EVOLUTIONARY IMPORTANCE OF IMMUNOLOGICAL TOLERANCE IN SYNGNATHID FISHES



Jamie Parker

MALE PREGNANCY AND THE EVOLUTIONARY IMPORTANCE OF IMMUNOLOGICAL TOLERANCE IN SYNGNATHID FISHES

DISSERTATION

Zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften

Doctor rerum naturalium

An der Mathematisch-Naturwissenschaftlichen Fakultät der Christian-Albrechts-Universität zu Kiel durchgeführt in der Pipefish Group am GEOMAR Helmholtz-Zentrum für Ozeanforschung Kiel

Vorgelegt von:

JAMIE PARKER

Kiel, December 2021

Gutacher: Prof. Dr. Olivia Roth

RAPPORTEURS

Prof. Dr. Olivia Roth (PhD advisor, Reviewer 1 and defence committee)

Dr. Sissel Jentoft (Reviewer 2 and defence committee)

Prof. Dr. Tal Dagan (defence committee)

Prof. Dr. Stanislav Gorb (chairperson)

Date of the oral examination: **4th February 2022 (14:00)**

CONTENTS

SUMMARY

Pregnancy has evolved upwards of 150 times across vertebrates and represents the reproductive strategy of the highest investment. Morphological and immunological tolerance adaptations evolved to facilitate the connection between embryo and parent during pregnancy, preventing embryonic rejection from occurring and maintaining an immunologically homeostatic environment. However, knowledge concerning the co-evolutionary relationship between pregnancy and the immune system is overwhelmingly restricted to mammalian species. In order to understand how pregnancy could have evolved to such a diversity as well as providing further insight into the specific adaptations that facilitated pregnancy evolution, research needs to transcend the mammalian system.

The syngnathid fish group, which includes the seahorses, pipefishes and seadragons, lays claim to the only evolutionary instance of male pregnancy in the animal kingdom. A number of different brooding forms have evolved with varying degrees of investment within the lineage. These range from pouchless external egg carrying skin integument (*Nerophinae*), muscular flap derived, semi-sealed pouches designed to envelop the eggs (*Syngnathus*) and the specialised sealed pouches with muscularised opening and closing capabilities (*Hippocampus*). However, the evolutionary oddities within the syngnathid group extend beyond their reproductive strategies. The functional loss of the Major Histocompatibility Complex class II pathway (MHC II) in the *Syngnathus* genus and related pathway components in the seahorse (*Hippocampus*) has raised questions regarding their immunological proficiency. Furthermore, the relevance of this loss to immunological tolerance levels and its potential influence on the evolution of advanced male pregnancy has been stipulated. In turn, this thesis explored the male pregnancy transcriptome at various stages of gestation in multiple species, while also assessing the role of the adaptive immune system and particularly the role of immunological tolerance in male pregnancy evolution.

The first chapter of this thesis examined the male pregnancy period, by utilizing comparative transcriptomics to assess brooding tissue expression patterns between different pregnancy forms and gestation stages (non-pregnant, early, late and parturition). Immune and pregnancy related mechanisms were compared with those in female pregnancy to see if similar evolutionary pathways were likely adopted using the same genetic components. Results suggested that a catabolic shift occurs leading up to parturition in male pregnancy, which is congruent with patterns found in female pregnancy. In addition, female pregnancy associated inflammation patterns during early gestation were also evident in each brooding species examined here, suggesting a potential role during egg implantation. The most significant discovery, however, can be attributed to the exclusive evocation of immunological tolerance

measures during early pregnancy in pouched syngnathids. This immune modulation was represented by the downregulation of MHC I genes, an expression pattern which was not reciprocated in the pouchless species. Findings suggested that increased feto-paternal intimacy found during early pregnancy in pouched syngnathids likely requires immune modulatory processes to circumvent embryo rejection. Furthermore, it supports the premise that immunological tolerance was important for the evolutionary development of advanced male pregnancy.

The allorecognition mechanisms of syngnathids with (*Syngnathus typhle*) and without (*Nerophis ophidion*) MHC II were investigated in chapter II by utilising fin transplants and differential gene expression analyses. Attempts were made to decipher which immune pathways are upregulated during transplantation, particularly in the absence of the MHC II, while also assessing immunological tolerance threshold differences within and between species. Autograft and allograft transplants were shown to trigger a number of immune and inflammation related genes in both species, while also exhibiting signs of tissue repair and remodelling activity. *Gzma*, a gene coding for a cytotoxic T-cell (CTL) implicated granzyme was shown to be upregulated in allograft tissue of *S. typhle* when compared with autografts. This could be indicative of a MHC I mediated immune response which may be compensating for absence of MHC II components. Signs of immunological tolerance were found in in *N. ophidion* autografts by way of MHC I gene downregulation. This chapter provided useful insight into the mechanisms evoked during transplantation and tissue responses to trauma, as well as establishing an effective protocol for fin transplantation.

Chapter III of this thesis aimed to characterise the immune cell repertoire of the MHC II devoid *S. typhle* by adopting single-cell transcriptomics. Questions concerning the potential ramifications of this genomic loss on the evolution of immune cell populations in *S. typhle* provided a fascinating topic for investigation. By utilising known immune cell markers, a number of key immune cell subtypes were highlighted including: T-cells, B-cells, neutrophils, macrophages and basophils. As in chapter II, *gzma* was also identified along with other T-cell subtype markers. This should give motivation to future studies, which should strive to isolate distinct immune cell subtypes by utilising a more enhanced resolution. While the absence of MHC II and CD4 related expression provides initial support for the predicted absence of $CD4^+$ T-cells in *S. typhle*. This chapter represents the first single-cell transcriptome driven immune cell characterisation of any syngnathid fish, laying the foundations for future single-cell and immunological assessments within the lineage.

ZUSAMMENFASSUNG

Schwangerschaft in Wirbeltieren hat sich im Laufe der Evolution mehr als 150 Mal evolviert und stellt im Tierreich die Fortpflanzungsstrategie mit der höchsten elterlichen Investitionsrate dar. Verschiedene morphologische und immunologische Anpassungen waren notwendig, um sowohl die Verbindung zwischen Embryo und Elternteil während der Schwangerschaft zu gewährleisten, als auch eine immunologisch homöostatische Umgebung aufrechtzuerhalten, die eine Abstoßung des Embryos verhindert. Das heutige Wissen über die koevolutionäre Beziehung zwischen Schwangerschaft und Immunsystem ist überwiegend auf Säugetiere beschränkt. Um zu verstehen, wie sich die Schwangerschaft in einer solchen Vielfalt evolvieren konnte, und auch um weitere Erkenntnisse über die spezifischen Anpassungen, die die Evolution der Schwangerschaft ermöglicht haben, zu gewinnen, muss sich die Forschung vermehrt auch auf andere Systeme im Tierreich fokussieren.

Die Gruppe der Syngnathiden, zu der die Seepferdchen, Seenadeln und Fetzenfische gehören, beanspruchen das einzige evolutionäre Beispiel einer männlichen Schwangerschaft im Tierreich für sich. Innerhalb der Gruppe hat sich eine Reihe verschiedener Brut- und Bruttaschenformen evolviert, welche von bruttaschenlosen, eiertragenden Seenadeln (*Nerophinae*), über, von Muskelklappen abgeleiteten, halbgeschlossenen Bruttaschen, die die Eier umhüllen (*Syngnathus*), bis hin zu spezialisierten, geschlossenen Bruttaschen mit kontrollierten Öffnungs- und Schließfunktionen (*Hippocampus*) reichen. Die evolutionären Eigenheiten innerhalb der Syngnathidengruppe gehen sogar noch über ihre Fortpflanzungsstrategien hinaus. Der Funktionsverlust des Haupthistokompatibilitätskomplexes der Klasse II (MHC II) bei der Gattung *Syngnathus* und ähnlicher Komponenten beim Seepferdchen (*Hippocampus*) hat Fragen zu ihren immunologischen Fähigkeiten aufgeworfen. Diese Besonderheit einer reduzierten, immunologischen Toleranz wird als möglicher Einfluss auf die Entwicklung einer fortgeschrittenen männlichen Schwangerschaft vermutet. In dieser Studie wurde das Transkriptom der männlichen Schwangerschaft verschiedener Syngnathiden Arten in verschiedenen Stadien der Schwangerschaft untersucht, um insbesondere die Rolle des adaptiven Immunsystems sowie die Rolle der immunologischen Toleranz bei der Evolution der männlichen Schwangerschaft aufzuklären.

Das erste Kapitel dieser Arbeit befasste sich mit der männlichen Schwangerschaft in verschiedenen Schwangerschaftsstadien. Die Genexpression des Brutgewebes innerhalb der Bruttasche wurde mit Hilfe vergleichender Transkriptomik bei verschiedenen Trächtigkeitsformen und Schwangerschaftsstadien (nicht trächtig, früh, spät und Geburt) analysiert. Immun- und schwangerschaftsbezogene Mechanismen wurden mit denen der

weiblichen Schwangerschaft verglichen, um festzustellen, ob diese ähnliche, evolutionäre Entwicklung mit denselben genetischen Vorgängen errungen wurde. Die Ergebnisse deuten darauf hin, dass es bei männlichen schwangeren Tieren vor der Geburt zu einer Verschiebung richtung Katabolismus kommt, die mit den Vorgängen während der weiblichen Schwangerschaft übereinstimmt. Darüber hinaus wurden bei allen hier untersuchten brütenden Arten während des frühen Schwangerschaftsstadiums hohe Entzündungswerte, die klassisch für die weibliche Schwangerschaft während der Einnistung der Eier sind, beobachtet. Die bedeutendste Entdeckung ist jedoch die Aktivierung und Verstärkung der Immuntoleranz während des frühen Schwangerschaftsstadiums bei bruttaschentragenden Syngnathiden. Diese Modulation des Immunsystems erfolgte durch die Herunterregulierung von MHC I-Genen, ein Vorgang, der bei den bruttaschenlosen Arten nicht zu beobachten war. Die Ergebnisse deuten darauf hin, dass die erhöhte feto-paternale Intimität, die während der frühen Schwangerschaft bei Syngnathiden mit Bruttasche auftritt, wahrscheinlich immunmodulatorische Prozesse erfordert, um die Abstoßung des Embryos zu verhindern. Darüber hinaus unterstützen sie die Annahme, dass immunologische Toleranz für die Evolution der fortgeschrittenen männlichen Schwangerschaft wichtig war.

Die Mechanismen der Allorekognition von Syngnathiden mit (*Syngnathus typhle*) und ohne (*Nerophis ophidion*) MHC II wurden in Kapitel II mit Hilfe von Flossentransplantaten und differenziellen Genexpressionsanalysen untersucht. Der Fokus lag auf der Frage, welche Immunwege während der Transplantation hochreguliert werden, insbesondere in Abwesenheit von MHC II, während gleichzeitig immunologische Toleranzschwellenunterschiede innerhalb und zwischen den Arten analysiert wurden. Es zeigte sich, dass Autotransplantate (Transplantate von eigenem Gewebe) und Allotransplantate (Transplantate von fremdem Gewebe) bei beiden Spezies eine Reihe von immun- und entzündungsbezogenen Genen aktivieren, während sie gleichzeitig Reparatur und Umbau des Gewebes anregen. *Gzma*, ein Gen, das für ein Granzym kodiert, das bei zytotoxischen T-Zellen (CTL) eine Rolle spielt, wurde nach Allotransplantaten im Gewebe von *S. typhle* im Vergleich zu autogenen Transplantaten hochreguliert. Dies könnte ein Hinweis auf eine MHC-I-vermittelte Immunantwort sein, die das Fehlen von MHC-II-Komponenten kompensiert. In *N. ophidion*-Autotransplantaten wurden Anzeichen für eine immunologische Toleranz durch eine Herunterregulierung der MHC I-Immunantwort festgestellt. Dieses Kapitel lieferte, neben einem wirksamen Protokoll für die Flossentransplantation, nützliche Einblicke in die Mechanismen, die bei der Transplantation und bei der Reaktion des Gewebes auf ein Trauma ablaufen.

Kapitel III dieser Arbeit zielte darauf ab, das Immunzellrepertoire des MHC-II-losen *S. typhle* durch die Anwendung von *single-cell* Transkriptomik zu charakterisieren. Der Fokus lag hierauf

den möglichen Auswirkungen dieses genomischen Verlusts auf die Evolution der Immunzellpopulationen in *S. typhle*. Durch die Verwendung bekannter Immunzellmarker konnte eine Reihe wichtiger Immunzellsubtypen identifiziert werden, darunter: T-Zellen, B-Zellen, Neutrophile, Makrophagen und Basophile. Wie in Kapitel II wurde auch *gzma* zusammen mit anderen Markern des T-Zell-Subtyps identifiziert. Dies soll ein Ansporn für künftige Studien sein, die verschiedene Immunzellsubtypen isolieren und untersuchen wollen. Das Fehlen von MHC II und CD4-verwandter Genexpression ist ein erster Beleg für die Abwesenheit von CD4+ T-Zellen in *S. typhle*. Dieses Kapitel stellt die erste Anwendung von *single-cell* Transkriptomik zur Immunzell-Charakterisierung eines syngnathiden Fisches dar und legt den Grundstein für künftige *single-cell*- und immunologische Untersuchungen dieser einzigartigen Tiere.

INTRODUCTION

Parental Investment

Parental investment - the dispensation of parental resources, both maternal and paternal - enhance the survival of the progeny while reducing the parental future reproductive potential (Trivers, 1972). There are many forms of parental investment, which include but are not limited to, gamete provisioning, placental nourishment and post-natal care, with parental contributions influenced by the animal's dyadic relationship (Trivers, 1972; Kleiman and Malcolm, 1981). From an evolutionary standpoint offspring can be perceived as an extension of their parents, and are solely responsible for extending the ancestral line. Selection thus favors a strong parental influence when it comes to ensuring offspring survival. The degree of investment is diverse among vertebrates and is tied to a number of reproductive processes. These include broadcast spawning and external egg laying (oviparity), which often rely on massive litter sizes to ensure the continuation of the parents genetic lineage, internalized egg incubation (ovoviviparity), pregnancy (viviparity) and intimate post-parturition parental care (Clutton-Brock, 1991). Investment is an important contributor to vertebrate reproduction evolution, with the historically accepted model of females investing more (genetic information, gestational and post-pregnancy nourishment), and males contributing less (only genetic) to brood development (Clutton-Brock, 1991). This, however, is not consistent with many bird and fish lineages which share parental duties more evenly and in some cases exhibit complete sex-role-reversals (Ahnesjö, 1989; Ketterson and Nolan Jr, 1994). In fact, when ranking parental care type prevalence in teleost fishes, male care ranks second above, bi-parental and female care (Sargent and Gross, 1986). This predominant form of care in teleosts, which is conceivably the evolutionary root accounts for roughly 60% of the total care instances, in some cases the male is completely independent in its duty (Gross and Shine, 1981; Blumer, 1982; Ahnesjö, 1989). The most frequently observed form of care among teleosts is nest guarding (95%), a system adopted by species such as sticklebacks and cichlids (Pressley, 1981; Goodwin et al., 1998). Other rearing roles that directly or indirectly support progeny survival include oral brooding and egg cleaning (Oppenheimer, 1970; Blumer, 1979).

Arguably the greatest beneficiaries of parental care are progeny that receive post-natal care such as nursing following internal gestation. The intricate adaptations that coalesced to enable internal gestation are likely correlated with advanced embryogenic assistance. In spite of its huge energy demands and the dangers that it poses to the brooders, viviparity surely constitutes the pinnacle of parental care and its independent evolution across various animal lineages has peaked the attention of many evolutionary biologists (Tinkle and Gibbons, 1977; Wourms and Lombardi, 1992; Blackburn, 1992).

The Birth of Viviparity

Viviparity, the reproductive term given to the internal retention of an embryo within a parent until embryogenesis is complete, is an evolutionary innovation that provides offspring with a nutrient supply, protection and platform for gas exchange (Blackburn, 1999). It is a product of a diverse set of evolutionary adaptations, tasked with roles including nutrient provisioning and immunity, culminating in a reproductive model of the highest investment. Phylogenetic research supports the notion that gestation holds oviparous (egg laying) roots; convergently evolving on numerous occasions to form a highly specialized form of reproduction (Blackburn, 2015). Within the commonly researched mammalian model, physiological and morphological adaptations such as the mammalian placenta can be considered evolutionary modifications of ancestral oviparous tissues and processes (Wourms, 1981). Placentas and there more intermediate forms have evolved independently, multiple times across vertebrate lineages (Reznick et al., 2002; Van Dyke et al., 2014). The placenta was formed through persistent apposition and eventual fusion of embryonic and parental tissue, procuring a physiological connection between offspring and parent. The resulting organ would come to mediate nutrient transfers and immunological processes ensuring the successful embryonic growth. It is clear that in every case of viviparity continuous reshaping and remodeling has occurred, with traits being lost and novelties being attained. Adaptations have been facilitated by genome duplications and rearrangements, lending to the formation of novel genes (Sitras et al., 2012) and functional modification of others (co-option) (Harlin-Cognato et al., 2006; Knox and Baker, 2008). The phenomenon, however, is not limited to mammalian species, having been found in almost every vertebrate class, with phylogenetic studies highlighting independent evolutionary emergences in over 150 vertebrate lineages (Weekes, 1935; Amoroso, 1968; Hogarth, 1976; Blackburn, 1982). Accordingly, the evolution of pregnancy is accredited with being one of the best examples of convergent evolution.

Primordial fish can lay tribute to the expansion of many morphological and physiological novelties such as the first functional jaw (Zhao and Zhu, 2010) and rudimentary adaptive immune system (Schluter et al., 1999; Boehm, 2011). Research also suggests that the first viviparous organisms were primitive fish, a group not often associated with parental care (Wourms, 1981). Notwithstanding the majority of modern teleost fishes are external fertilizers and contribute very little to progeny survival post-fertilization. However, despite this fact fishes still boast the highest diversity of reproductive strategies and consequently attract significant research attention due to their overarching importance in reproduction evolution (Baylis, 1981; Kolm, 2019). As with reptiles and mammals viviparity has convergently evolved a number of times in fishes and their developmental diversity of maternal-embryonic relationships surpasses those found in terrestrial groups (Turner, 1947; Baylis, 1981). However, many

anatomical, evolutionary adaptations found between the teleost gestation models and their mammalian counterparts differ, such as the lack of a true uterus and an allantoic circulatory system (Turner, 1947). Viviparous teleost examples include sharks and rays of which advanced viviparity is the dominant form of reproduction (Wourms and Demski, 1993), rockfishes which adopt a basic lumenal form of live-bearing (Wourms, 1991) and poeciliids which utilize a follicular pseudo-placenta to live-bear young (Thibault and Schultz, 1978) (Fig. 1). Viviparity in fish can be broadly divided into two types: Histotrophic viviparity, whereby fish develop in the oviduct of the parent and attain nutrients through means of oophagy and adelphophagy, and hemotrophic viviparity, in which the mother directly provides nutrients usually through a placenta (Wourms, 1981). The evolutionary path from external fertilization of gametes to highly specialized placental-pregnancy is a gradual process, with many intermediary branches. The advent of live-bearing offspring accompanied the reduction in egg number and ultimately litter size, but compensated by rigorous protection and in cases such as the ancient coelacanth by drastically increased yolk supplement (Wourms et al., 1991).

Figure 1. Teleost species exhibiting varying forms of viviparity; **(a)** whale shark (Rhincodontidae), **(b)** rockfish (Scorpaenidae) and **(c)** guppy (Poeciliidae). Photo credits: Simon Pierce (a), Dave Stafford (b).

Parental contributions such as feeding, nest building and protection are acknowledged behavioral characteristics shared across the animal kingdom, but come with an energy investment cost for the caring sex. When it comes to pregnancy and genetic influence, however, the paternal investment for mammals was long thought to be limited to gamete contribution. This has since shown to be an underestimation, with studies observing the transfer of paternally derived epigenetic motifs that influence offspring gene expression (Jablonka and Lamb, 2014; Wei et al., 2014). Conceivably, the most devoted males can be ascribed to the Syngnathidae fish family (seahorses, pipefishes, pipehorses and seadragons), which have developed an array of brooding structures, some of which are comparable to those found in eutherian mammals.

Male Pregnancy – An Evolutionary Anomaly

The most heavily invested male parents are the syngnathids, a fish family characterised by the unique evolution of male pregnancy (Herald, 1959; Dawson, 1985). Syngnathids are the only male organisms that have evolved specialized, placenta-like, brooding structures analogous to those found in mammalian maternal systems; with the female transferring eggs to the male prior to fertilization (Breder and Rosen, 1966). Among the 300 species, brood encapsulation tissue and structure varies greatly, with brooding males initially being subdivided into trunk (gastrophori) and tail brooders (urophori) (Herald, 1959). The trunk brooding group consists of the two genera *Nerophinae* and *Entelurus*, both of which exhibit external egg carrying structures with no physical protective function (Wilson et al., 2001). Tail brooders, which comprise the remaining genera (*Syngnathus*, *Hippocampus*, *Kaupus*, *Phyllopteryx* and *Corythoichtys*), have developed a diverse set of brood pouch morphologies (Hamilton et al., 2017) (Fig. 2). In particular, *Syngnathus* possess an inverted semi-sealed pouch, while *Hippocampus* have evolved the most advanced sealed pouch structure (Fig. 3) (Vincent et al., 1995; Wilson et al., 2001; Stölting and Wilson, 2007). A number of syngnathid species have evolved placenta-like structures similar to those found in eutherian mammals (Stölting and Wilson, 2007). Accordingly, aside from embryo protection, brooding structures are accredited with roles in osmoregulation, gas exchange, nutrient support, immunity and aeration during pregnancy (Haresign and Shumway, 1981; Partridge et al., 2007; Ripley and Foran, 2009; Ripley, 2009; Roth et al., 2012; Goncalves et al., 2015). Interspecies variation with regards to pouch morphology has led to studies examining the link between brooding type and degree of investment (Berglund et al., 1986; Masonjones, 2001; Carcupino et al., 2002); with a growing consensus that increased pouch complexity associates with greater investment. The aforementioned morphological adaptations and remodeling of the oviparous machinery played an integral role in pregnancy evolution across all species concerned. Additional cell system and gene expression changes were also crucial to overcome predicaments involving parental-fetal immune interaction (Aluvihare et al., 2004). Immune modulation was key to the evolution of the most advanced, intimate forms of gestation and the intriguing co-evolutionary relationship between these two conflicting systems has helped accrue a wealth of research (Billingham et al., 1953; Mor and Cardenas, 2010) .

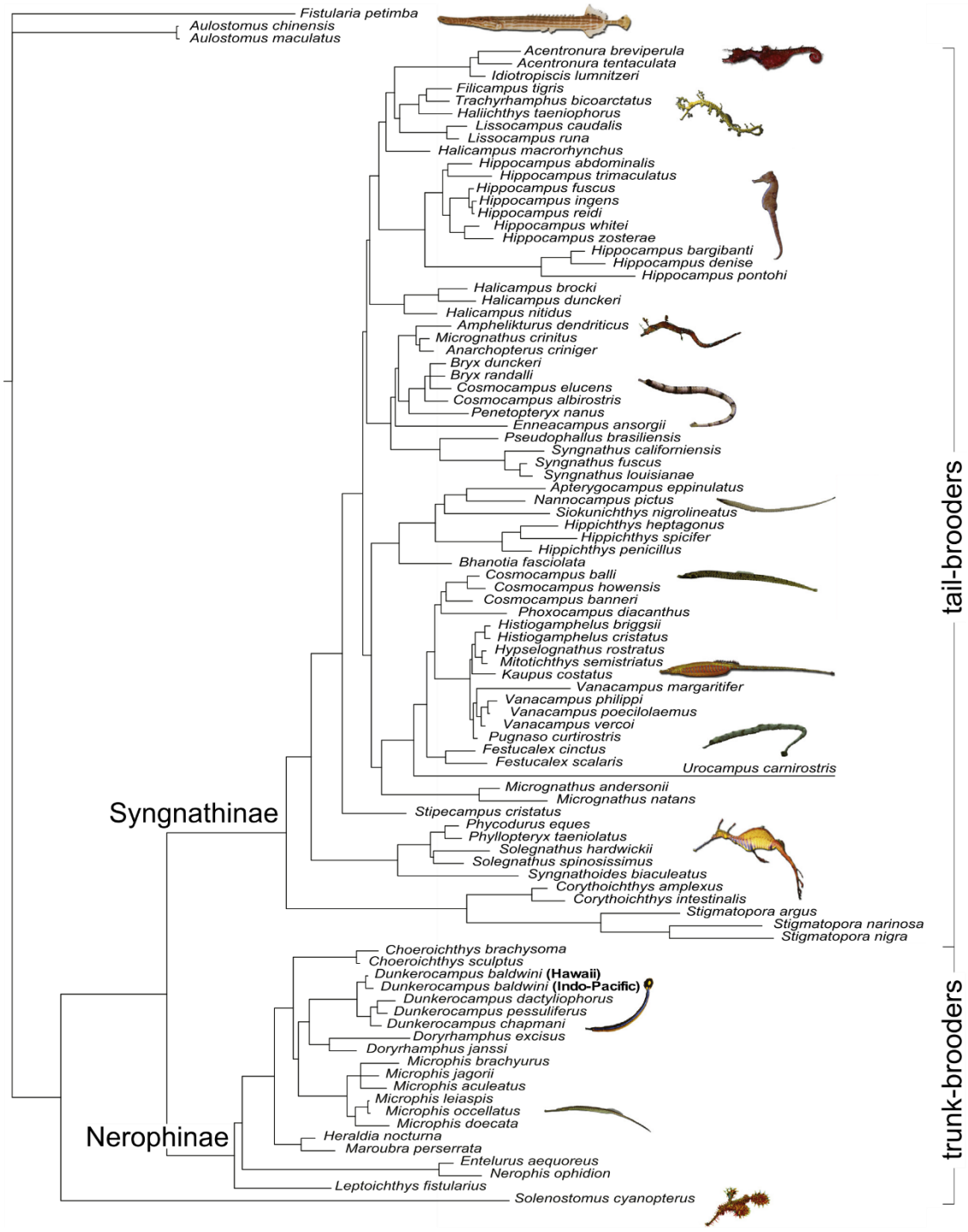


Figure 2. Phylogenetic visualisation highlighting Syngnathiformes and their brooding strategies (tail or trunk brooding). *Fistularia petimba* and *Aulostomus sp*. are without male pregnancy (adapted from Hamilton et al., 2017).

Figure 3. Syngnathid brooding types: (a) *Nerophis ophidion* (external egg-gluing/pregnancy), (b) *Syngnathus typhle* (inverted brood pouch) and (c) *Hippocampus erectus* (sealed brood pouch).

Co-evolutionary Relations – Pregnancy and the Adaptive Immune System

Thomas (1959) postulated that one of the keys to understanding immune system evolution is through the phenomenon of viviparity and in doing so was one of the first to allude to the connection between the two biological systems. The gastrointestinal region of a primordial jawed fish class (placoderms) was one of the first proposed origins of the adaptive immune system (Matsunaga and Rahman, 1998). This was thought to be as a result of increased internal infection that was inadvertently caused by the jaw adaptation and change in feeding habits. However, more recently others have shared Thomas's premise, propounding that the adaptive immune system emanated in large predatory fish that produced few young, as an additional form of defense to improve offspring survival (Flajnik and Kasahara, 2010). Moreover, propoundings that placoderms were internal fertilizers further support this claim (Long et al., 2009). Currently, it is generally agreed that the expansion of a number of vertebrate systems accommodating organism physical growth, likely coincided with adaptive immune system evolution (Kasahara, 2013).

The morphological and gene expression changes, such as immune gene modulation, are now known to be integral to a successful gestation (Moffett-King, 2002; Zenclussen et al., 2006;

Hedlund et al., 2009). However, pregnancy and immune system co-evolution creates a physiological dilemma, concerning the avoidance of embryo rejection through immune modulation and still maintaining a vigilant maternal immune function (La Rocca et al., 2014). These issues have been resolved in mammalian systems through gene expression changes at the onset and during pregnancy, specialized uterine/placental tissues and specific immune cell mechanism localizations (Moffett and Loke, 2006; Hedlund et al., 2009). Similar suppression of the adaptive immune system has been noted in syngnathid pregnancy, with the diversity downregulation of major histocompatibility complex I (MHC I) genes and even the complete loss of MHC II, which appears a striking solution to immune regulation when compared with the less drastic gene downregulation (Roth et al., 2020). MHC I and II, are key players in determining self from non-self and modulation of their expression during pregnancy is vital for progeny survival. The unusual functional loss of MHC II has been confirmed in the *Syngnathus* genus and loss of the associated MHC II invariant chain (CD74) in *Hippocampus* has stimulated conversation regarding their evolutionary links to advanced pouch pregnancy evolution (Roth et al., 2020). In conjunction with the presence of MHC II in the pouchless pipefish forms (eg. *Nerophis ophidion*) it is conceivable that the functional loss of MHC II may have facilitated the evolution of pouched pregnancy in syngnathids.

The assembly and analysis of a number of syngnathid genomes has been achieved over the last half decade. The work of Small et al. (2016) on *Syngnathus scovelli* and Lin et al. (2016) on *Hippocampus comes* helped unearth the genomic undertones that shaped many of the morphological changes found in syngnathids, such as the loss of ribs, pelvic fins and mineralized teeth. Furthermore, they built on previous work that identified the presence of “male pregnancy genes” such as an astacin metalloprotease named *patristacin* which may pay functional importance to brood pouch tissue (Harlin-Cognato et al., 2006; Small et al., 2013). Furthermore, *patristacin* represents one of the first examples of non-duplicative, male pregnancy specific gene co-option, which likely occurred during the evolution of the male brood pouch (Harlin-Cognato et al., 2006). Previous seahorse studies have sequenced the gestation transcriptome highlighting the potential activity of brood pouch specific, egg protection peptides and role of the leptin system on seahorse metabolism during pregnancy (Zhang et al., 2003; Zhang et al., 2016). Whittington et al. (2015) was the first to examine multiple stages of the seahorse pregnancy, documenting the upregulation of genes involved in tissue remodeling, immune modulation, and inferences that support that male and female pregnancy share a number of physiological evolutionary traits. Further transcriptome studies uncovered peculiarities in *Syngnathus typhle* immune defense repertoires, citing the functional loss of MHC II and its associated adaptive immune pathway components (Haase et al., 2013). This dominance of innate immune processes compared with adaptive was later reciprocated in *S.*

scovelli (Small et al., 2016). The confirmed genomic loss of MHC II genes was reported by Roth et al. (2020), reinforcing the previous functional findings and was the first to comparatively assess remodeling and evolution of multiple syngnathid immune repertoires. Crucially, these findings strongly supported the proposal that the trade-off of immunological tolerance and embryonic rejection partnered the evolution of male pregnancy and highlighted the remarkable flexibility of the vertebrate immune system.

The knowledge on male pregnancy and pregnancy convergent evolution as a whole has expanded rapidly in recent times and provided increasingly nuanced insights into their function and the pathways that molded them. Transcending the mammalian pregnancy model is essential to provide a more rounded understanding of this reproductive form and incorporating comparative studies between closely related species may help uncover the more subtle evolutionary changes that shape this phenomenon. Additional pathways and gene groups that contribute to the male pregnancy process are undoubtedly primed for discovery in this still comparatively under-researched pregnancy type. To this end, the syngnathid fish family and male pregnancy provide an ideal platform to explore such evolutionary characteristics, and grasping the mechanics of key concepts such as immunological tolerance could be beneficial to facets of biology that go beyond pregnancy altogether.

THESIS OUTLINE

To further the current understanding of the evolutionary pathways that shaped pregnancy as a reproductive system, research needs to delve into forms of pregnancy that transcend the mammalian model. The work outlined in this thesis aimed to adopt this approach by investigating the morphological and molecular characteristics of brooding syngnathid males, with the intention of highlighting key physiological pathways and immune system considerations that ensure a successful gestation period. The Syngnathidae male pregnancy presents an ideal system to carry out evolutionary associated research as it includes basal (*Nerophinae*), inverted pouch (*Syngnathus*) and advanced sealed pouch (*Hippocampinae*) brooding forms on a positive investment gradient. Furthermore, by all sharing a recent common ancestor, syngnathids provided the opportunity to utilize methods such as comparative transcriptomics to analyse the evolution of male pregnancy.

Chapter I – Immunological tolerance in the evolution of male pregnancy

In chapter I of this thesis a comprehensive multi-species approach was adopted to cross-examine gene expression patterns at sequential pregnancy stages, between Syngnathiformes species of varying brooding types. Comparisons with maternal pregnancy systems were used to ascertain whether homologous genes have convergently evolved within viviparous systems, while attempts were made to identify genes specific to paternal gestation. In a broader sense, clustering and gene ontology analysis were utilized to extract expression trends, while assessments were made concerning the influence of the immune system on the gestation period.

The first hypothesis of this investigation was that **(i)** replicates would cluster into their respective stages, within species, based on their gene expression profiles. Secondly, **(ii)** replicates of four closely related syngnathid species could be grouped based on pregnancy stage specific gene expression. Thirdly, **(iii)** influence of the immune system during gestation could be found in pouched brooders but not in external brooders. Next, **(iv)** immune suppression/modulatory processes were predicted to be more pronounced in brooding tissue during early pregnancy compared with late. Lastly, it was hypothesized that **(v)** homologous female pregnancy-related genes and pathways known from mammals would also be expressed in syngnathid male brooding tissue, while additional pathways specific to male gestation would also be found.

Results indicated a number of intriguing gene expression characteristics associated with male pregnancy. The upregulated expression of pro-inflammation genes during early pregnancy

suggest a facilitatory role during implantation, while metabolic processes appear to a catabolic state as pregnancy progresses. Signs of immunological tolerance measures in pouched syngnathids are evident during early pregnancy by way of downregulated MHC I related genes, leading to the proposal that immunological tolerance was crucial for the evolution of advanced male pregnancy. This was further substantiated by the absence of this trend in the most basal brooding form (*N. ophidion*) assessed here. Lastly, the reactivation of the adaptive immune system, particular in *H. erectus*, during late/parturition likely coincides with the opening of the brood-pouch prior to offspring expulsion. It is postulated that this opening is potentially due to a reduction in the feto-paternal connection at that time and to protect the highly vascularized internal tissue from external pathogens.

Chapter II – Comparative assessment of immunological tolerance in pipefish with and without the major histocompatibility complex class II

The MHC II functional peculiarities found within pouch bearing syngnathids poses many questions regarding the robustness, adaptability and efficiency of their immune systems. Moreover, the evolutionary impact of these changes on the ability to evoke crucial immunological tolerance measures and the possible implications regarding male pregnancy evolution provides a basis for a tantalizing study. By utilizing surgical fin-transplantations, chapter II aimed to examine the immunological tolerance and differential gene expression in syngnathid species with and without the MHC II pathway. In addition, this section aimed to highlight potential compensatory immune pathways involved in MHC II's absence.

The first hypothesis of this investigation was that from a visual standpoint **(i)** stronger signs of inflammation would be exhibited in *N. ophidion* allografts compared with the MHC II devoid *S. typhle* owing to the perceived increased immunological tolerance in *S. typhle*. The second hypothesis was that through gene expression data, **(ii)** a higher degree of allograft rejection would be shown in *N. ophidion* compared with *S. typhle*. Histocompatibility differences considered between autografts and allografts, it was predicted that within species rejection signs would be greater in allografts compared with autografts. Finally, **(v)** a higher degree of immunological tolerance to allogenic tissue was predicted to be expressed in *S. typhle* compared with *N. ophidion* based on the absence of MHC II.

Successful attachment of fin-transplants was achieved in *S. typhle* and *N. ophidion*, with visual observations two weeks post-surgery suggesting a more acute immune response in the pipefish with MHC II (*N. ophidion*). Differential gene expression analysis highlighted a number of upregulated immune related genes across both species; however, differences between allograft and autograft replicates were minimal suggesting an influence from surgical trauma.

The interesting upregulation of the cytotoxic T-cell related *gzma* in *S. typhle* allografts hints at an MHC I related response, potentially compensating for the MHC II loss, while the downregulation of MHC I in *N. ophidion* (autografts) hints at an immunological tolerance response.

Chapter III – Characterisation of pipefish immune cell repertoire through single-cell transcriptomics

Immune cell expression profiling and repertoire characterisations have been carried out on a number of model species (Carmona et al., 2017; Han et al., 2018). The advance of next generation sequencing methods and single-cell technologies have allowed scientists to unearth unique and profound discoveries that were otherwise unattainable using bulk profiling methods. Chapter III aimed to elucidate the potential consequences of these gene losses on the syngnathid immune system function and immune cell line populations. By utilizing single-cell RNA sequencing, it was possible to perform rapid transcript profiling of thousands of individual immune cells, culminating in the first characterisation of the broadnosed pipefish immune cell repertoire.

A number of key immune cell types were identified by using expressed gene markers accrued from previous research and immune cell characterization studies. Isolated cell types included, macrophages, neutrophils, thrombocytes, T and B lymphocytes, while other types such as erythrocytes and connective tissue cells were also identified. Moreover, in line with the evolutionary loss off MHC II in *S. typhle*, no indications of the associated $CD4^+$ T-cells were identified supporting the potential cell population loss in the species. The upregulation of T-cell subtype markers associated with cytotoxic and regulatory T-cells, encourages the need for further cell characterization studies to distinguish individual cell types at a higher resolution.

MODEL ORGANISMS – The Syngnathiformes

This thesis focused on four species of the Syngnathiformes order for research into the immune system processes and reproductive physiology. Syngnathiformes are among some of the most morphologically peculiar and physiologically distinct species; inhabiting a broad range of tropical and temperate coastal environments. Moreover, they boast the only instance of paternal pregnancy in the animal kingdom, within the Syngnathidae family. This unique viviparity example has encouraged considerable research into its evolutionary origins, with species such as *Syngnathus typhle* (broadnosed pipefish) and *Hippocampus kuda* (yellow seahorse) becoming increasingly popular for behavioral and molecular studies (Mobley, 2011;

Rosenqvist and Berglund, 2011). The following species were chosen for this thesis based on a gradient of increased parental investment and brood pouch complexity (Fig. 4):

Nerophis ophidion (straightnose pipefish) is the only representative of the gastrophori (abdominal brooder) used in this investigation, possessing the most basal form of brooding apparatus (Breder and Rosen, 1966). Embryos are glued to the male's ventral surface and fully exposed to the parent's environment (Herald, 1959). It is well camouflaged living among the eel/grass beds dispersed along the European coastal regions of the Mediterranean, Baltic and Black Sea (Dawson, 1986). Species of the *Nerophinae* subfamily have been found to possess a fully functional MHC II repertoire (Roth et al., 2020).

Syngnathus typhle (broadnosed pipefish) forms an embryo incubation system consisting of inverted epithelial tissue, isolating the brood from the environment (Breder and Rosen, 1966). The males are capable of incubating eggs from multiple females at the same time, a reproductive term known as polygynandry (Jones et al., 1999). Commonly found throughout the British Isles, Baltic and Mediterranean Sea among coastal vegetation (Dawson, 1986). *S. typhle* has been the subject of a significant number of recent molecular and behavioural studies, with fascinating discoveries showing the complete loss of the MHC II adaptive immune system pathway (Haase et al., 2013; Roth et al., 2020) and the links to MHC I status with mate choice (Roth *et al*., 2014). MHC genes have been shown to be downregulated during pipefish pregnancy while genes that show upregulation in early pregnancy include the pro-inflammatory TNF and the tolerance mediator TAP1 (Roth et al., 2020).

Syngnathus rostellatus (lesser pipefish) possesses a similar morphology and inverted brood pouch as *S. typhle*. It is endemic to the shallow coast regions of the Baltic Sea, British Isles and North Sea (Dawson, 1986). Recent studies have also confirmed the loss of the MHC II pathway in *S. rostellatus* (Roth et al., 2020).

Hippocampus erectus (lined seahorse) is prevalent along the west Atlantic coast from Nova Scotia to the Caribbean (Lourie et al., 1999; Carpenter and De Angelis, 2002), thriving in warmer tropical waters. As with other seahorse species, males evolved the most specialized brooding structure within the syngnathid lineage, a sealed pouch with placenta-like structures located on the tail. Unlike the majority of fish species, *Hippocampus spp.* were recently found to possess a solitary MHC IIβ gene, a key component of the adaptive immune system (Bahr and Wilson, 2011). The suggestion that the pot-bellied seahorse could be immunologically deficient in terms of the traditional adaptive immune repertoire, were supported by the genomic loss of the important MHC II invariant chain (CD74) (Roth et al., 2020).

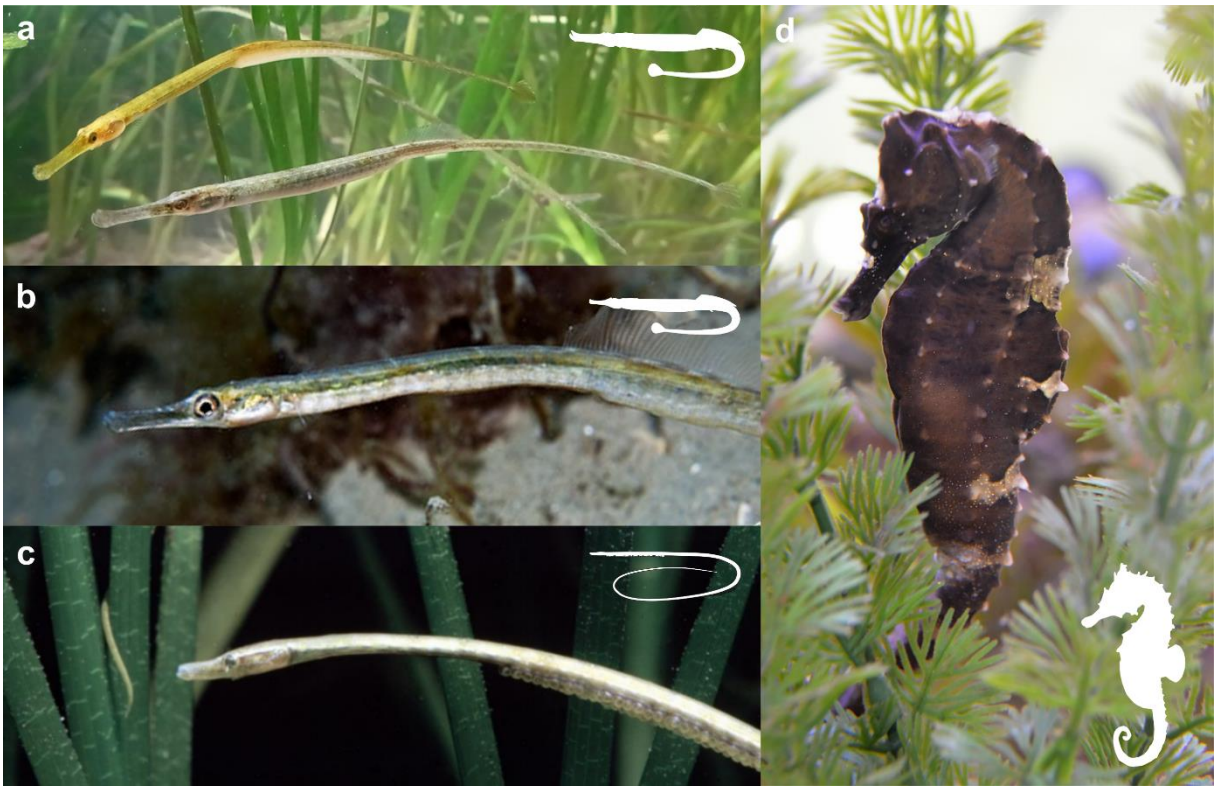


Figure 4. All four syngnathid species used in this thesis: (a) *Syngnathus typhle*, (b) *Syngnathus rostellatus*, (c) *Nerophis ophidion* and (d) *Hippocampus erectus*. Photo credits: Uli Kunz (a, d), Paul Kay (b) and Anders Berglund (c).

CHAPTER I

Immunological tolerance in the evolution of male pregnancy

Jamie Parker[a], Arseny Dubin[a], Ralf Schneider[a], Kim Sara Wagner[a], Sissel Jentoft[b], Astrid Böhne[c], Till Bayer[a] & Olivia Roth[a]

[a]Marine Evolutionary Ecology, GEOMAR Helmholtz Centre for Ocean Research Kiel, D-24105 Kiel, Germany; [b]Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, NO-0371 Oslo, Norway; [c]Center for Molecular Biodiversity Research, Zoological Research Museum Alexander Koenig, D-53113 Bonn, Germany.

Accepted with minor revisions, final revision submitted in *Molecular Ecology*

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 (non-pregnant, 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 feto-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: Immunity, male pregnancy, syngnathidae, immunological tolerance, evolution, transcriptomics

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 (Gittleman, 1981; Wourms, 1981; Bainbridge, 2014). 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 non-self can be partly attributed to the diverse set of major histocompatibility complex (MHC) genes (Ljunggren and Kärre, 1990; Edwards and Hedrick, 1998). The implantation of the semi-allogeneic fetus 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 co-activator (CIITA) expression are absent in invading embryonic trophoblast cells in mammals (Von Boehmer and Kisielow, 1990; Murphy and Tomasi, 1998), while MHC I is downregulated and non-classical MHC I upregulated to help modulate the first immune interactions between fetus and mother (Hiby et al., 2004; Murphy et al., 2009). Mammalian regulatory T-cells and natural killer cells control immune responses across the feto-placental 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 (Mor et al., 2011; Chavan et al., 2017).

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 (Garnica and Chan, 1996; Bell and Ehrhardt, 2002). Maternal metabolic processes that fluctuate during pregnancy, support the stage-specific growth demands imposed by the embryo and help maintain maternal homeostasis (Naismith and Morgan, 1976; Denne et al., 1991; Alvarez et al., 1996; King, 2000). Metabolic rate fluctuations during pregnancy have been reported in lizards (Beuchat and Vleck, 1990; DeMarco, 1993), mammals (Reynolds et al., 1986; Lain and Catalano, 2007; 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 and 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 (Herald, 1959; Dawson, 1985). 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 (Wilson et al., 2001; Carcupino et al., 2002; Ripley et al., 2010). *Nerophinae*, however, develop integument tissue, which swells in order to help hold and partially immerse the eggs (Carcupino et al., 2002) (Fig. 1).

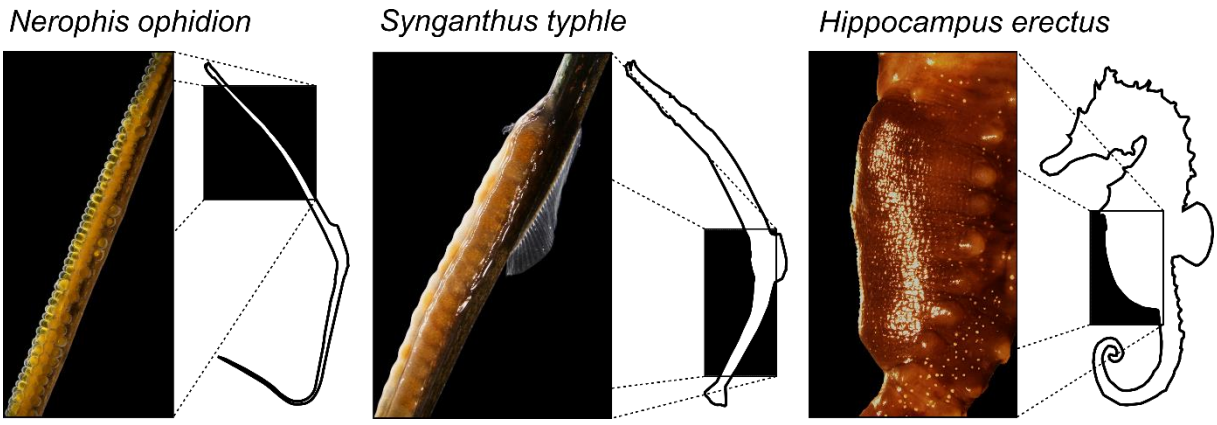


Figure 1. Syngnathid brooding types: *Nerophis ophidion* (external egg-gluing/pregnancy), *Syngnathus typhle* (inverted brood pouch) and *Hippocampus erectus* (sealed brood pouch).

Syngnathid research has highlighted genetic and morphological congruencies found between male and eutherian mammal reproductive systems (Stölting and Wilson, 2007; Small et al., 2013; Whittington et al., 2015; Roth et al., 2020), with special interest being funnelled into immunological and nutrient transfer processes (Ripley and Foran, 2009; Roth et al., 2020; Skalkos et al., 2020). Similarly, several studies have characterised brood pouch tissue gene expression at multiple pregnancy stages in *Hippocampus abdominalis* (Whittington et al., 2015; Lin et al., 2017) and more recently two *Syngnathus* species (Small et al., 2013; Keller and Roth, 2020; Roth et al., 2020), 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 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 non-pregnant, 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. Secondly, **(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.

MATERIAL & METHODS

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.

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 use in this experiment, lab-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,Roth (2016). All fish were kept in species-specific breeding groups in 100L 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.

Tissue sampling

Four pregnancy stages were targeted in this investigation: **Non-pregnant**, **early** and **late pregnancy,** and **parturition** (Fig. 2). Based on in-lab 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 non-pregnant 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.

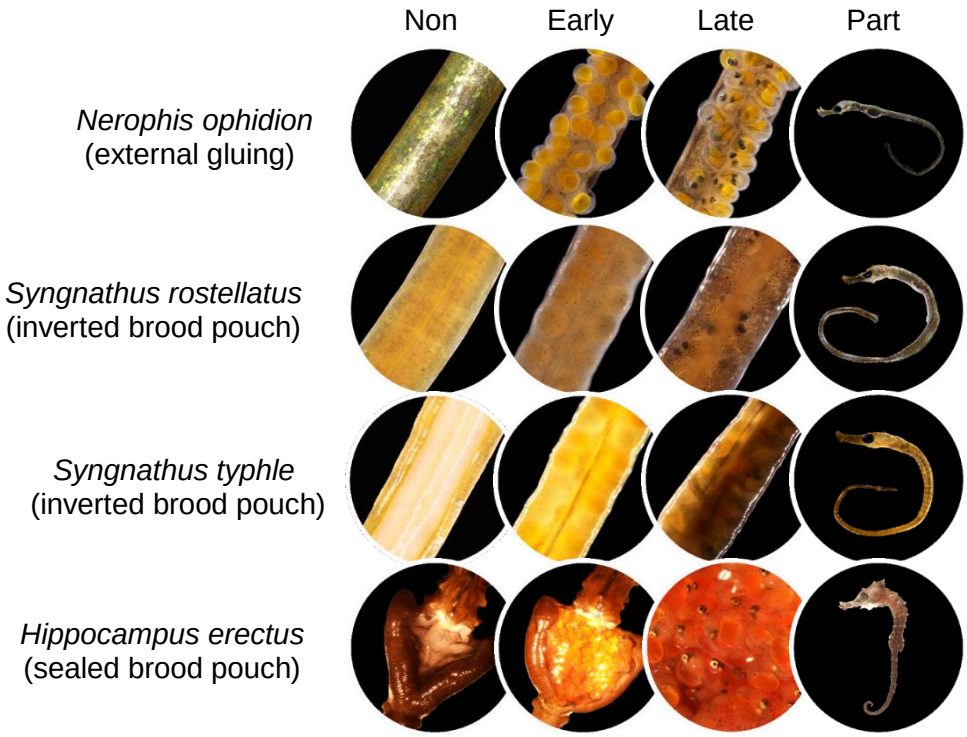


Figure 2. Visualisation of the different syngnathid brooding types used in this investigation and pregnancy stages at which brooding tissue was extracted. Gestation lengths: *N. ophidion* (24-28 days), *Syngnathus* (26-30 days) and *H. erectus* (14-16 days).

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

For RNA extraction, tissues were removed from the RNAlater, homogenized and RNA extraction was conducted according to manufacturer's recommendations using an RNeasy Tissue Mini Kit (Qiagen, Hilden Germany). Initial sample RNA quality was evaluated using NanoDrop-1000 spectrophotometer (NanoDrop) and Fragment Analyzer (Agilent Technologies) prior to library preparation. Library preparation was carried out using the Illumina TruSeq mRNA stranded kit with Illumina Unique Dual Indexes and the quality control of the libraries was performed using the Fragment Analyzer NGS standard sensitivity kit on a 12 capillary Fragment Analyzer (Agilent Technologies). Next, libraries were paired-end sequenced (Illumina, HiSeq 4000, 150bp reads). The library preparation and subsequent

sequencing were conducted at the Norwegian Sequencing Centre (NSC; https://www.sequencing.uio.no), University of Oslo, Norway. All resultant raw reads from all species were adapter trimmed using Trim galore! (v0.6.5) (Krueger, 2015) and quality checked using FastQC (v0.11.9) and MultiQC (v1.9) (Andrews, 2010; Ewels et al., 2016).

For transcriptome assembly, raw reads were first processed as follows: errors were corrected using the Rcorrector package with standard settings (v1.04) (Song and Florea, 2015). Due to the large number of reads in the dataset, they were normalized using Bignorm (v0.01) with the recommended settings (Wedemeyer et al., 2017). The normalized reads for all replicates within species were assembled to a transcriptome with the Trinity package version 2.8.5, with the normalization option turned off, but otherwise using default settings (Haas et al., 2013). To annotate them, the contigs were further analysed with the TransDecoder and Trinotate tools which are part of the Trinity package, versions 5.5.0 and 2.0.0 respectively, as recommended by the authors (Bryant et al., 2017). The TransDecoder prediction step was run with the options '—retain_pfam_hits' and '–retain_blastp_hits'. The Pfam-A, Rfam and Uniprot databases needed by these tools were downloaded in November 2019 (v2019_11).

Species-specific transcript abundance quantification was achieved by aligning the raw reads from each species to their respective transcriptomes using RSEM (v1.3.3) (Li and Dewey, 2011) and bowtie2 (v2.4.2) (Langmead and Salzberg, 2012). Abundance estimates were transferred using tximport (v1.18.0) (Soneson et al., 2015) in preparation for downstream gene expression analyses. Genes with raw count values < 5 that were represented in less than three replicates were excluded from downstream analyses.

Multispecies ortholog comparisons

Transcriptome assemblies underwent gene orthology analysis (OrthoFinder; v2.4.0) (Emms and Kelly, 2015; Emms and Kelly, 2019), utilizing 11 fish species as references (including the four used in this study) (supplementary; Table S1). Multiple sequence alignment was achieved using DIAMOND (v0.9.21) (Buchfink et al., 2015) and MAFFT (v7.475) (Katoh and 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 orthologs. To compare expression of each ortholog between stages and across species, transcripts per million (TPM) values for each ortholog were extracted, standardized to account for small differences in replicate TPM sum totals and log(n+1) transformed. Multiple copies of the same ortholog were tallied prior to standardization and transformation.

Ortholog comparison: Statistical analysis

Principal component analysis (PCA) was carried out first on orthologs 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 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; $P < 0.05$).

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 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.

Ortholog comparison: Functional group enrichment analysis

Gene ontology (GO) functional group annotation analyses were carried out on the top 675 most influential orthologs, using DAVID (v6.8) (Sherman and 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 orthologs for *Nerophis ophidion* were also subjected to GO functional enrichment analysis for comparative purposes only.

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: **Non-pregnant vs. parturition**,

early vs. parturition, and **late vs. parturition**. Resulting *p*-values were corrected for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Only genes with an adjusted *p*-value < 0.05 and an absolute log-2fold change expression of > 1 were considered for downstream analyses.

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 and 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.

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) (Mungal, 2003; Boyle et al., 2004) 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 and 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, allowinor 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.

RESULTS

Ortholog species comparisons

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

Principal component analyses

Within-species principal component analyses (PCA) demonstrated stage-specific sample clustering (Fig. 3). Multivariate analyses of variance (MANOVA) confirmed a significant stage (PCs; used: PC1-PC8) effect for each syngnathid species (supplementary; Table. S2), with *S. typhle* and *N. ophidion* displaying the strongest stage significance. Analysis of variance (ANOVA) and post-hoc Tukey tests highlight significant stage-specific differences within each PC, for each species (supplementary; Table. 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 non-pregnant and early pregnant replicates, while non-pregnant and late pregnant replicates differences were also shown to represent distinct clusters (Fig. 3a). Early pregnancy stage replicates were significantly different from both non-gravid 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 (Fig. 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 non-pregnant individuals (Fig. 3c). Lastly in *N. ophidion* (PC1), differentiation of samples belonging to the non-pregnant replicates from those associated with early stage was observed, while PC3 exhibited significant differences between early and the two non-gravid stages (non-pregnant and parturition) (Fig. 3d). Other PCA plot comparisons for each species were also conducted as a reference (supplementary; Fig. S2-S5).

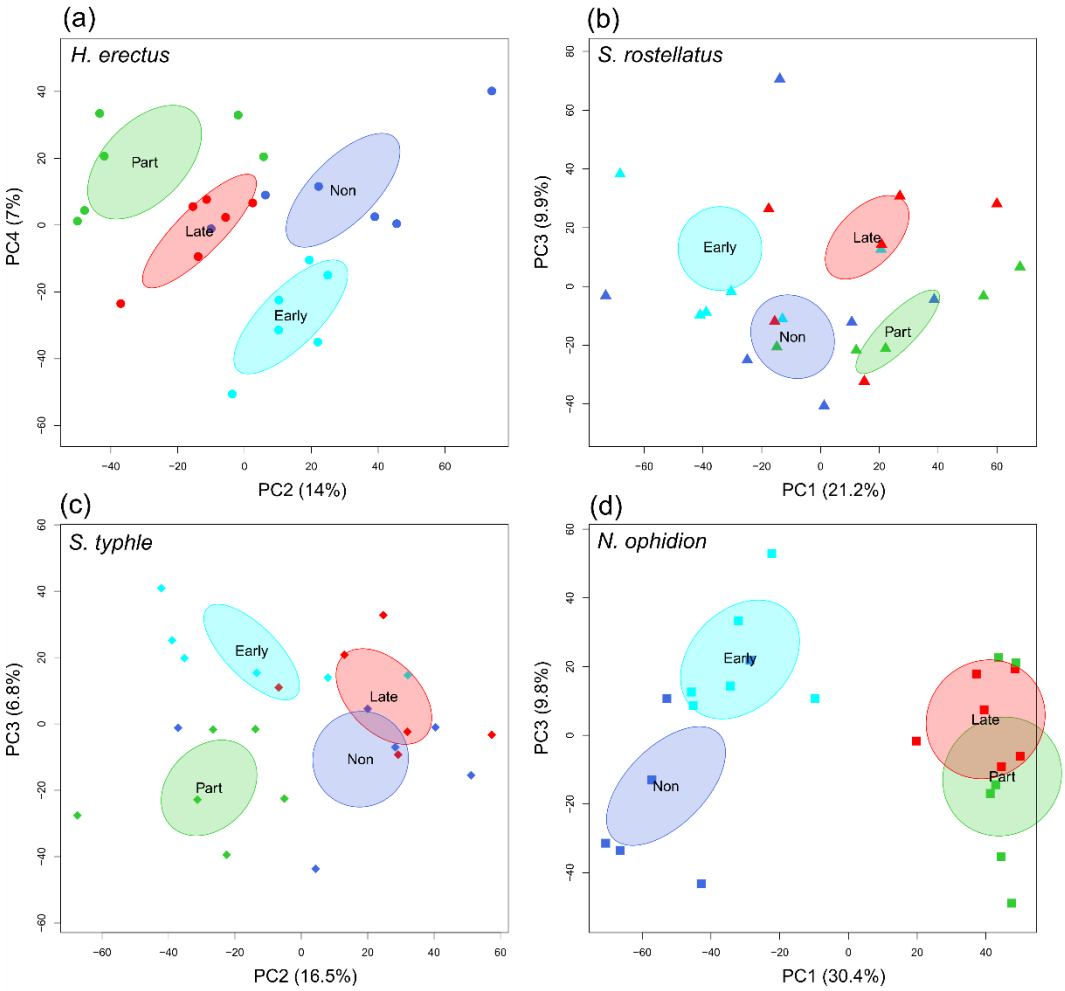


Figure 3. Principal component analysis for (a) *H. erectus*, (b) *S. rostellatus*, (c) *S. typhle* and (d) *N. ophidion* replicates based on ortholog loadings. Replicates pregnancy stage identification: non-pregnant (blue), early (cyan), late (red) and parturition (green). Ellipses represent 70% confidence. PCs represent the two most significant PCs for each species, for other comparisons see supplementary.

MANOVA on the residuals data set (species effect removed) identified significant stage differences within the first eight principal components (supplementary; Table. S5). PCA plots of the residuals suggested stage-specific separation of replicates (Fig. 4a, b, and c). Three PCA comparisons were used to distinguish differences between combined replicate groups; however, it was not possible to discriminate each pregnancy stage independently. Instead, PC2 (~12.9% variation) and PC4 (~4.6% variation) accounted for the starkest contrast found between the “initial” (non-pregnant 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 testing

(supplementary; Table. S6, S7). PC7 (~2.3% variation), appeared to account for the separation of non-gravid (non-pregnant and parturition) and gravid (early and late) replicates. ANOVA and post-hoc Tukey tests of PC7 yielded supporting significance of this separation (supplementary; Table. 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% (Fig. 4d) of the variation, respectively, with PC3 separating non-pregnant/early replicates from late/parturition, while PC4 divided gravid (early/late) from non-gravid (non-pregnant/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 testing. All PC score data used in this investigation can be accessed in the supplementary (supplementary data 1).

The top 675 highest-ranking (10%) orthologs were either assigned a positive or negative loading value (supplementary; Table. S8). These values can be used to determine how close the expression of an ortholog is associated to a particular gestation stage. Influential orthologs of interest driving the separation of gestation stages were highlighted for their potential roles during pregnancy in other viviparous species (Fig. 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 ortholog gene references for this section can be acquired in the supplements (Tables S9-S12).

In the “all species” comparison (Fig. 5), a number of MHC I pathway components (*mr1*, *ap1m1, erap2, sec24d, b2m, psma3*), transporters (*chmp4b, rab5a, slc9a2*), angiogenesis (*adam9*) and catabolism genes (*abhd12*, *casp8*) were among the orthologs whose expression was associated with the later stages (late/part). Additionally, immune genes (*ticam1*, *zc3h12a, cyld*) were also associated with the gestation’s concluding phases, while *plcd1* (placenta) was also present. Of the orthologs exhibiting expression that is positively associated with the beginning of pregnancy (non/early) *fgl1* stood out with its immune suppressive function and *cd82* (immune signaling). Other highly influential genes include those involved in angiogenesis and development (*plxdc1*, *mmp2*), placental formation (*emilin1*), muscle function (*tnni1*, *tnni2*, *tnnt3*) and inflammation (*csf1*). Strongly influential orthologs along the PC4 axis (late/parturition) include a number of proteasomes (*psme3*, *psmd13*, *psma4*, *psmc4*) involved in catabolism, as well as *tnip3* involved in inflammation. Notably, the proteasome *psma3*, which was among the most influential orthologs along the PC2 axis (late/parturition), was also influential along PC4 in the same stage direction. In the direction of non-pregnant/early stages,

orthologs such as *inpp5k* (cytoskeletal organization) and *sord* (metabolism) were also shown to be influential.

As with the all species analysis, a number of orthologs involved in MHC antigen presentation (*psma4*, *kifap3*, *ap1s2*) and inflammation (*tnip3*) were among the most influential in pouched species comparisons during the gestation climactic stages (Fig. 6). A strong influence was held by other immune genes during the later pregnancy stages, such as *irf2* (immune regulation) and *ppp6c* (innate immunity), as well as *gale* (catabolism) and *pycr1* (metabolism), all of which were specific to pouched species. *Ticam1* (innate immunity) and *psmd13* (catabolism) were shown to have a similar influence in the pouched species, as they did in the all species data set. As with the “all species” data set, *cd82* (immune signaling) showed an upregulation during the beginning of pregnancy in pouched species, while *gramd4* (innate immunity), and *emp2* (angiogenesis) were specifically influential during the infancy of pregnancy in pouch brooders. As in all species, cytoskeletal organization (*inpp5k)* and angiogenesis (*plxdc1*) and tissue repair genes, such as *mmp2*, were among the most influential genes characterizing the beginning of gestation. In pouched species, when considering the influential orthologs of PC4, a number of genes closely associated with the non-gravid stages (non-pregnant/parturition), namely *trim25* (immunity), *bcr* (GTPase regulation) and *adipor* (metabolism), were identified. Conversely, among the pouched species orthologs connected with the gravid stages (early/late) were the immune gene *csf1* (inflammation) and the cell cycle genes *ccnb2*, and *ruvbl1*. The placenta associated *emilin1*, which was linked with the initial pregnancy stages in all species, also showed gravid stage association among pouched brooders. Lastly, both *abcc1* (transport) and *emp2* (angiogenesis) were among the most influential orthologs of PC3 and PC4, and consequently, their direction suggests a strong association with early pregnancy. The influence of *psme3* (catabolism) and *kdrl* (angiogenesis) was also observed and were both connected closely with the late gestation stage replicates.

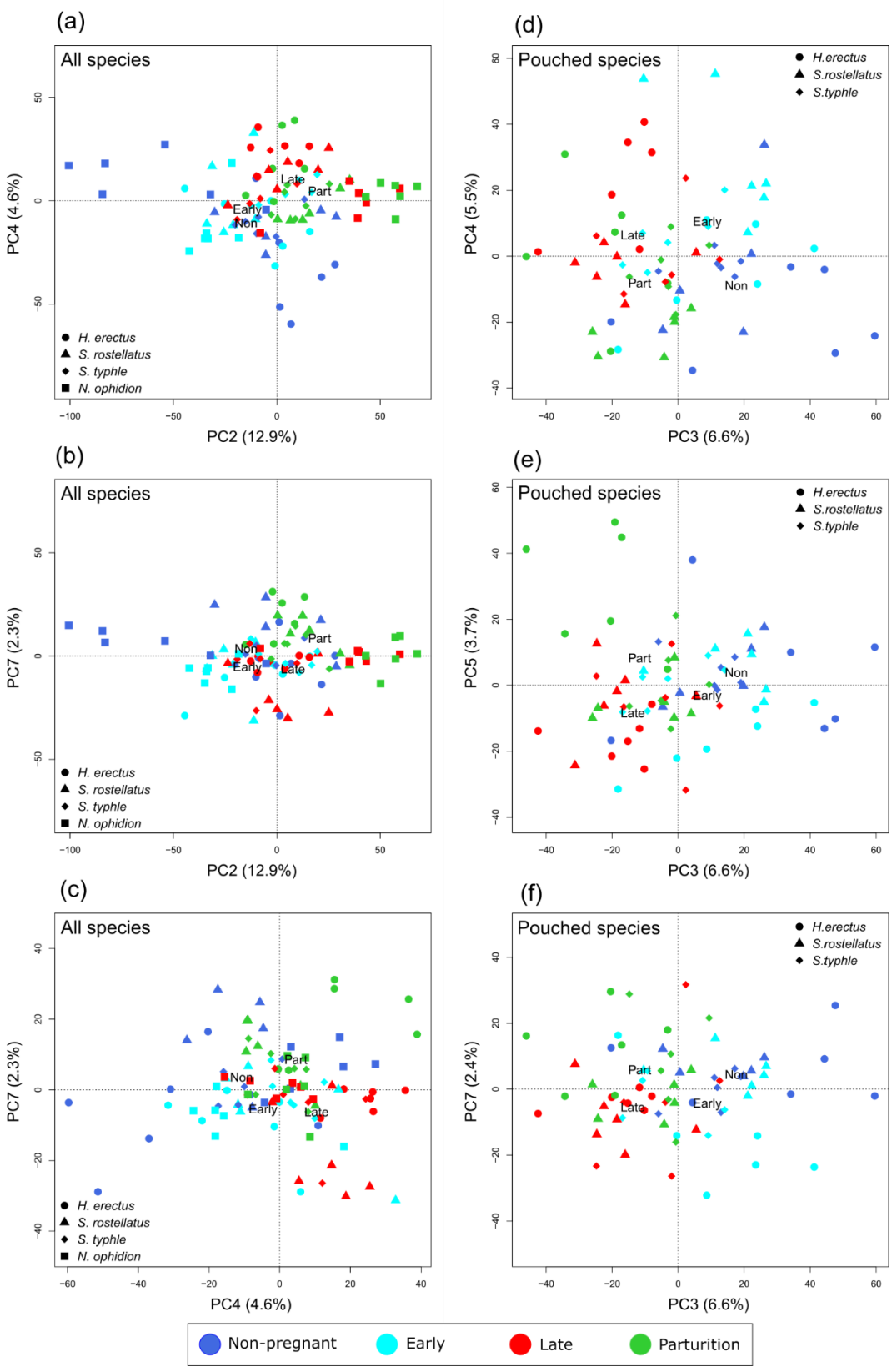


Figure 4. PCA component visualizations for all species combined (a, b, c) and pouched species only (d, e, and f). Stage text labels represent stage means.

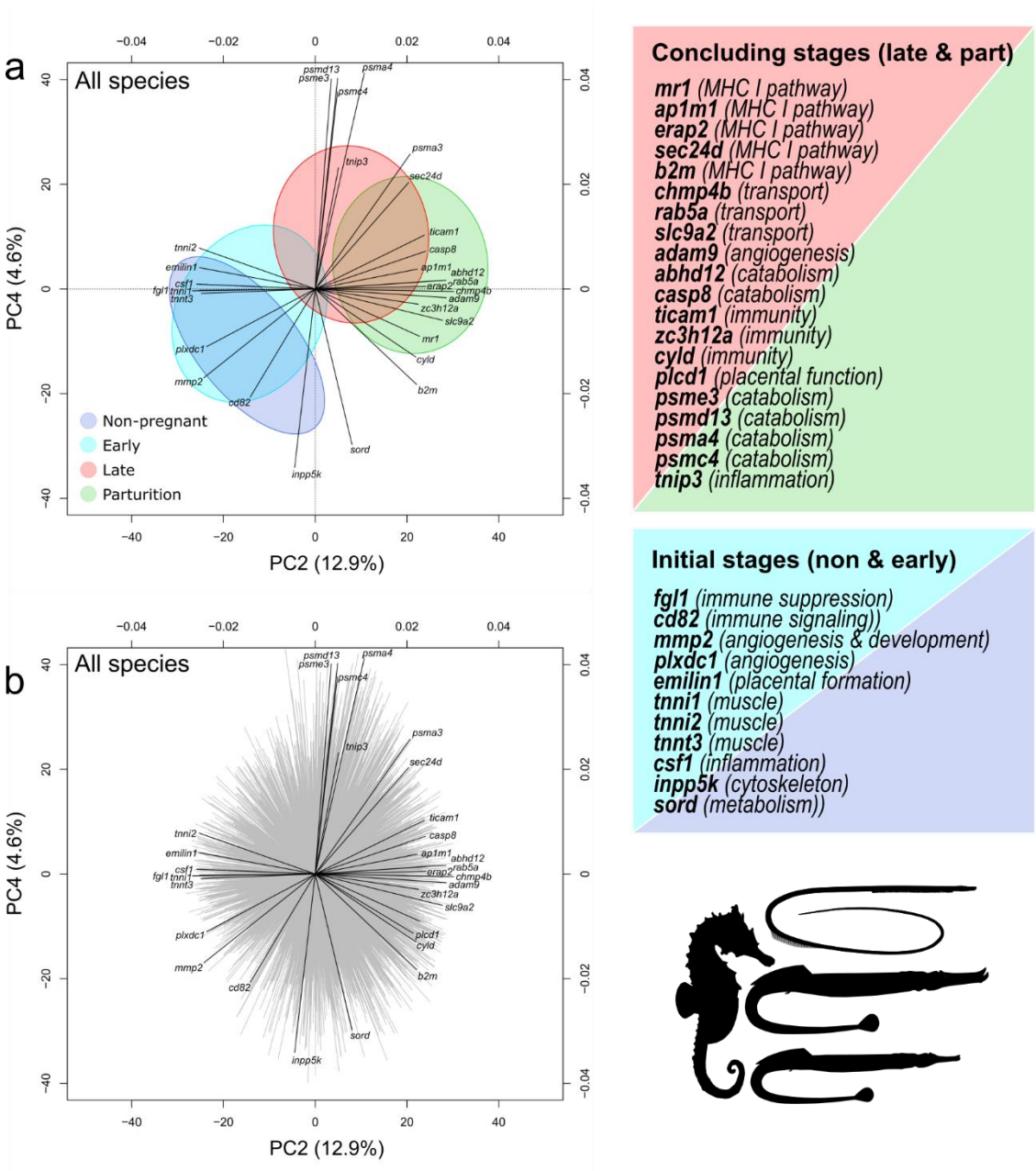


Figure 5. (a) PCA component plots for all species data set with genes of interest within the top 675 most influential orthologs and pregnancy stage ellipses. **(b)** PCA component plots with genes of interest and all other ortholog loadings used in analyses (grey). Ellipses represents 70% confidence.

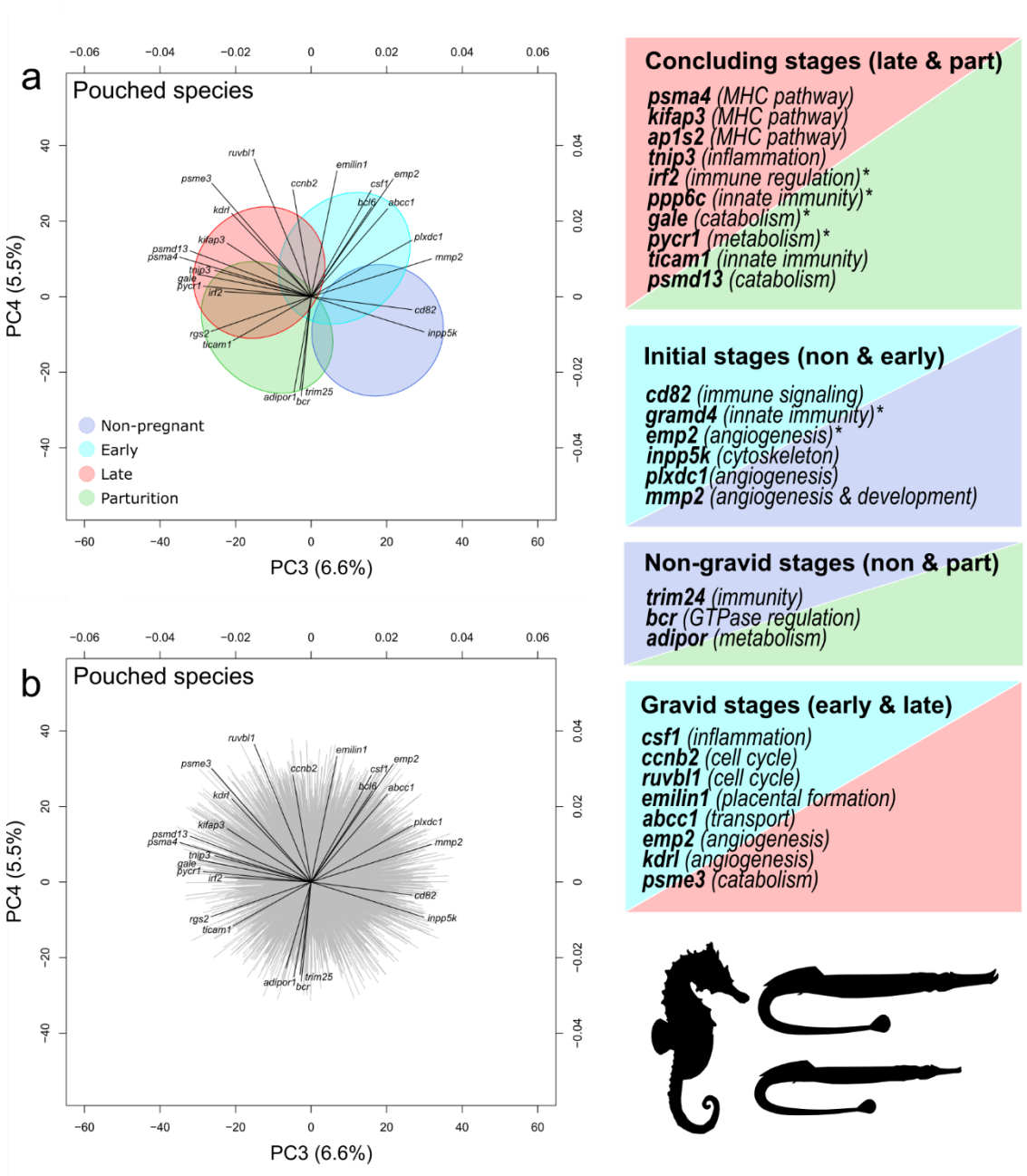


Figure 6. (a) PCA component plots for pouched species data set with genes of interest within the top 675 most influential orthologs and pregnancy stage ellipses. **(b)** PCA component plots with genes of interest and all other ortholog loadings used in analyses (grey). Ellipses represents 70% confidence.

GO functional annotation

GO functional enrichment analysis of the most influential orthologs highlighted a number of upregulated physiological pathways. When considering the “all species” data set (supplementary; Table. S13-S16), metabolic processes were shown to retain significance following FDR correction during the initial, non-pregnant/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 (supplementary; Table. S17-S20).

Human background GO functional enrichment, uncovered similar pathway enrichment findings, but with a higher degree of significance (supplementary; Table. S21-S28). This background was used to provide a perspective on the ortholog 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 orthologs characterising the two most significant stage defining PCs (PC1 and PC3) for *Nerophis ophidion* did not highlight any upregulated antigen processing and presentation processes (supplementary; Table. S29-S32).

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*, 2,088 genes in *H. erectus*, 3,196 genes in *S. rostellatus* and 1,158 genes in *S. typhle* (Fig. 7). All gene reference sources referred to in this section can be found in the supplements (Table. S33-36). 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 (Fig. 8).

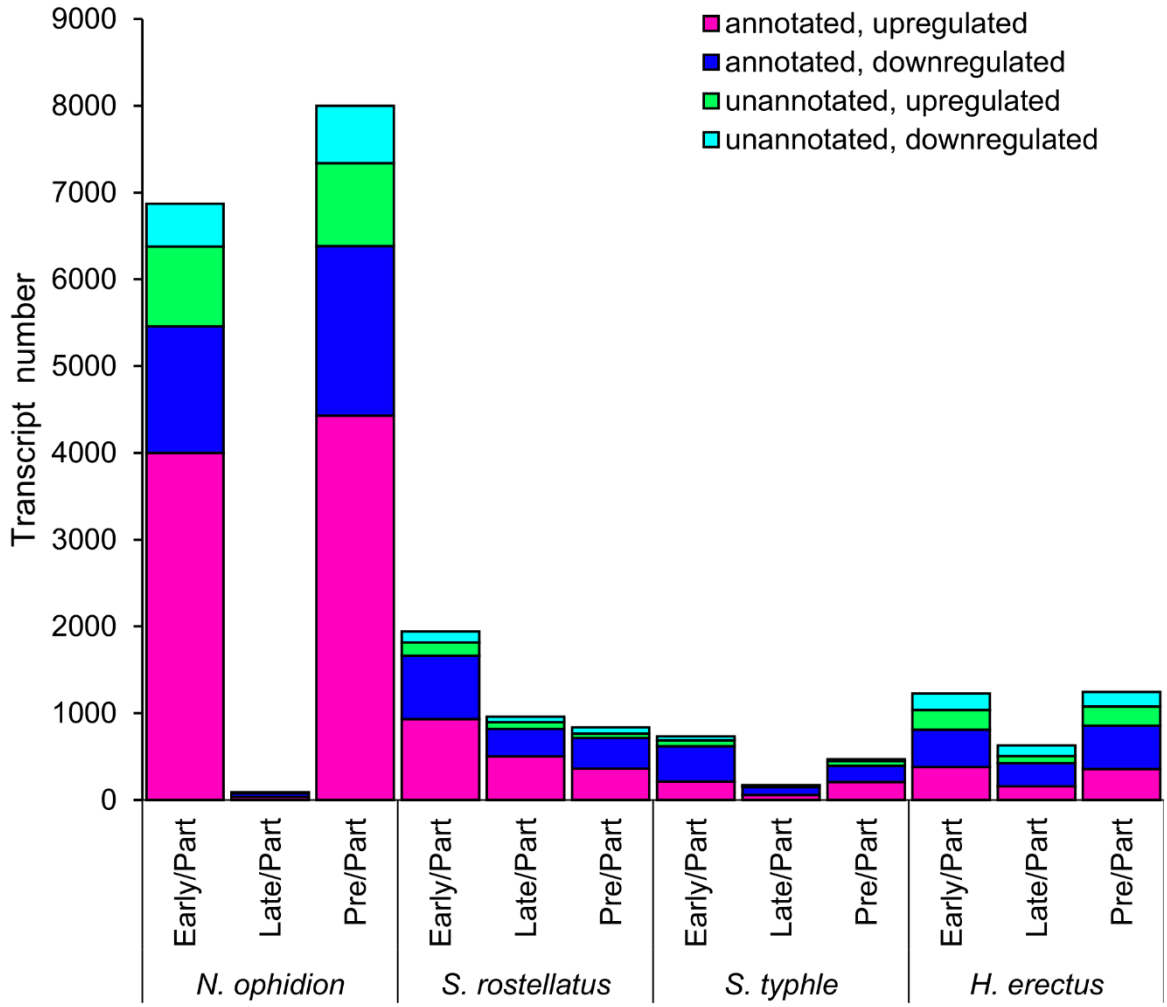


Figure 7. Differentially expressed transcript numbers for pregnancy stage pairwise comparisons (upregulated/downregulated).

A number of shared and distinctly expressed genes were observed across *Hippocampus erects* (H), *Syngnathus typhle* (T), *Syngnathus rostellatus* (R) and *Nerophis ophidion* (N). 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 (supplementary; 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 likely represent an alternative function. *N. ophidion* expressed a larger array of MHC I and MHC II related genes in non-pregnant 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.

The transport related *Apoa1* (metabolism) was upregulated in all species during pregnancy. All pouched species during early pregnancy exhibited the downregulation of *il-13ra2* (anti-inflammation), *xcr1* (immune signalling) and *tnf* (inflammation). The pro-inflammatory *il-17* receptor expression was upregulated in *H. erectus* and *S. rostellatus*, whilst being downregulated in the non-pouched *N. ophidion*. *Fkbp8* (apoptosis) was differentially expressed only in pouched species, with downregulations during non-pregnant (H, T, R), early (T) and late (R) stages. *Bcl6* (immune regulation) was among the few upregulated genes during non-pregnant, early and late stages in all three pipefish species, while the cytokine *il-12b* (pro-inflammation) exhibited upregulated expression during early pregnancy in the *Syngnathus* species.

A number of progesterone related receptors (*paqr3*, *paqr4, paqr5a*, *paqr6*, *paqr8, paqr9*) were differentially expressed (H, S, N), as well as prostaglandin proteins present in all species (*ptgfr*, *ptgis*, *pnpla8*, *ptgdr2*) and during pregnancy (*ptgfr*, *ptgis*, *pnpla8*, *ptgdr2*). The gene coding of the zinc-finger protein PRDM1 was downregulated during late pregnancy in *H. erectus*, throughout pregnancy in the *Syngnathus species*, but upregulated in the basal pregnancy of *N. ophidion* (early). All species differentially expressed one of either of the egg envelope proteases *lce* and *hce* during pregnancy, with consistent upregulation only being observed in early gestation (H, S, N).

Sealed brood pouch (Hippocampus erectus)

Among the most upregulated genes found during pregnancy in *H. erectus* were those involved in cytoskeletal/tissue remodelling (*capn2*, *rac1b*, *chi3l1*), apoptosis (*pawr*), angiogenesis (*angptl1*, *cmlkr1*) and adaptive immunity (*chi3l1*, *il-17r*) and innate immunity (*msrb1*, *crp*, *trim35*). *H. erectus* was the only species to exhibit consistent upregulation of *ccn1* (angiogenesis/tissue remodelling) during pregnancy. The anti-inflammatory interleukin *il-10* was upregulated in non-pregnant stages and during early gestation. The inflammation related protease inhibitor, *a2m,* was upregulated in post-parturition but downregulated in late pregnancy, while binding protein S100A12 showed elevated expression during early gestation. The most downregulated gene recorded in *H. erectus* during early pregnancy was the inflammation co-coordinator *selplg*. In addition, genes involved in catabolic processes (*cel*, *psmc2*, *ggact*) were strongly downregulated during the beginning of male gestation, while *mmp13* and *mmp9* were highly downregulated during non-pregnant, early and late stages. Immunologically significant downregulations in early and late stages of a number of adaptive (*cd274*, *bcl6b*, *csk*, *il-17r*) and innate (*tlr5*, *spon2*) immune related genes were observed in the

seahorse brood pouch. Other notable genes including *epx* (eosinophil & placental function) and *cd276* (T-cell modulation) were downregulated during early pregnancy and parturition stages, respectively.

Inverted brood pouch (Syngnathus typhle)

Genes exhibiting the most significant degree of upregulation during pregnancy in *S. typhle* included those with roles in RNA processing (*ahnak2*), DNA-binding (*supt6h, pif1, pol*), catabolic processes (*mep1a*, *mrc2*), viral function (*rep*, *erv3-1*) and vascular remodelling (*ereg*)**.** The tissue-remodelling gene *mmp13* was upregulated during parturition. Other prominently expressed genes include the chemokine receptor *ccr7* and *chia* (adaptive immunity), which were upregulated in early and the entirety of the gestation period, respectively. During early pregnancy, a number of upregulated inflammation genes were identified (*ly75*, *il-18r1, il-18rap*). A number of heat shock proteins (*hspa1l*, *hsp83*, *hspa5, hspd1, hspa9*) and *casp1* (pro-inflammation) were among the most downregulated genes during *S. typhle* pregnancy, as were a number with immune function (*coro1a*, *lgals3, lcp1*) during the initial stages of gestation. Unlike in *H. erectus*, the angiogenic *cmlkr1* was found to be downregulated during early pregnancy.

Inverted brood pouch (Syngnathus rostellatus)

The gene encoding for the key fertilization protease, *fetub*, was the most upregulated gene found during pregnancy in *S. rostellatus*; other upregulated genes include those with putative functions in transcription (*sall4*, *satb2*), metabolism (*apob*), development (*prtga***,** *fibin, col2a1*), and recombination (*rag1*). *S. rostellatus* pouch tissue exhibited an upregulation of *a2m* during early gestation, which was then followed by sharp downregulation during late pregnancy, as found in *H. erectus.* In addition, the tissue remodelling related genes *mmp2*, *ddr2* and *plg* were all upregulated during pregnancy. Furthermore, similarly to *S. typhle*, there was an upregulation throughout pregnancy of the multi-purpose vascular remodelling protein (*ereg*), which has additional suggested functions in tissue repair, wound healing and vascular remodelling. In congruence with the other pouched species, a selection of upregulated immune related genes was uncovered; standouts include *chia,* which was as highly expressed as in *S. typhle*, and the multi-functional interleukin, *il-6*. One of the most downregulated genes codes for the pro-inflammatory protein *casp1***,** which matched the expression trend of its functional relatives (*casp3*) and associated protein (*nlrp1b*) within the onset of pregnancy. *Epx* (eosinophil & placental function), as with *H. erectus*, was shown to be downregulated during early pregnancy, while the anti-inflammatory apoptotic *tnfrsf21***,** receptor subunit *il-10ra* and *cmlkr1* (angiogenesis) were all also downregulated during early gestation in *S. rostellatus*.

No pouch (Nerophis ophidion)

Among the most upregulated genes in gravid *N. ophidion* are those involved in muscle and collagen function (*mybpc1*, *casq2*, *obscn*, *myom1*, *col6a3*, *mbnl1*, *ttn*), fertilization (*fetub*), cell differentiation (*anxa13*, *egfl6*), development (*itgb8*, *sfrp1, klhl41, fhl1*) and inflammation (*cxcl8*, *cd44*, *tnfaip6*, *il-17rb, csf1, cd81*). Other significant findings regarding immunological function include the *cd40* receptor (antigen binding) and *ccl17* (T cell activation). Dominant downregulated genes include *cathepsins l-2*, which differs from the upregulation shown in *H. erectus*, a number of developmental (*fxr1*, *prtga*) and inflammation genes (*gps2*, *prxl2a*, *il-17rd, il-17rc*). Non-pregnant and early brood-pouch tissue downregulation of *epx* matched that of *H. erectus. Cmlkr1* (angiogenesis) expression as with *H. erectus* and contrary to both *Syngnathus* species was shown to be upregulated during early pregnancy.

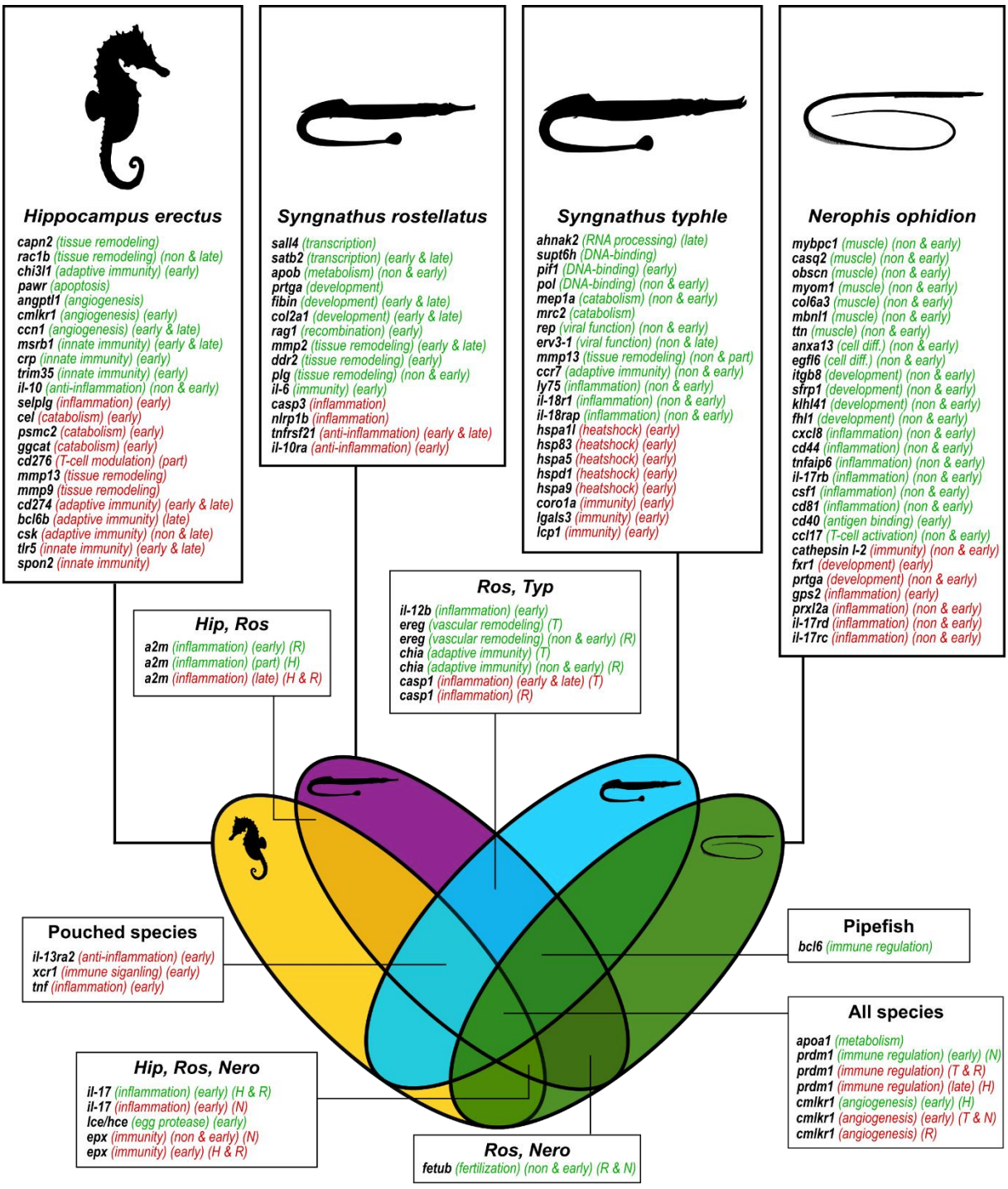


Figure 8. Schematic diagram depicting differentially expressed genes of interest in *Hippocampus erectus*, *Syngnathus rostellatus*, *Syngnathus typhle* and *Nerophis ophidion*. Genes highlighted are selected based on high and low expression and/or their roles in immunity and pregnancy. Upregulated genes 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).

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* (1,990 genes), *S. rostellatus* (481 genes), *S. typhle* (80 genes) and *N. ophidion* (10,500 genes). Clustering divided 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) (Fig. 9-12)(supplementary; 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 non-pregnant 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.

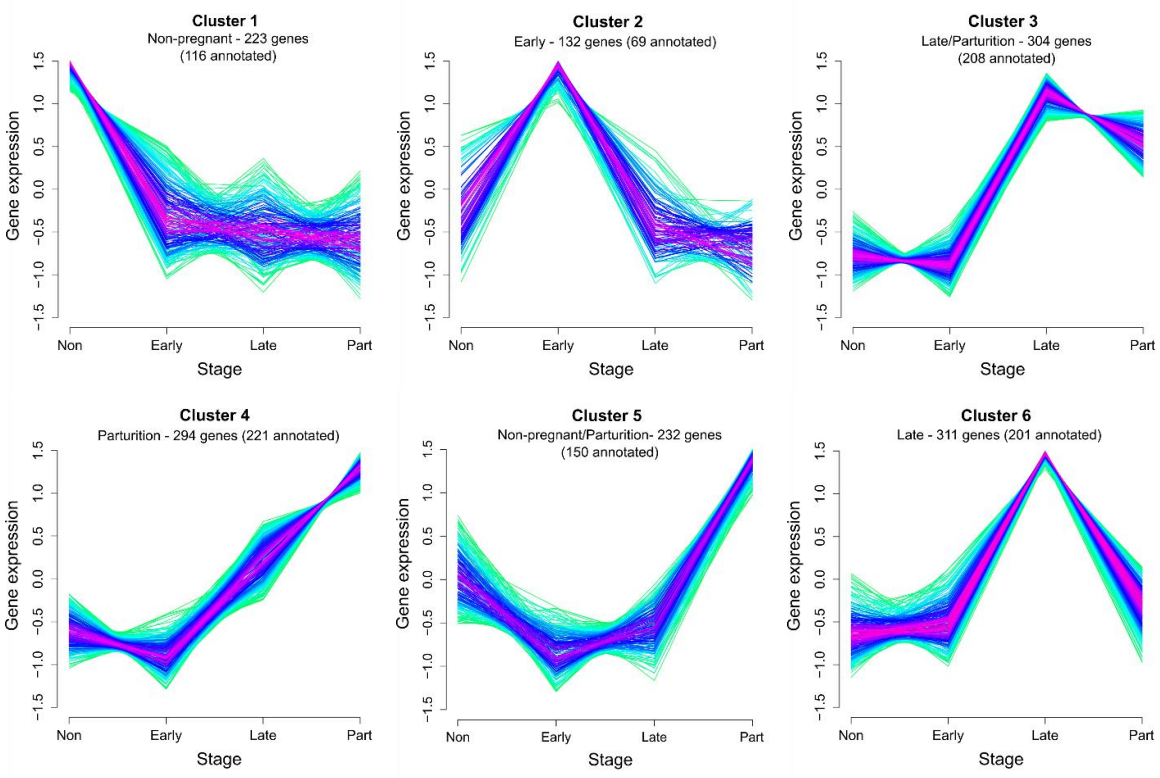


Figure 9. Soft-clustering of differentially expressed genes, characterising specific male pregnancy stages in *Hippocampus erectus.*

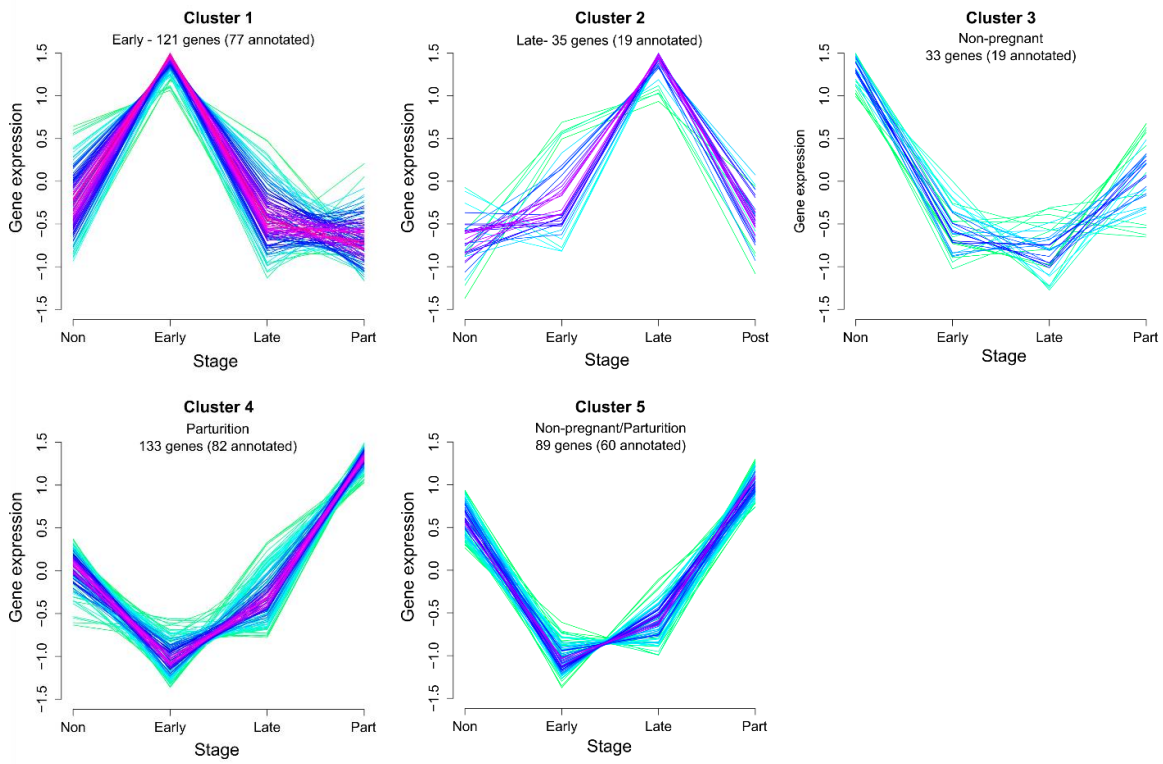


Figure 10. Soft-clustering of differentially expressed genes, characterising specific male pregnancy stages in *Syngnathus rostellatus.*

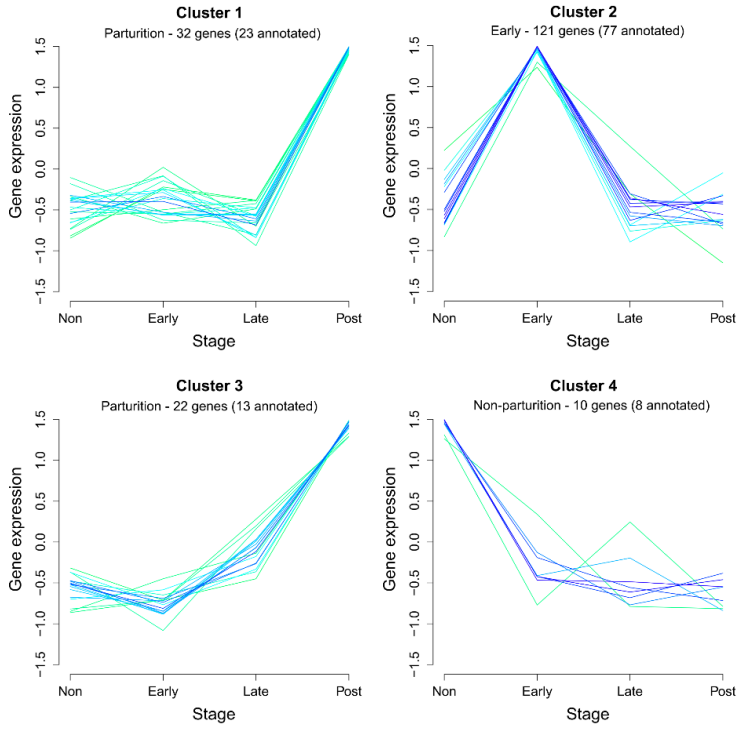


Figure 11. Soft-clustering of differentially expressed genes, characterising specific male pregnancy stages in *Syngnathus typhle*.

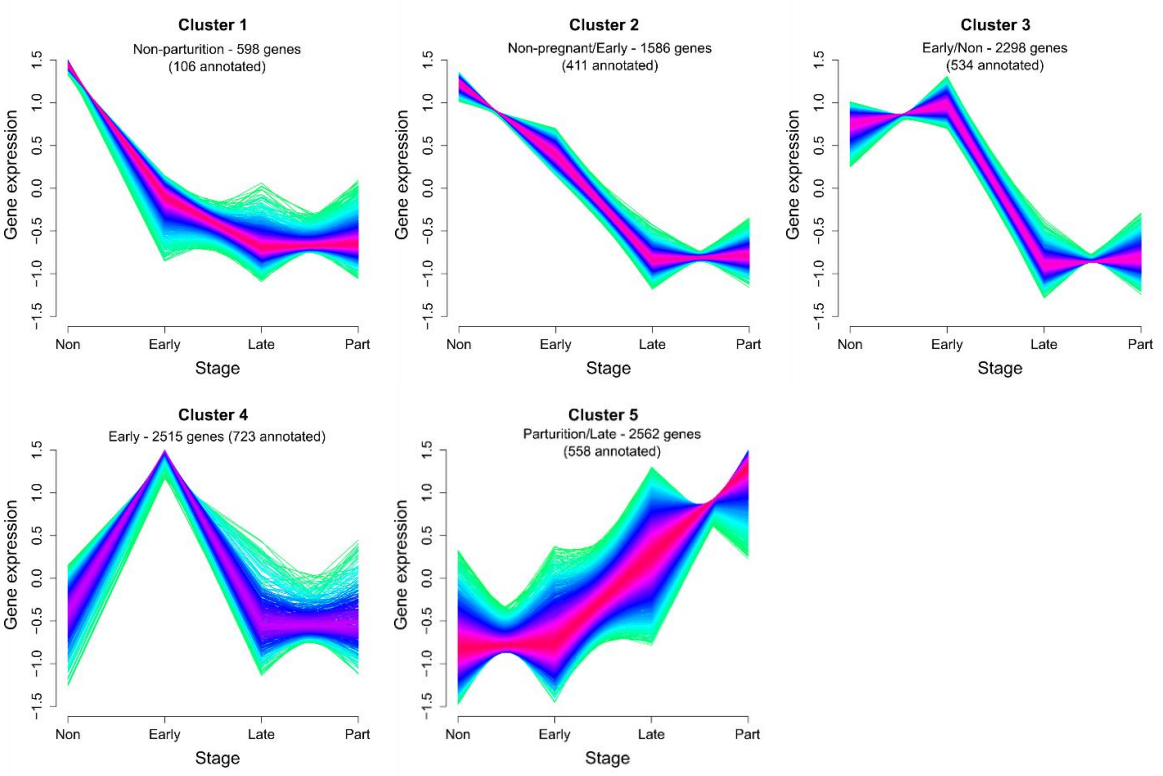


Figure 12. Soft-clustering of differentially expressed genes, characterising specific male pregnancy stages in *N. ophidion*.

GO functional annotation (mFuzz)

Broad GO slim term mapper annotations produced a number of patterns when comparing functions between pregnancy stages. Immune system processes were overrepresented in post-parturition and early upregulated compared with background levels in *H. erectus*, while cellular nitrogen compound metabolism (late, late/part) and biosynthetic processes (late/part) also showed an upregulation (supplementary; Table. S39). In addition, the genes associated with the parturition and non-pregnant/parturition clusters showed an eminent enrichment of transport proteins in *H. erectus* and *S. rostellatus*. Lastly, *H. erectus* and *N. ophidion* late/parturition clusters exhibited an upregulation in cellular nitrogen compound metabolism related genes (supplementary; Table. S39).

Functional gene annotations also unearthed a number of pathways specific for each cluster (supplementary; Table. 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 non-pregnant 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 non-pregnant 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* (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 non-pregnant-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 non-pregnant/parturition cluster (cluster 5) highlighted a number of genes involved in metabolic processes (*sgpp2*, *galc*).

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 and 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 (Helguera et al., 2009; Griffith et al., 2013; 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 (Small et al., 2013; Whittington et al., 2015; Roth et al., 2020) 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 and 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 (Whittington et al., 2015; Roth et al., 2020).

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 (non-pregnant/early) vs concluding (late/parturition) pregnancy stages is of particular strength in syngnathid fish. This brings to light shared orthologs 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 and 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 (Wilson et al., 2001; Carcupino et al., 2002; Stölting and Wilson, 2007). Nutrient provisioning from father to offspring has been suggested in pouched syngnathid species (Ripley and Foran, 2009; Kvarnemo et al., 2011; 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* likely 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; Laksanawimol et al., 2006; Dudley et al., 2021), 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 II and Cullum, 2014), and could also therefore be important for successful male pregnancy.

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 non-pouched 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 and Friesen, 2020), supported by active pouch defense mechanisms in seahorse pregnancy (Zhang et al., 2019; Wu et al., 2021). 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 and 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 (Watanabe, 1999; Carcupino et al., 2002), 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.

N. 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 and Shumway, 1981; Ripley and Foran, 2009; Kvarnemo et al., 2011), there is little evidence suggesting that syngnathids with external pregnancy are physiologically connected to the progeny during gestation (Kronester-Frei, 1975; Berglund et al., 1986). Accordingly, the reduced degree of feto-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 non-pregnant *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 non-pouched 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 clarify further if non-pouched 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 (Roth et al., 2011; Lin et al., 2016). Immune system states of pouch bearing syngnathids fluctuate owing to the shifting feto-paternal demands, and a consensus is growing that immune modulation is present and necessary (Whittington et al., 2015; Keller and Roth, 2020; Li et al., 2020; Roth et al., 2020). 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 6). **(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 feto-paternal tolerance in general, immunological mechanisms during male pregnancy on a stage-specific level are likely more nuanced. In humans, the upregulation of non-classical MHC I genes surrounding the feto-maternal interface has been recognised (Rapacz-Leonard et al., 2014), with some being reported in peripheral blood of pregnant women and invading extravillous cytotrophoblasts (Kovats et al., 1990; Hamai et al., 1999). 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 non-classical MHC I orthologs 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 (Mor, 2007; Orsi and Tribe, 2008; Challis et al., 2009). 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 (supplementary 1).

Signs of inflammation during human pregnancy are associated with the stages encompassing implantation and parturition, while the intermediary development/growth stage is characterised by anti-inflammatory processes (Mor, 2007; Mor et al., 2011). Successful embryo implantation

is believed to be heavily dependent on inflammation induced tissue swelling (Barash et al., 2003; Chavan et al., 2017); such swelling can also be observed during pouched syngnathid pregnancy to help maximise the surface area for the large number of embryos (Carcupino et al., 2002). Indications of pouched pipefish pro-inflammatory genes being upregulated, have been observed during pouch development (Roth et al., 2020), including those attributed with roles in mammalian embryo implantation, namely, *tnf*, prostaglandins and *il-6* (Griffith et al., 2017). The latter has been shown to regulate inflammation genes in pufferfish and pipefish, propounded to mediate pouch-specific inflammation in gravid seahorses (Bird et al., 2005; Roth et al., 2020; Wu et al., 2020), and was upregulated in the pouched *S. rostellatus* in this study. Another protein implicated in seahorse pouch immune function is the proteinase inhibitor, Alpha-2 macroglobulin (*a2m*) (Wu et al., 2020), which was found to be upregulated in pouch brooders in this study. Owing to its physiological influences when binding with TNFα, PDGF, IFNγ and IL-6 (Umans et al., 1999; Tayade et al., 2005; Tronco et al., 2020) and the prominence of these associates found in pipefish pregnancy (Roth et al., 2020), strong upregulation of *a2m* and *pzp* (*a2m*-like) in *S. rostellatus* (early), and *H. erectus* (*a2m*, early cluster; and parturition) may suggest it holds a similar initial, pro-inflammatory role in pouched syngnathids as it does in mammals. The downregulation of the anti-inflammatory receptor, *il-13ra2*, during early pregnancy in all pouched species only, and the upregulation of pro-inflammatory *il-12b* in both *Syngnathus* species, also supports this occurrence.

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 pro-inflammatory 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 pro-inflammatory processes during this time.

The pro-inflammatory orthologs, *ticam1* and *tnip3,* shared among all species was influential within the concluding pregnancy stage replicates and remained influential following the removal of *N. ophidion*. This may indicate that at least in pouched species pro-inflammatory processes help drive parturition.

The inflammatory role of prostaglandin hormones during mammalian reproduction include assistance with blastocyst implantation, maintenance of pregnancy, luteolysis and parturition

(Kennedy, 1977; Kelly, 1994; Poyser, 1995). Several prostaglandin related genes were found to be differentially expressed across all pregnancy types in this study. Progesterone is another key hormone accredited with pregnancy maintenance function, a role in the suppression of the maternal immune system to fetally derived antigens, and priming the endometrium for implantation (Rothchild, 1983; Spencer et al., 2004). Progesterone expression has also been cited previously in pouch brooding syngnathids and its external influence on seahorse testis and pouch development have been suggested (Roth et al., 2020; Qin et al., 2020). The upregulation of a number of progesterone receptor genes in *H. erectus* could infer similar importance for supporting gestation; however, contrary expression in *S. rostellatus* suggests it may be specific to the most advanced form of male pregnancy.

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; Wegmann et al., 1993; Raghupathy et al., 2000). 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 and 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 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; Lee et al., 2008; Lee et al., 2011; Komi et al., 2016). Roth et al. (2020) highlighted the downregulation of *chia* in developed non-pregnant *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 feto-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 pro-inflammatory 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 likely 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 (Ripley, 2009; Kvarnemo et al., 2011; Skalkos et al., 2020), is limited in non-pouched species (Kronester-Frei, 1975; Berglund et al., 1986). Considering the deductions made here regarding the lack of feto-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 (Wilson et al., 2001; Carcupino et al., 2002; Ripley et al., 2010) 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 likely a driver of these expression disparities. Only the ortholog-based dataset 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 orthologs 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 and 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 pro-inflammation 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 multi-species 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.

CHAPTER II

Comparative assessment of immunological tolerance in pipefish with and without the major histocompatibility complex class II

Jamie Parker[ab] & Olivia Roth[ab]

[a]Marine Evolutionary Ecology, GEOMAR Helmholtz Centre for Ocean Research Kiel, D-24105 Kiel, Germany,
[b]Marine Evolutionary Biology, Christian-Albrechts-University, D-24118 Kiel, Germany.

Submitted for review in *Developmental and Comparative Immunology*

ABSTRACT

The major histocompatibility complexes (MHC) are famous for their pivotal functions in the vertebrate adaptive immune system, charged with roles in allorecognition and T-cell antigen presentation. To this end, the genomic loss of the MHC II pathway in the *Syngnathus* pipefishes raises questions regarding their immunological vigilance and in particular their ability to recognise non-self antigens. By utilising allograft and autograft fin-transplants, we investigated and compared the allorecognition immune responses of two pipefish species, with (*Nerophis ophidion*) and without (*Syngnathus typhle*) a functional MHC II system. Transcriptome-wide assessments were conducted to explore the immunological tolerance and potential compensatory measures occupying the role of the absent MHC II. Visual observations suggested a more acute rejection response in *N. ophidion* allografts compared with *S. typhle* allografts. A number of differentially expressed genes involved in inflammation and innate immunity were identified among transplantees, as well as genes with roles in vascularisation and tissue recovery. The intriguing upregulation of the cytotoxic T-cell implicated *gzma* in *S. typhle* allografts, suggests a prominent MHC I related response, which may compensate for the MHC II and CD4 loss. MHC I related downregulation in *N. ophidion* autografts hints at an immunological tolerance related reaction. These findings indicate alternative measures evolved to cope with the MHC II genomic loss enabling the maintenance of appropriate tolerance levels. This study provides some intriguing insights into the immune and tissue recovery mechanisms associated with syngnathid transplantation, and can be a useful methodological reference for future studies focussing on transplantation transcriptomics in non-model systems.

Keywords: immunological tolerance, syngnathidae, MHC, immune system, adaptive immunity, transplant

INTRODUCTION

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

The discovery of MHC proteins in teleost fish arrived many years after their identification in mammals (Hashimoto et al., 1990), with ancestral genome duplications and rearrangements in early teleosts giving rise to a vast diversity of MHC structures (Grimholt, 2016; Wilson, 2017). For years MHC II was believed to be synonymous among teleosts and mammals, however, the discovery of complete loss of the MHC II in *Syngnathus* and a functional loss in *Hippocampus* has ever since challenged this idea (Haase et al., 2013; Roth et al., 2020). These findings brought into question the fishes' immunological capacity with particular concerns over its ability to determine self from non-self.

Allorecognition can be assessed using allogeneic transplants, with historical research carried out on mammals highlighting the importance of MHC in mediating allograft rejection and determining histocompatibility (Snell and Higgins, 1951; Dausset, 1981; Snell, 1981). Fish related transplantation experiments have also provided sufficient grounding for evaluating allorecognition, however, none have investigated fish with natural MHC II deficiency and the majority have been unable to utilise next generation sequencing advances to substantiate physical phenotypic observations (Kallman and Gordon, 1957; Hildemann and Haas, 1960; Kallman, 1970; Hildemann, 1972; McKinney et al., 1981; Cardwell et al., 2001). Owing to the vast array of effector mechanisms adopted by the immune system to eliminate invading

pathogens, it is crucial that the immune system avoids promoting an immune response against self, a process known as self-tolerance (Goodnow et al., 2005). Recognition process complications often contribute to the development of autoimmune diseases (Nepom and Erlich, 1991; Fernando et al., 2008). As MHC are important mediators of self-tolerance, it stands to reason that the evolutionary loss of MHC II in *S. typhle* may have impacted its ability to determine self from non-self and facilitated an increased level of immunological tolerance.

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

MATERIALS & METHODS

Ethics Statement

All aquaria maintenance methods and experimental procedures used in this investigation meet the guidelines issued by the German Animal Welfare Association (Permit no. V242 –

35167/2018) and are in accordance with German Law. No endangered species were used in this investigation.

Animals

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

Fin Transplants

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

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

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

Tissue sampling

Fish were monitored daily for 14 days post-surgery documenting changes in behaviour and movement. At day 15, all fish were euthanized with an overdose of MS-222 (Tricaine, 500 mg

L^{-1}; Sigma-Aldrich, Munich, Germany) and the surgical area tissue was removed, without the transplant, as well as the gills, before being immersed in RNAlater. Samples were then immediately incubated at 4 °C for one week to allow for percolation, before relocating them to -20 °C in preparation for RNA extraction. Tissues were thawed and homogenised using a tissue shredder (Qiagen) prior to total RNA extraction utilising the RNeasy Tissue Mini Kit (Qiagen, Hilden Germany).

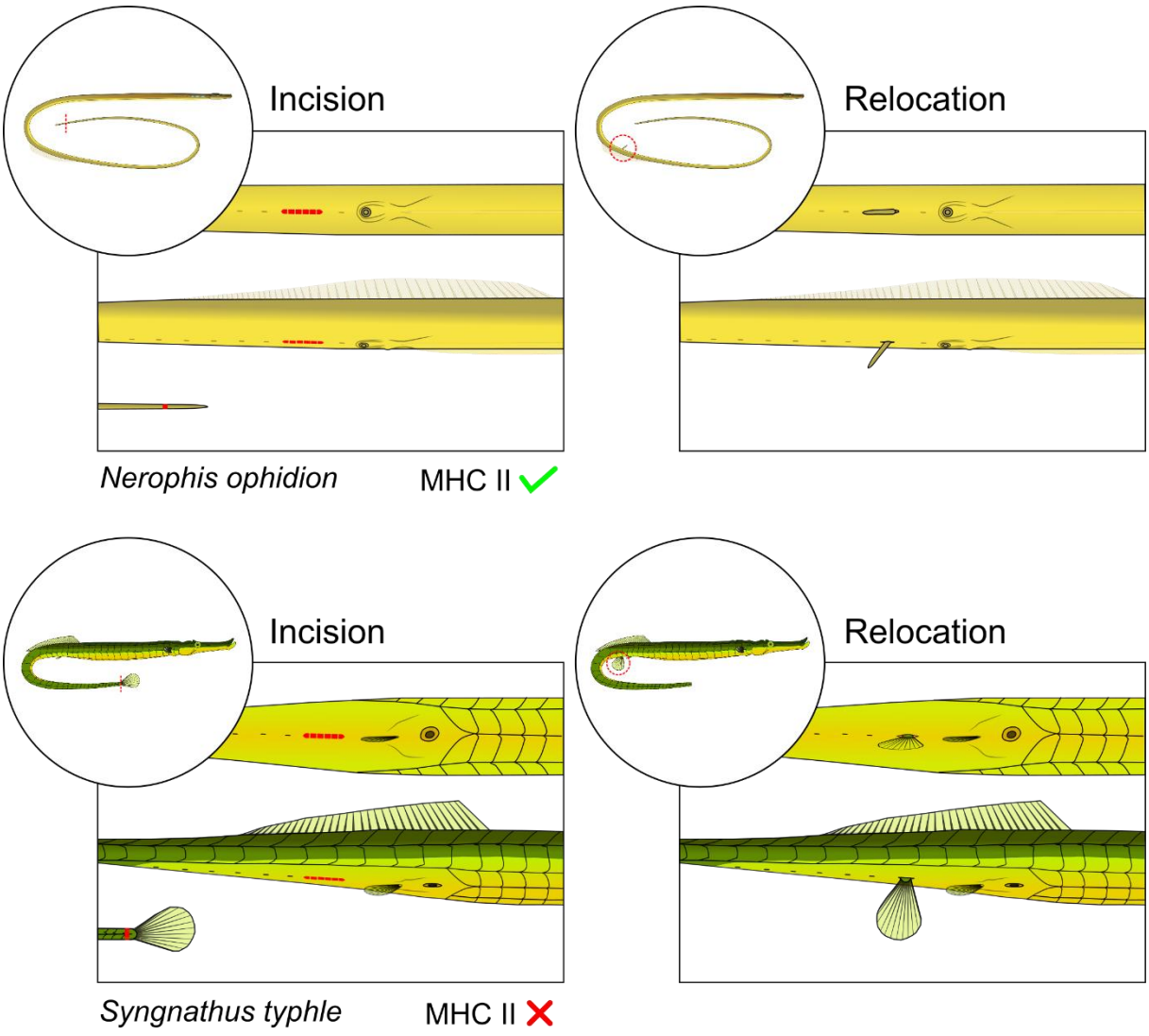


Figure 1. Fin transplant incisions and relocations for both syngnathid species, *N. ophidion* and *S. typhle*. Green MHC II indicates presence of MHC II and red indicates loss of gene and function. Red dotted lines at day 1 show the incision location and dotted red circles indicate the relocation area.

Transcriptome sequencing and de novo assembly

Extracted RNA quality was assessed using the NanoDrop-1000 spectrophotometer (NanoDrop) and Fragment Analyzer (Agilent Technologies) before library preparation. Libraries were prepared using an Illumina TruSeq stranded mRNA kit, before being paired-end sequenced (Illumina, NovaSeq 6000, 2x150bp reads) at the Competence Centre for Genomic Analysis (CCGA) in Kiel. Adapters were trimmed from all resultant raw reads using Trim galore! (v0.6.5) (Krueger, 2015) prior to FastQC (v0.11.9) and MultiQC quality checks (v1.9) (Andrews, 2010; Ewels et al., 2016).

Processing raw reads for transcriptome assemblies were carried out as follows: Rcorrector package (v1.04) with standard settings was used to correct for errors (Song and Florea, 2015). Quality and adapter trimming were carried out on corrected reads using the fastp program (v0.20.0) (default settings) (Chen et al., 2018). Owing to the large number of reads, Bignorm software was utilized for normalization, using the recommended settings (Wedemeyer et al., 2017). Using default settings and disabling the normalization option, the normalized reads were assembled to the transcriptome using the Trinity package (v2.8.5) (Haas et al., 2013). For annotation, contigs were analysed with the Trinity package associated tools, TransDecoder (v5.5.0) and Trinotate (v2.0.0), under the author's recommendation (Bryant et al., 2017). The TransDecoder prediction step was conducted using the options '—retain_pfam_hits' and '–retain_blastp_hits'. The required databases (Pfam-A, Rfam and Uniprot) for these tools were downloaded in November 2019.

Transcript abundance and pairwise differential gene expression analysis

Deduction of transcript abundance was conducted by utilising RSEM v1.3.3 (Li and Dewey, 2011) and bowtie2 v2.4.2 (Langmead and Salzberg, 2012) to align reads to each respective transcriptome assembly. Tximport (v1.18.0) (Soneson et al., 2015) was used to transfer abundance estimates prior to downstream differential gene expression analysis. Data filtering was carried out, excluding genes with an expression value of < 5 and present in < 3 replicates. All remaining counts were transformed using mean variance stabilizing transformation (VST), before principle component analysis (PCAs) and uniform manifold approximation projection UMAP (McInnes et al., 2018) were adopted to reveal unusual outliers. The DESeq2 package (v.1.22.2) was used for all differential gene expression analysis (Love et al., 2014) in R (v3.6). Three pairwise comparisons were carried out between the following pairs incorporating the non-surgical controls: **allograft vs control (ALLO/C)**, **autograft vs control (AUTO/C)** and **surgical control vs control (SC/C)**. In an attempt to remove the influence of surgical trauma associated immune activity, two more pairwise comparisons were carried out; **allograft vs surgical control (ALLO/SC)** and **autograft vs surgical control (AUTO/SC).** Lastly, a

pairwise comparison between **allograft vs autograft (ALLO/AUTO)** was conducted in an attempt to highlight genes strongly associated with the two transplant types. Multiple testing corrections were implemented using the Benjamini and Hochberg adjustment (Benjamini and Hochberg, 1995). Genes with a log-2fold change expression of > 1 and adjusted *p*-value < 0.05 were used for downstream analysis. The same process was carried out on the sequenced RNA extracted from gill tissue as an immunological reference to assist with downstream analyses. The gill was chosen based on its richness of blood and therefore presence of circulating immune cells.

RESULTS

Visual assessments

Each fish was assessed visually throughout the post-surgery period and all pre-mature fish mortalities were documented. A number of fatalities were observed in allograft (*N. ophidion*: 9, *S. typhle*: 2) and autografts (*N. ophidion*: 6, *S. typhle*: 3). Fin 'sloughing', described as the shedding or loss of tissue from transplant, was observed at varying degrees in both species which is congruent with previous transplant studies on Xiphophorin fishes (Kallman and Gordon, 1957). The complete loss of fin transplant was also observed in some instances likely owing to ineffective tissue connection (supplementary; Fig. S1). In an initial trial carried out on *S. typhle* individuals, two transplantees were shown to maintain allograft transplants following 30 days after surgery (data not shown). Initial observations following 14 day period suggested a more acute immune reaction in *N. ophidion* (Fig. 2) with MHC II compared with *S. typhle* where MHC II has been lost (Fig. 3). Evidence of possible revascularisation can be observed in both species suggesting the transfer donor blood to host and establishment of a histological connection.

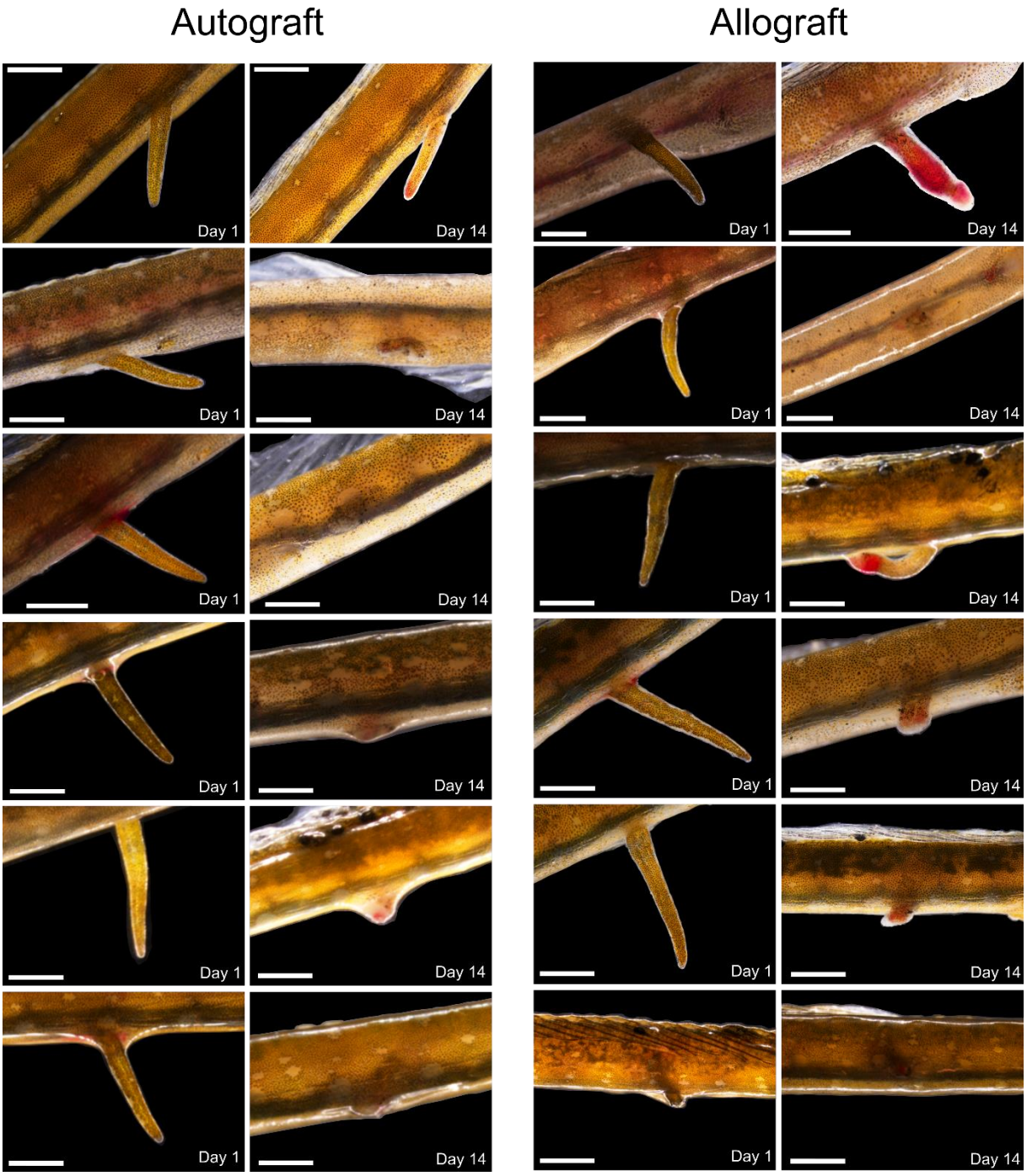


Figure 2. Surgical transplant images of autograft and allografts in *Nerophis ophidion* directly after surgery (day 1) and prior to dissection (day 14). Scale bars represent 1mm.

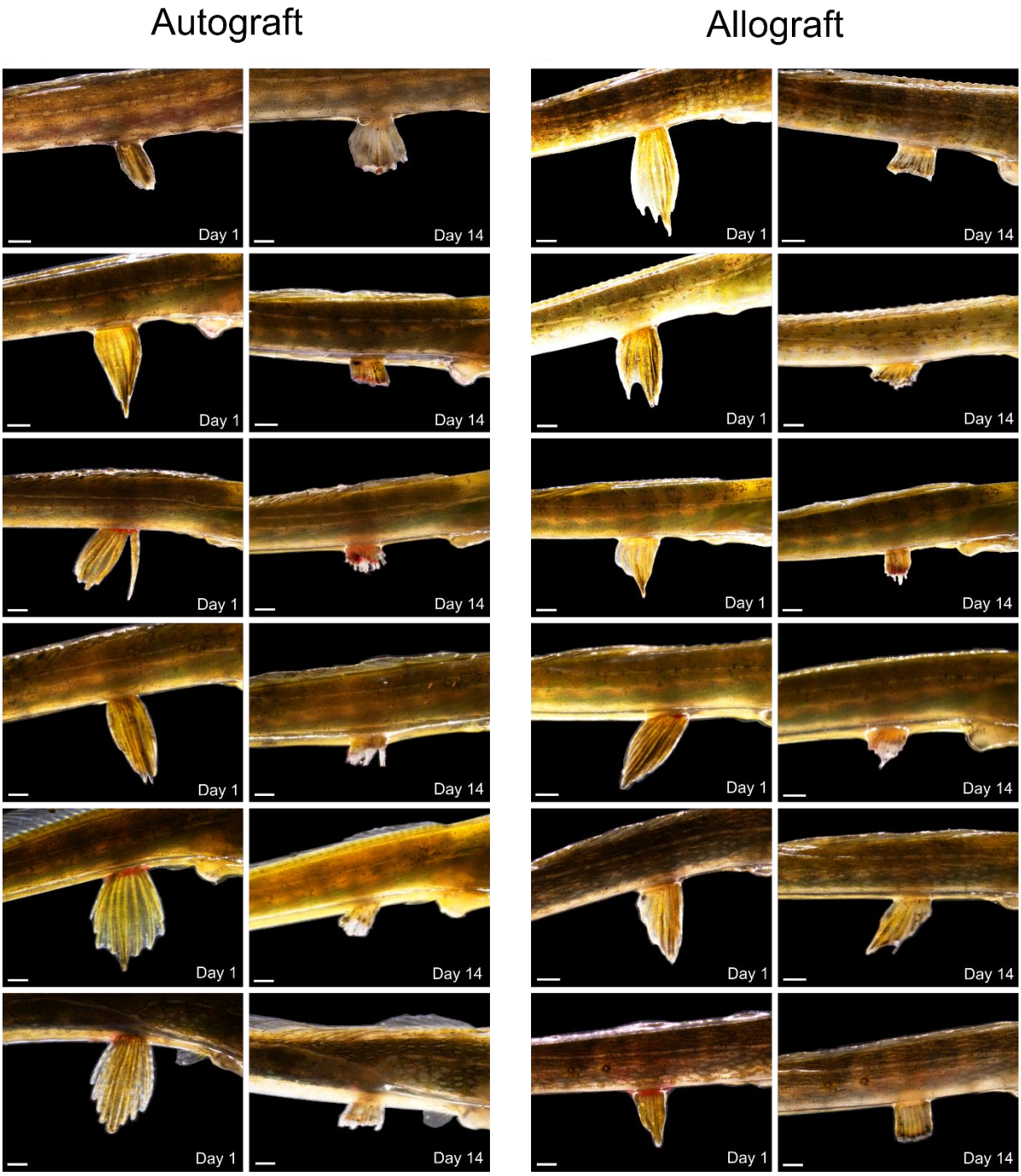


Figure 3. Surgical transplant images of autograft and allografts in *Syngnathus typhle* directly after surgery (day 1) and prior to dissection (day 14). Scale bars represent 1mm.

Differential gene expression of surgical area tissue

Differential gene expression analysis was carried out on the extracted surgical area tissue from two syngnathid pipefish species, *S. typhle and N. ophidion*. Four surgical types; allograft **(ALLO)**, autograft **(AUTO)**, surgical control **(SC)**, control **(C)**, each with 6 replicates were carried forward for gene expression assessments. 222-349 M paired-end reads per surgical type with an average of 23.5 M per sample. A total of 2,550 and 1,233 annotated genes were differentially expressed across all replicate pairwise comparisons in *S. typhle* and *N. ophidion* surgical tissue, respectively (supplementary; Fig. S2). Genes of interest were selected based on their relevance to processes such as immune system function, tissue remodelling, wound healing, and regeneration. The six pairwise comparisons used in this investigation were as follows; allograft vs control **(ALLO/C)**, autograft vs control **(AUTO/C)**, surgical control vs control **(SC/C),** allograft vs surgical control **(ALLO/SC),** autograft vs surgical control **(AUTO/SC)** and allograft vs autograft **(ALLO/AUTO).**

In both species the majority of annotated differentially expressed genes are shared between allograft (ALLO/C) and autograft (AUTO/C) comparisons within species, when using the non-surgical control (C) replicates for pairwise comparisons (Fig. 4) (supplementary; Table S1-2). Similarly, a number of genes were shared between the *S. typhle* transplant types and their equivalences in *N. ophidion*. Shared upregulated allograft genes in the ALLO/C comparison, included *ctsk* (inflammation), *tnnl1* (muscle function), *mmp13* (collagen degradation) and *gas2* (apoptosis), with the latter three also showing upregulation in autograft replicates in both species. Downregulated genes shared few similarities among allograft subjects, with one notable gene (*vwa7*) which is present within the MHC III region and thought to control lung tumour susceptibility (Snoek et al., 2000). Further notable upregulated genes specific to autograft, when compared with control replicates in both species included, *tlr1* (innate immunity), *cmlkr1* (anti-inflammation and pro-inflammation) and *adam12* (muscle regeneration). *Ahnak* (neuronal development), *myh10* (cytokinesis) and *slc4a1* (transport) were also among the few genes that exhibited a shared downregulation in autografts across species. Surgical control (SC) replicates in *S. typhle* and *N. ophidion*, when compared to C, did not share any differential expression similarities.

Syngnathus typhle - species-specific gene expression

Shared gene expression among transplant types

Species-specific gene expression observations for *S. typhle* uncovered a number of shared similarities between ALLO/C, AUTO/C and SC/C pairwise comparisons with control replicates (Fig. 4). The transcriptional activator, *irf1*, which is implicated in innate and adaptive immune

processing, was one of the most upregulated genes in all three transplant types, implying a general upregulation of immune responses upon surgery. Other shared genes exhibiting upregulated expression included two transporter genes (*mcoln3*, *pitpnc1*) and the transcription factor, *tcf12*. Notable genes that were upregulated in ALLO and AUTO tissue compared with C include some with immune function (*tyrobp*, *clec4d*, *nfatc1*, *havcr1*), vascular remodelling (*cthrc1*, *mmp19*), transport (*slc37a2*, *ncs1*), development (*sfrp2*, *olfml3a*, *thbs4b*), cell adhesion (*itgb3*, *itgb8*) and apoptosis (*dram1*), while *gzma* was upregulated in both ALLO and SC replicates but not differentially expressed in AUTO.

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

ALLO/SC and AUTO/SC comparisons, using SC as the comparative control produced a large number of differentially expressed genes in *S. typhle*. Ribosomal processing and translation (*eef1a*, *eef-2*, *rpl3*), immunity (*tyrobp*, *il17ra*, *nlrp12*, *tnfrsf11a*) and tissue remodelling (*mmp2*, *mmp14*) genes, were all upregulated in ALLO and AUTO when compared with SC, alongside the immune regulator *fstl1*. Conversely, *fcer2* (immunoglobulin receptor), was jointly downregulated in ALLO and AUTO samples, when compared with the surgical control, along with immune genes *polr3e* (innate immunity), *nfil3* (T-cell activity) and *tcim* (apoptosis).

Allografts

A number of upregulated genes with immune function were identified in *S. typhle* ALLO replicates when compared with C including *igfbp3* (growth factor), *ildr2* (T-cell regulation) and the chitinase coding *chia* (adaptive immunity). Incidentally, *chia* was also shown to be upregulated in allografts when compared with the surgical control. Conversely, a different chitinase (*chi3l1*) with adaptive immune function, was shown to be strongly downregulated in ALLO in both C and SC comparisons. Additional immune genes found to be upregulated in ALLO when compared with SC included; *prf1* (apoptosis), *prdm1* (immune and tolerance function), *dbnl* (adaptive immunity), *pappa* (wound healing) and an alternative *fcer2* ortholog (immunoglobulin receptor). While allografts exhibited downregulated expression of cracr2a (adaptive immunity), c7 (innate immunity) and *samsn1* (adaptive immunity regulator), when compared with the surgical control. Among the most downregulated genes in the aforementioned comparison were *dusp1* (stress response), *ntf2* (transport) and *aip* (signalling).

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

Autografts

As in allografts a number of immune system related genes were upregulated in autografts when compared with control including, *il-17ra* (inflammation) and *cd84* (adaptive and innate immunity), while others such as *fcrl5* (B-cell development), *trim5* (viral defense), *il22ra1* (immunity) were shown to be downregulated. Functions for other upregulated genes among autograft replicates included transportation (*slc12a9*, *atp2a1*, *slc26a10*), angiogenesis (*angptl3*, *pgf*, *flt1*), development (*foxd3-a*, *hoxd12a*) and collagen interaction (*pcolce*, *mmp16*). A number of downregulated genes with muscular (*stac3*, *mef2c*, *tpm4*), erythrocyte/developmental (*hba1*, *cahz*, *klf1*, *bpgm*) and apoptotic (*peg10*) function, were identified specifically in AUTO compared with SC. The AUTO/SC pairwise comparison highlighted number of immune genes, namely the immune regulator *cd276* and surface protein *cd53* (adaptive immunity), which were both upregulated in AUTO, while inflammation implicated (*ccr6*, *ly75*, *c1qbp*), innate immunity (*irak1*) and cytotoxic (*gzma*) related genes, showed the opposite expression direction. The cytolytic *prf1*, which was similarly upregulated in allografts in the ALLO/SC comparison, also exhibited upregulated expression in autografts in AUTO/SC. Highly downregulated expression of *nexn* (cytoskeleton organisation), *mcm2* (cell cycle) and *srrt-b* (RNA processing) was found in AUTO compared with SC, while upregulated expression was exhibited by the solute-carriers *slc16a9* and *slc6a8*.

Surgical controls

The amount of differentially expressed genes in the SC/C pairwise comparison exceeded those in both ALLO/C and AUTO/C. Notably, a number of genes involved in ribosomal processing were shown to be upregulated (*bop1*, *wdr12*, and *ebna1bp2*) and downregulated (*rpl21*, *rpl-7*, *rpl12*, *eif5a*) in surgical control tissue compared with control replicates, while a couple of apoptotic genes (*mcl1*, *tnfrsf11b*) also showed positive upregulation. As with allograft and autograft tissue there was an upregulation of immune genes such as *il10* (anti-inflammation), *il18r1* (pro-inflammation) and *il1r2* (innate immunity). Equally, other immune genes, which were predominately innate related, were shown to be downregulated in SC replicates compared with C, including; *icoslg* (T-cell proliferation), gimap8 (apoptosis), *zc3h12a* (inflammation), *il20rb* (immune modulation), *nlrp12* (anti-inflammation). The only antigen processing and presentation related genes to be differentially expressed in *S. typhle* were *h2-k1* (MHC I), *canx* (adaptive immunity) and *cd48* (adaptive immunity), which were all

downregulated in SC in comparison with C. In contrast to ALLO, the chitinase *chia* (adaptive immunity) was downregulated in SC when compared with C.

Nerophis ophidion - species-specific gene expression

Shared gene expression among transplant types

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

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

Allografts

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

(signaling), which were both upregulated in ALLO, while *trim55* (muscle), *pxk* (actin-binding), *sgms1* (apoptosis) *ywhab* and *cbl* (signaling) were conversely downregulated.

Autografts

Overall, in the AUTO/C comparison *N. ophidion* autograft replicates showed a general downregulation of antigen processing and presentation related genes including, *h2-d1, popy class I histocompatibility antigen, b2m*, *tap1*, *cd48* and *cd3e* which incidentally were not downregulated in allograft replicates. Despite these downregulations, *N. ophidion* AUTO replicates also upregulated, compared with C, inflammation (*cmlkr1*, *abcc1*, *ccr3*, *ackr3*) and T-cell activity genes (*gpr183*, *hlx*). The AUTO/SC pairwise comparison unearthed a few differentially expressed immune genes tasked with roles in neutrophil chemotaxis (*lect2*) and blood coagulation (*f13b*), both of which were upregulated in autografts. Genes with alternative functions such as angiogenesis (*esm1*, *plxnd1*, *thbs4*) and development (*gdf6a*, *dkk3*, *prrx1*) were also upregulated in AUTO tissue compared with C, while the growth factors *gdf6a* and *htra1a* were upregulated in AUTO tissue compared with SC.

Surgical controls

Among the genes exclusively expressed in SC replicates the immune regulators, *apoa1* and *sec14l1*, were stand outs concerning upregulated genes with immunological function when compared with the standard control.

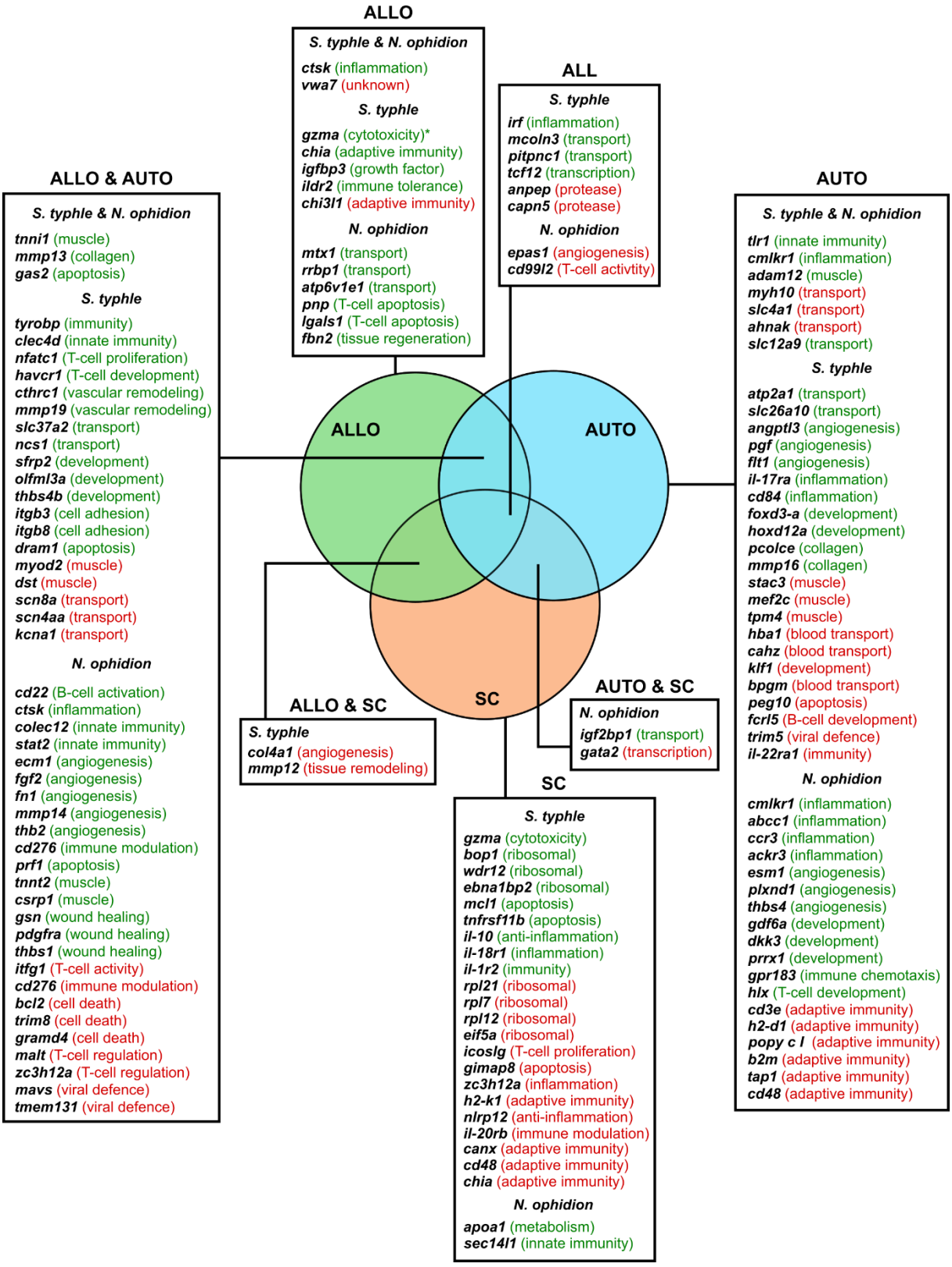


Figure 4. Schematic diagram showing differentially expressed genes of interest in allograft (ALLO), autograft (AUTO) and surgical control (SC) replicates. Upregulated (green) and downregulated (red) genes are in relation to control replicates. Genes shown possess roles in immunity and/or have high/low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

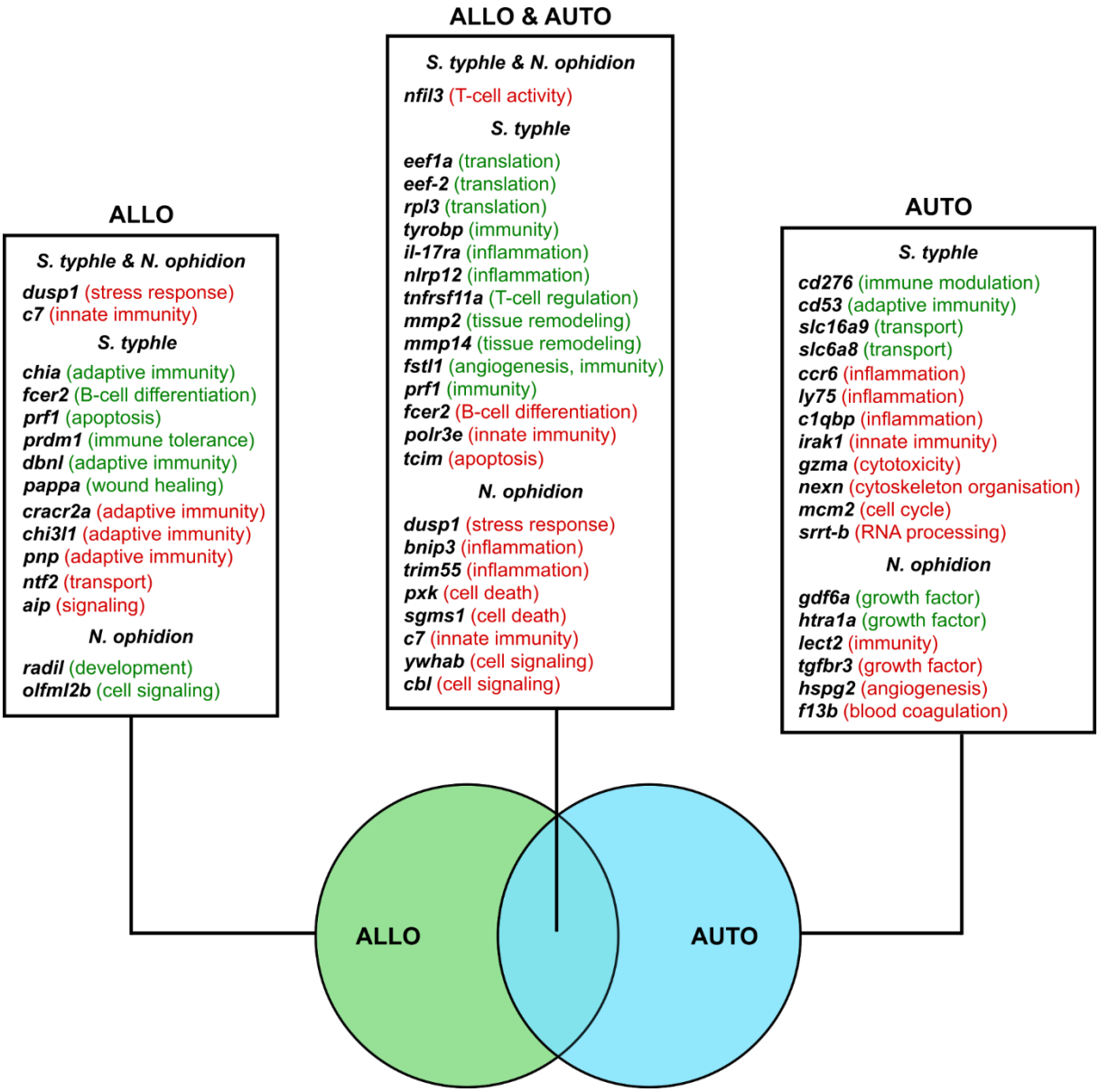


Figure 5. Schematic diagram showing differentially expressed genes of interest in allograft (ALLO) and autograft (AUTO) replicates. Upregulated (green) and downregulated (red) genes are in relation to surgical control replicates. Genes shown possess roles in immunity and/or have high/low expression levels.

DISCUSSION

This investigation was the first transcriptome-wide assessment of the immunological capacity of a fish species devoid of the MHC II pathway; utilising allograft and autograft fin-transplants to understand the mechanisms that govern allorecognition, rejection and tissue recovery.

Investigating the immunodeficient *S. typhle* provides a fascinating opportunity to understand the impact MHC II loss on self non-self recognition, as well as the potential to shed new light on compensatory measures involved in pipefish allorecognition. Comparing *S. typhle* with *N. ophidion*, a pipefish with a fully functional MHC II pathway, can provide some crucial evolutionary context for the adaptive immune system rearrangements. Immunological findings in this investigation were also accompanied by a number of intriguing physiological and tissue recovery processes associated with transplantation.

Historically, allogeneic transplantation studies were used to assess histocompatibility and led to the discoveries of the MHC molecules, with experiments carried out on mice (Snell, 1948; Snell and Higgins, 1951; Auchincloss Jr and Winn, 2004), lizards (Cuellar and Smart, 1977; Manríquez-Morán and Méndez-de la Cruz, 2008) and fish (Cardwell et al., 2001) varying in degrees of success. MHC I and MHC II are both recognised mediators of transplant rejection (Snell and Higgins, 1951; Ayala García et al., 2012), however, the comparative importance of MHC I from MHC II in this process is difficult to differentiate. Allograft rejection has been reported previously in fish (Šećerov, 1912; Sauter, 1934; Nardi, 1935), while interstrain platyfish have shown signs of rejection and tissue sloughing up to 15 days following surgery (Kallman and Gordon, 1957; Kallman and Gordon, 1958). While these previous studies were aware that the genetic constitution of the host and fin donor are involved in graft rejection, a detailed inspection of the underlying molecular pathways in play were not explored. Visual signs of rejection induced by fin transplants were also documented here, with *N. ophidion* allografts appearing to evoke a more acute rejection response than *S. typhle* (Fig. 2), supporting the first hypothesis of this study **(i)**. The increased mortality among *N. ophidion* allografts compared with *S. typhle* also provides support for this rejection effect. However, following a molecular examination the role of MHCs in transplant rejection in both species is not as prominent as would have first been thought.

Appropriately, MHC II related genes were not detected in *S. typhle* in this study, however, perhaps more surprising was the lack of MHC II expression in *N. ophidion*. MHC II is exclusively expressed on antigen presenting cells such as dendritic cells and mononuclear phagocytes (Stastny et al., 1986). The lack of MHC II expression in *N. ophidion* could be due to a low number of these associated immune cells in the examined tissue. To see if any MHC II expression could be observed elsewhere, *N. ophidion* gill tissue was also examined. Gills are likely to harbour a greater number of antigen presenting cells, based on their role as an immunologically active mucosal interface between the vascular system and environment. However, the absence of MHC II related gene expression also in gills remains difficult to interpret. As of yet, it is not possible to exclude that the *N. ophidion* MHC II pathway despite its genomic presence (Roth et al. 2020), might have lost its function. The functional loss of

MHC II was recently suggested for seahorses for which sequences of the MHC II pathway remained largely unaffected but a lost exon of the MHC II invariant chain CD74 was observed (Roth et al. 2020). This previous study also emphasised the flexibility of the vertebrate immune system and challenged the preconceptions of what immune system requirements are needed to survive. With future work focusing on comparative single-cell expression approaches (Parker et al., In revision) across syngnathids combined with morphological assessments of immune cells the aim is to shed light on these open questions.

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

In addition to their role in antigen presentation MHC are charged with maintaining immunological tolerance (Von Boehmer and Kisielow, 1990). Brood pouch related immune modulation has been exhibited in syngnathids previously through the downregulation of MHC I (Parker et al., In revision; Roth et al., 2020). Expression findings here in autograft replicates in both species also appear to have adopted similar mechanisms with regards to the suppression of the MHC I pathway. The downregulation of the aforementioned *gzma* in

autografts compared with surgical control (*S. typhle*) contrasts its upregulated role in allogenic transplant rejection. While the downregulation of a number of MHC I related genes (*h2-d1*, *popy c l*, *tap1*) in *N .ophidion* autografts could confirm a transplant and host tissue cell recognition of self, resulting in a downregulation to avoid an autoimmune response. This promising immune modulation provides support to the third hypothesis of this investigation **(iii)**. Moreover, this MHC downregulation and the upregulated involvement of *gzma* in *S. typhle* allograft provides additional support to the fourth hypothesis here, as they both indicate expression disparities between autograft and allografts within species **(iv)**. Disentangling the specific drivers and importance of the MHC in immune modulation and transplant rejection in pipefish is an intriguing challenge. Based on these results it appears that MHC I does participate in these immunological functions, however, additional observations suggest that allogenic rejection may transcend the diverse set of MHC loci.

The innate immune system and inflammatory processes are recognised influences involved in transplant rejection (Mori et al., 2014; Braza et al., 2016). These associations were supported by a number of gene expression trends identified in this study. Chitinases in humans are expressed prominently in innate phagocytes (macrophages and neutrophils) at sites of infection, inflammation and tissue repair (van Eijk et al., 2005; van Eijk et al., 2007). Specifically, chitinases have been associated with M2 macrophages (Hartl et al., 2009), a subtype thought to have been identified in *S. typhle* previously (Parker et al., In revision). M2 macrophages possess an anti-inflammatory role with activities believed to induce wound healing and tissue remodelling (Gordon, 2003; Xiao et al., 2008; Murray and Wynn, 2011). Similar processes may be occurring in *S. typhle*, with the upregulation of *chia* in allografts, however, the converse downregulation of *chi3l1* renders the specific role of individual chitinases in *S. typhle* difficult to explicate. It is possible that these chitinases in pipefish simultaneously perform different immunological and tissue regenerative roles upon allograft transplantation. Whereby the downregulation of *chi3l1* may stimulate an immune response while an upregulation in another activates wound healing. The highlighted presence of these innate immune components in previous pipefish male pregnancy studies (Parker et al., In press; Small et al., 2013; Roth et al., 2020) reinforces their relevance in terms of immune modulation in syngnathids. Further in depth functional analyses should be encouraged to help elucidate the specific function of chitinases within the Syngnathiformes order.

Irf1 has a multi-scale role in the immune system lending assistance to both innate and adaptive branches (Kröger et al., 2002; Wang et al., 2020). *Irf1* has been implicated in apoptotic processes which may be particular prevalent around the incision site (Kim et al., 2004; Gao et al., 2010). Apoptosis is also closely linked to wound healing (Wu and Chen, 2014) and would likely explain why *irf1* upregulation in this study is only found in fish which underwent surgery.

The absent expression of *irf1* in *N. ophidion* is surprising as it also underwent the same transplant procedure. To clarify this absence in *N. ophidion*, further genomic evaluation would be required, as it is plausible that *irf1* may have only been conserved in some syngnathid species.

Angiogenesis is a crucial process involved in wound healing, stimulating the formation of blood vessels, ensuring the perfusion of oxygenated blood to assist the healing tissue (Tonnesen et al., 2000; Rajnoch and Viklický, 2004; Li et al., 2013). It is also a known immune inflammation characteristic with significance in a number of pathologic and transplant related reactions (Folkman, 1995; Ferrara, 2000; Lemström et al., 2002). In line with this, angiogenic processes appear to be upregulated in both autograft and allograft transplants in both pipefish species, based on the upregulation of genes such as *mmp19*, *angptl3* and *flt1*. This trend is perhaps unsurprising considering that both transplant types required incisions. Taken with the upregulated expression of tissue regeneration, wound healing and vascular remodelling related genes, positive angiogenic activity appears to be important for the recovery of tissue following transplant attachment. Furthermore, the upregulation of a number of metallopeptidases in this study, known to exhibit elevated expression following tissue injury, are also implicated in muscle and fin regeneration processes (Bai et al., 2005; Bellayr et al., 2009; Lei et al., 2013; Mohammadabadi et al., 2021), supporting the active occurrence of tissue healing in these fishes.

Alongside the immune system peculiarities that evolved among syngnathid members, the group is also defined by their unique evolution of the enigmatic male pregnancy (Herald, 1959; Dawson, 1985). Recently, pregnancy related research in syngnathids proposed that immunological tolerance during pregnancy was more important for the evolution of pouched brooders, such as *S. typhle*, compared with external egg carriers like *N. ophidion*, due to demands of increased intimacy and physiological connection with the offspring (Parker et al. In press; Roth et al., 2020). Some reports have even suggested that the MHC II loss in *S. typhle* may have helped facilitate the evolution of advanced male pregnancy, as a proxy for immunological tolerance (Roth et al., 2020). From a conceptual point of view, semi-allogeneic embryos are similar to allograft transplants in that they both possess genetic material from a different individual and should in principle evoke an immune response. The downregulation of MHC I related genes in this investigation in *N. ophidion* autografts is similar to the immunological tolerance related expression patterns observed in pouched syngnathids during pregnancy (Parker et al., In press). However, this link may be somewhat tenuous as *N. ophidion* was the one species in that study, which was not expected to evoke immunological tolerance measures, due to its basal and most likely less intimate brooding strategy. Based on findings here, it is suggested that immunological tolerance measures evoked during pregnancy

and transplantation are fundamentally different and should thus be considered different phenomena. Moreover, the structure and physiology of the two tissue types (surgical area and brooding tissue) are likely to react differently to foreign tissue, therefore could require alternative immune mediation. Syngnathid immunological tolerance remains a fascinating concept when considering the tolerance variation across brooding types, its links to male pregnancy evolution, and the impact caused by MHC II loss. Findings here reinforce the complexities encompassing this immune system facet, how it has evolved in syngnathids and the challenges that accompany it when attempting to assess these modulatory processes.

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

The immune processes that facilitate transplant rejection and destruction are highly dynamic. Immune cells that drive rejection impart their influence at various stages during the immune response making the timing of each response difficult to interpret. Skin grafts in mice highlighted the CTL accumulation in the surrounding area and allograft tissue 10 days following surgery (Celli et al., 2011). Despite the sampling taking place in this investigation 4 days later, the congruencies concerning CTL function at this progressive stage of rejection is promising. Zebrafish have been reported to have an active T-cell response 14 days following infection (Covacu et al., 2016), while transplant studies in fish have highlighted a number of different allograft rejection timings. Some report rejection between 5 to 20 days following surgery (Kallman and Gordon, 1957; Kallman and Gordon, 1958; Nakanishi, 1987), while carp skin grafts between siblings were reportedly rejected between 10-14 days (Kaastrup et al., 1989). These studies give further support to the adopted 14 days sampling time used here, however, it cannot be ruled out that there were immunological factors involved in transplantation that were not detected due to sampling time. Elucidating the temporal dynamics of the rejection

process in these fishes would be an interesting proposition, as it could shed additional light on the specific agents and sequence of events that shape an adaptive immune response.

This study highlights the role of MHC I in transplant rejection and immunological tolerance in pipefish species, with and without MHC II. The fascinating loss of MHC II in *S. typhle* requires additional investigation to determine whether it has an influence on immunological tolerance processes. However, this investigation provides some strong indications that MHC I related CTL function acts as a form of MHC II compensation. Immunological tolerance measures were observed in *N. ophidion* with the downregulation of MHC I, but based on the location and tissue structure differences these measures likely differ from those adopted in male pregnancy. Beyond the realms of MHC, details of physiological responses presented here involving angiogenesis, tissue regeneration and wound healing, impart compelling insight into how pipefish respond to transplantation and tissue trauma. Overall, this study expands on the growing knowledge concerning the complicated immunological workings and related evolution of the syngnathid lineage, while highlighting the subject of CTL activity as an interesting avenue to explore further in future experimental studies.

CHAPTER III

Characterization of pipefish immune cell repertoire through single-cell transcriptomics

Jamie Parker[1,4], Naomi Croft Guslund[2,3], Sissel Jentoft[3], and Olivia Roth[1,4]

[1] Marine Evolutionary Ecology, GEOMAR Helmholtz Centre for Ocean Research Kiel, D-24105 Kiel, Germany, [2] Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, NO-0371 Oslo, Norway, [3] Department of Immunology, Institute of Clinical Medicine, University of Oslo, NO-0372 Oslo, Norway, [4] Marine Evolutionary Biology, Christian-Albrechts-University, D-24118 Kiel, Germany.

Submitted for review in *Frontiers in Immunology*

ABSTRACT

Teleost adaptive immune systems have evolved with more flexibility than previously assumed. A particularly enigmatic system to address immune system modifications in the evolutionary past is represented by the Syngnathids, the family of pipefishes, seahorses and seadragons. These small fishes with their unique male pregnancy have lost the spleen as an important immune organ as well as a functional major histocompatibility class II (MHC II) pathway. How these evolutionary changes have impacted immune cell population dynamics have up to this point remained unexplored. Here, we present the first immune cell repertoire characterization of a syngnathid fish (*Syngnathus typhle*) using single-cell transcriptomics. Gene expression profiles of individual cells extracted from blood and head-kidney clustered in twelve putative cell populations with eight belonging to those with immune function. Upregulated cell marker genes identified in humans and teleosts were used to define cell clusters. While the suggested loss of CD4+ T-cells accompanied the loss of the MHC II pathway was not supported, the upregulation of specific subtype markers within the T-cell cluster indicates subpopulations of regulatory T-cells (*il2rb*) and cytotoxic T-cells (*gzma*). Utilising single-cell RNA sequencing this report is the first to characterise immune cell populations in syngnathids and provides a valuable foundation for future cellular classification and experimental work within the lineage.

Keywords: single-cell transcriptomics, syngnathidae, pipefish, immune system, immunity, cell profiling, immune cell.

INTRODUCTION

The vertebrate immune system has evolved into an extremely diverse, layered network of defense mechanisms capable of combatting a wide variety of agents on a specific (adaptive) and generic (innate) level (Cooper and Alder, 2006). The first adaptive immune system evolved in the descendants of the ray-finned fishes (Teleostei) ~450 million years ago (Flajnik and Kasahara, 2010). Teleosts comprise a diverse group of fishes made up of over 30,000 species, ranging in morphology, physiology and immune system constituents (Star et al., 2011; Zhu et al., 2013; Ravi and Venkatesh, 2018; Dubin et al., 2019; Roth et al., 2020).

Among them, representatives of the syngnathids (seahorses, pipefishes and seadragons) are conceivably the most morphologically bizarre fishes, boasting the unique evolution of male pregnancy (Herald, 1959; Dawson, 1986; Stölting and Wilson, 2007). The peculiarities extend to the syngnathid immune system, with *Syngnathus* and *Hippocampus* missing a functional Major Histocompatibility Complex II (MHC II) pathway (Haase et al., 2013; Roth et al., 2020). Until recently this pathway was thought to be synonymous across vertebrates, it has been postulated that the evolution of full pregnancy with placenta-like structures in syngnathids may have been facilitated by this adaptive immune rearrangement (Roth et al., 2020). Yet, the presence of a fully functional MHC II repertoire in other pipefish species (eg. *Nerophinae*), with basal male pregnancy without placenta-like structures, presents the lineage as an intriguing subject for immune and evolutionary studies. These immunological adaptations within the group were suggested to have coevolved with male pregnancy permitting immunological tolerance towards the semi-allogenic embryo (Parker et al., In press; Roth et al., 2020). Whilst recent studies have helped elucidate the syngnathid immune networks and processes, the evolutionary repercussions of such immune deficiencies in the immune cell populations remain unknown, with no established immune cell characterization of any syngnathid species.

Assessing immune cell populations historically relied on established cell surface markers receptive to specially designed antibodies, a practice almost exclusively reserved for model organisms such as humans and mice (Perfetto et al., 2004; Pitsillides et al., 2011). The advent of single-cell mRNA sequencing (scRNA-seq), the practice that facilitates the expression assessment of individual cells (Tang et al., 2009), is less restricting than using pre-designed antibodies. It can thus be applied in non-model species. Combined with advanced cell separation techniques such as Drop-seq microfluidics (Macosko et al., 2015), scRNA-seq can characterise thousands of cellular gene expression profiles, from which cell type and functional groups can be deduced.

A number of teleost studies have utilised cell isolation and single cell transcriptomics to assist with the identification of brain (Raj et al., 2018; Alemany et al., 2018), kidney (Brown et al.,

2021), intestine (Wen et al., 2021) and embryonic (Wagner et al., 2018; Farrell et al., 2018) derived cell populations. Moreover, further work identifying teleost immune cell repertoires in a number of model fish species (Dee et al., 2016; Carmona et al., 2017; Hernández et al., 2018; Guslund et al., 2020; Niu et al., 2020; Perdiguero et al., 2021), have established a catalogue of putative immune cell markers, that extend beyond those used in mammalian cell identification.

The immune related genomic rearrangements that have arisen within the syngnathid fish group provide an exciting opportunity to explore the immune cell evolutionary repercussions which may have occurred due to immune pathway loss. Moreover, the discovery of immune cell type identifiers could help lay the foundation for future immunological experiments and characterising studies in the future. By employing Drop-seq micro-fluidics and scRNA-seq this study's aim was to carry out the first characterisation of the broadnosed pipefish (*Syngnathus typhle*) immune cell repertoire. Genomic alterations have resulted in the loss (*aicda*, *ciita*) or functional redundancy (*cd74*) of several MHC II pathway related genes (Roth et al., 2020). Hence, observed immune cell population distinctions were be expected when comparing pipefish with other teleosts, such as the absence of the MHC II related $CD4^+$ T-cell subset. Furthermore, this investigation wanted to determine whether this adaptive immune system absence could have given rise to alternative or compensatory innate immune cell types that adopted MHC II's functional role.

MATERIALS AND METHODS

Ethics Statement

All aquaria set-ups and dissection methods meet the guidelines issued by the Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Ditgitalisierung (MELUND) (Permit number V 242 – 57983/2018) and are in accordance with German animal welfare law.

Animals

Aquaria-bred *Syngnathus typhle* reared in the GEOMAR aquaria facilities were transported to the Centre of Ecological and Evolutionary Synthesis in Oslo, Norway. Fish were kept under the standard conditions used by Beemelmanns and Roth (2016). All fish were fed live and frozen mysids twice a day and fish were starved for 24 hours prior to dissection.

Tissue dissection and dissociation

Fish (n=3) were euthanized with an MS-222 overdose (500 mg/l, Sigma-Aldrich) prior to blood extraction. Blood was suspended in 1x PBS/0.01% BSA after massaging sufficient blood from the severed tail vein. Head kidney was removed and placed in a petri dish containing 1x PBS/0.01% BSA medium and a 35-40µm cell strainer, before carefully massaging cells through the filter using a syringe plunger. Both blood and head kidney cells were centrifuged at 300 x g for 5 min at 4 °C and re-suspended in 1ml 1x PBS/BSA (0.01%). All cells were kept on ice for the duration of the protocol. Cell sample concentration and quality were assessed under the stereomicroscope and diluted until cell concentrations met the required 200 cells/µl needed for microfluidic processing.

Microfluidic cell capture (Drop-Seq)

This investigation adopted the original Drop-Seq methodology set out by Macosko et al. (2015) and its subsequent amended protocol (v3.1) with useful advice from Evan Macosko, Melissa Goldman and Steve McCarroll (URL:dx.doi.org/10.17504/protocols.io.mkbc4sn). Individual suspended cells and micro-particle beads were coalesced within a singular nanoliter oil droplet (80 µm) using the microfluidic droplet generator (Dolomite, UK). Beads are equipped with oligonucleotides consisting of four sections: (1) priming site, (2) “cell barcode”, (3) Unique Molecular Identifier (UMI) and (4) reverse transcription primer (30-bp oligo dT sequence). Each bead possesses multiple primers (10^8) with identical barcodes but unique UMIs for downstream identification.

Each cell is lysed following successful droplet formation stimulating mRNA hybridisation with the bead primers and the formation of single-cell transcriptomes attached to microparticles (STAMPs). STAMP reverse transcription is then carried out prior to cDNA amplification and Tn5-mediated tagmentation. To facilitate the multiplexing of multiple cell populations within the same sequencing library, unique sample barcodes were incorporated within the adapter primers during the post-tagmentation PCR procedure.

scRNA-Seq and gene quantification

PCR amplification success was assessed on the Agilent BioAnalyzer High Sensitivity Chip (Agilent Technologies, Oslo, Norway) prior to library preparation. Library preparation and high output paired-end sequencing were carried out at the Norwegian Sequencing Centre (Illumina, NextSeq500, 75bp) (NSC; www.sequencing.uio.no), University of Oslo, Norway. A custom adapter sequence (Read 1, 20bp, GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAC) and standard Illumina adapter sequence (Read 2, 60bp) were used. Using the Drop-Seq Core Computational Protocol

(v2.0.0) (Nemesh, 2016) resultant reads were mapped to the *S. typhle* genome using STAR (Dobin et al., 2013) following quality checks (FastQC v0.11.9 and MultiQC v1.9) (Andrews, 2010; Ewels et al., 2016) and adapter removal. A digital expression matrix was constructed exhibiting transcript number (per gene, per cell) by grouping all reads by the cell barcode and UMI for each gene. The first 600-5,000 STAMPS, organised into decreasing read number order, from each sample were used for downstream analysis.

Cell and gene filtering

Data from all blood and head kidney samples was merged in preparation for gene expression analysis, utilising the R package Suerat (v4.0.3) to create a singular Seurat object. To remove partially sampled/dying cells and potential doublets/multiplets, detection parameters were set only to include cells with > 150 genes and < 1300 genes and 3000 molecules. These guidelines are supported by the clustering analysis tutorial (Butler et al., 2018) and previous fish species utilising the same methodology (Guslund et al., 2020).

Cell clustering

Expression count data was scaled and normalized across all cells by library size and then log2 transformed (Seurat; “LogNormalize” method), prior to principal component analysis (PCA) of the 2,000 most variably expressed genes. Heatmaps and elbow plots were used to assess heterogeneity amongst PCs and to determine the optimal number of PCs to carry forward for further analysis (supplementary; Fig 1 and 2). Shared nearest neighbour (SNN) modularity clustering was implemented using the Seurat “FindCluster” extension (PCs 1:23, resolution 0.35), followed by the use of Uniform Manifold approximation and projection (UMAP) for cluster visualization (McInnes et al., 2018).

Differential gene expression analysis and functional identification

Differential gene expression analysis was carried out on genes that were expressed in ≥ 25 cells within a cluster, using the “Findmarkers” Seurat extension, which is based on the Wilcoxon rank sum test. Post-hoc statistical testing was carried out using the Bonferroni multiple comparison correction. The most influential differentially expressed genes for each cluster were extracted and genes with an adjusted $p > 10^{-50}$ were considered for cell marker assignment and cell type classification. Annotated gene functions were deduced through independent literature searches, utilising the Universal Protein Knowledgebase (UniProt) (Consortium, 2021) and The Human Protein Atlas v20.1 (Pontén et al., 2008).

RESULTS

From three *S. typhle* pipefish individuals a total of 12,129 genes across (8658 cells) cells were accrued following filtering and quality control measures, while UMAP projection helped distinguish 12 distinct cell type clusters characterised using shared gene expression profiles (Fig. 1). Clusters ranged in size from 4,508-12 cells. 2,196 cell were derived from blood and 6,462 were derived from the head kidney.

Hematopoietic cells, erythrocytes and connective tissue cells

Differential gene expression analysis provided the foundation for putative cell type identification by characterising UMAP clusters by their significant gene expression profiles. Significant genes with immune system roles and relevant for cluster identification can be found in Figure 2 and 3 and in the supplementary material (Table S1-S2). The putative function and classification for cells belonging to the largest cluster central to the UMAP visualization, was hematopoietic or progenitor immune cells (4,508 cells). This was owing to the lack of strong immune markers and presence of genes involved in cell cycle processes (*hsp90ab1*) and protease inhibition (*plcp1*). Additionally, two genes (*pabpc1, krt8)* have been shown to be strongly upregulated in hematopoietic cells previously (Salvagiotto et al., 2008) as well as a number of ribosomal related genes. In relation to hematopoietic cells, the hematopoietic lineage cell-specific protein (*hcls1*) was expressed significantly in three other clusters (B, T and Neutrophils) but not in the overall hematopoietic cell cluster itself, despite sporadic expression being visible within the cluster (Fig. 4). The majority of cells making up the hematopoietic cluster could be attributed to those extracted from the head kidney (supplementary; Fig. 3). Two closely related clusters that showed distinct separation from the other groupings were identified as erythrocytes (1,332 and 714 cells), based on their dominant upregulation of haemoglobin subunits (*hba1* and *hbb2*). Additionally, there was a distinct upregulation of ribosomal related transcripts as well as a few genes with immune related function that were identified (*h2-d1* and *ifi27*). Converse to the hematopoietic cluster, erythrocyte clusters were dominated by blood derived cells (supplementary; Fig. 3). Lastly, one small cluster perceived to be related to structural/connective tissue (29 cells) was identified based on the strong upregulation of fibrous binding (*fbn1* and *fn1*), muscle regeneration (*adamts15*) and collagen (*col5a2*) related genes. The remaining eight clusters were assigned to different immune cell types.

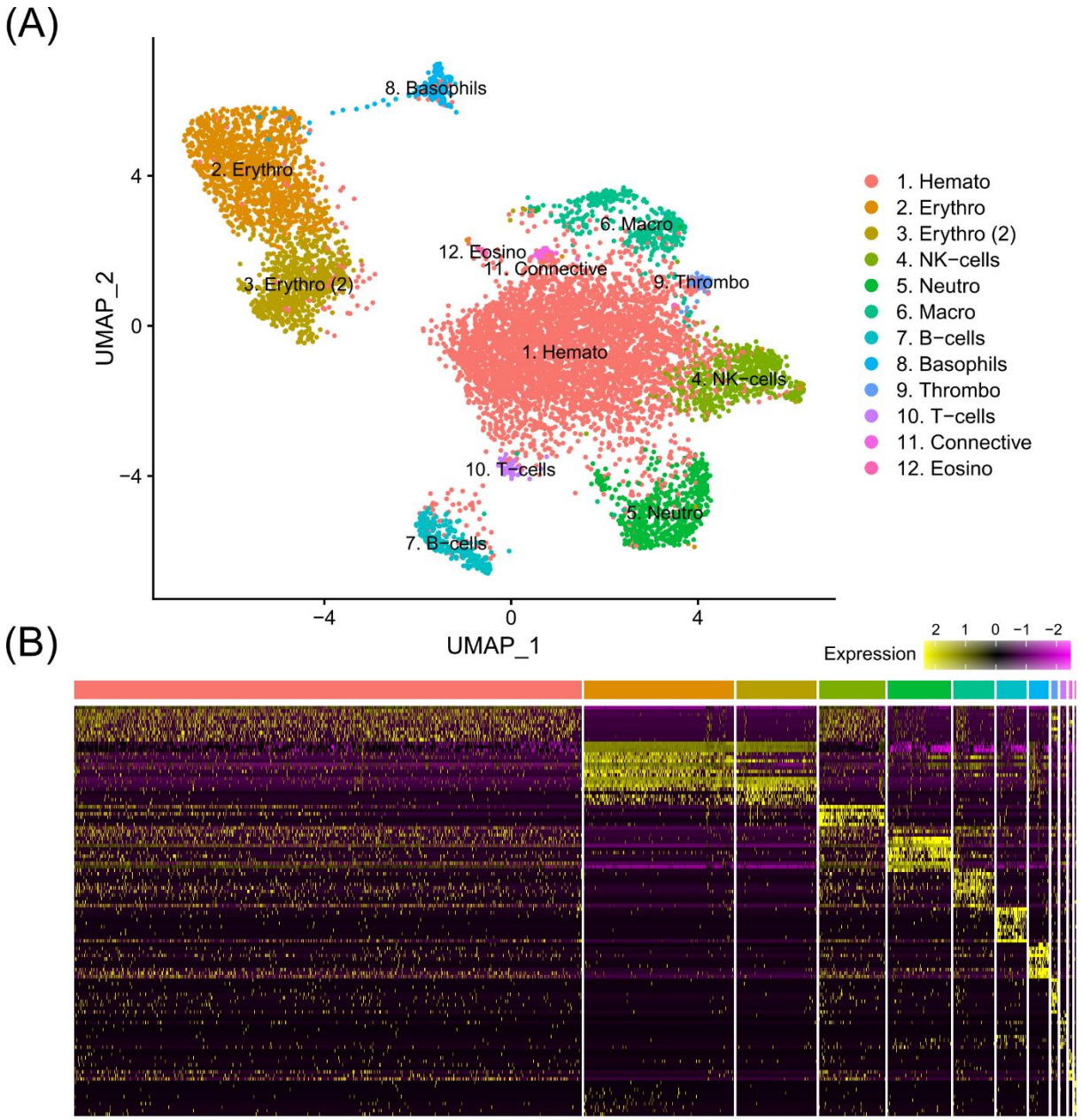


Figure 1. (A) Visual representation of isolated blood and head kidney extracted cell types of *Syngnathus typhle* using Uniform manifold approximation projection (UMAP). Cell clusters characterized by differentially expressed gene markers associated with specific cell sub-types. All markers associated with immune function previously reported in humans and/or fish species. Two erythrocyte clusters are represented with the second highlighted as such (2). Full names for all cluster are as follows: 1. Hematopoietic cells, 2 and 3. Erythrocytes, 4. Natural killer cells, 5. Neutrophils, 6. Macrophages, 7. B-cells, 8. Basophils, 9. Thrombocytes, 10. T-cells, 11. Connective tissue cells, 12. Eosinophils. (B) Differential gene expression heat map highlighting top 10 marker genes, with genes representing rows and cells representing columns.

Natural killer cells

The largest cluster was identified as putative natural killer-like cells (NK-cells) (586 cells), largely due to the expression of *hps3*, a lysosome related protein implicated in NK-cell function (Gil-Krzewska et al., 2017). This cluster exhibited a reduced number of significantly differentially expressed genes when compared with all other clusters, providing fewer candidates for identification. A number of granular related serine proteases (*prss1*, *chymotrypsin b*), an immune implicated cytoskeleton regulator (*msn*) (Ramoni et al., 2002) and a cytoplasmic adapter associated with NK-cell receptors (*shc1*) (Umehara et al., 2002) were present but at reduced significance.

Neutrophils

The next largest cluster, which was named as the neutrophils (560 cells), provided a number of strongly upregulated immune genes and specific markers. One of the most prominent was the enzyme *lce* (egg hatching) which was previously used as a neutrophil marker in codfish (Guslund et al., 2020). *Cebpe*, a gene encoding an enhancer binding protein thought to be essential for neutrophil development (Lekstrom-Himes and Xanthopoulos, 1998) and the neutrophil cytosol factor 4 (*ncf4*) were also highly expressed. Other indication markers include *il-1r*, which is an important neutrophil receptor (Futosi et al., 2013), the inflammation factor *aif1,* which is expressed in neutrophils and monocytes (Monaco et al., 2019), and the neutrophil cell migration-inducing *mmp9* (Bradley et al., 2012).

Macrophages

As with the neutrophil cluster, the perceived macrophage cluster was supported by an array of easily identifiable upregulated gene markers (364 cells). These include, *csf1r2* (macrophage differentiation), a member of a group of receptors recognised as macrophage markers in teleost fish (Roca et al., 2006; Katzenback and Belosevic, 2012; Chen et al., 2015), *mrc1* (mannose receptor) a macrophage marker in Atlantic cod and humans (Rőszer, 2015; Guslund et al., 2020), and *clec4e*, a pattern recognition receptor shown to highly expressed in macrophages (Miyake et al., 2013). *Cd209*, a receptor found on antigen presenting cells which promotes MHC II presentation of the HIV virus and was one of the most upregulated genes found within the cluster. Additional gene contributors that helped define the cluster include the macrophage related protein, *mpeg1*, *lysozyme g*, and the macrophage capping protein, *capg*.

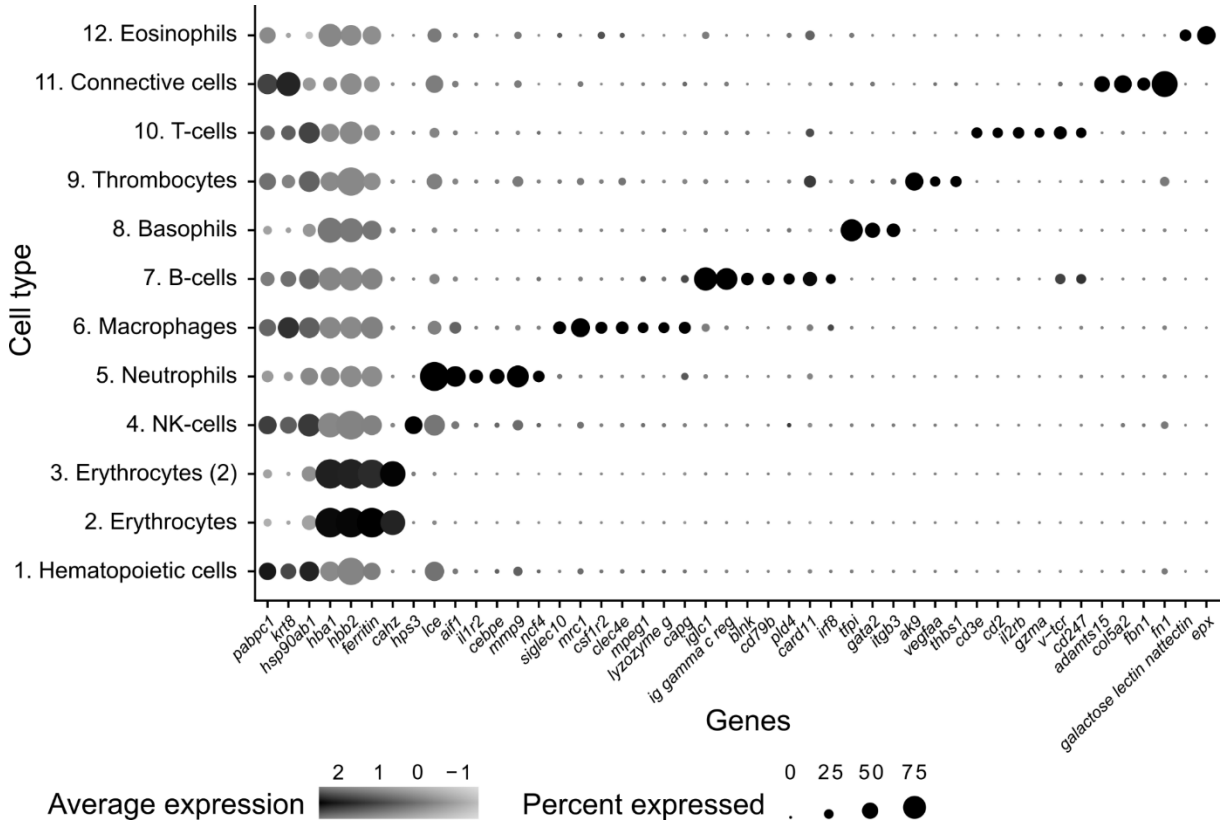


Figure 2. Dot plot visualization of deduced cell markers used to define cell clusters in *Syngnathus typhle*. Dot size denotes the percentage of cells expressing the gene within each cluster and the mean expression level of active expressional cells is indicated by the colour intensity.

B and T-cell lymphocytes

A large cluster of B-cells (265 cells) was identified by the upregulation of a high number of immunoglobulin components (eg. *iglc1*, Ig lambda chain, *ig heavy chain*, *ig kappa chain*) which are exclusively released by B-lymphocytes. Further support was provided by the upregulation of *irf8*, *cd53* (B-cell differentiation and development), *swap-70, blnk* (B-cell activation), *card11* (B-cell signalling) and the B-cell antigen receptor *cd79b.* In addition, two T-cell receptors were among the top 20 differentially expressed genes within this cluster (*cd247* and *v-tcr*).

A population of T-cells were also strongly represented (cluster 10) (53 cells) with the upregulated expression of four universal T-cell receptors (*cd2*, *cd3e, v-tcr and cd247*), as well as the interleukin receptor subunit *il2rb.* Incidentally, IL2R has been shown to play central immune suppressive role when expressed by regulatory T (T_{reg}) cells (Chinen et al., 2016). Lastly, the cytotoxic protease *gzma* was also highlighted for its immune related activity and previously used as a marker for cytotoxic cells in cod (Guslund et al., 2020).

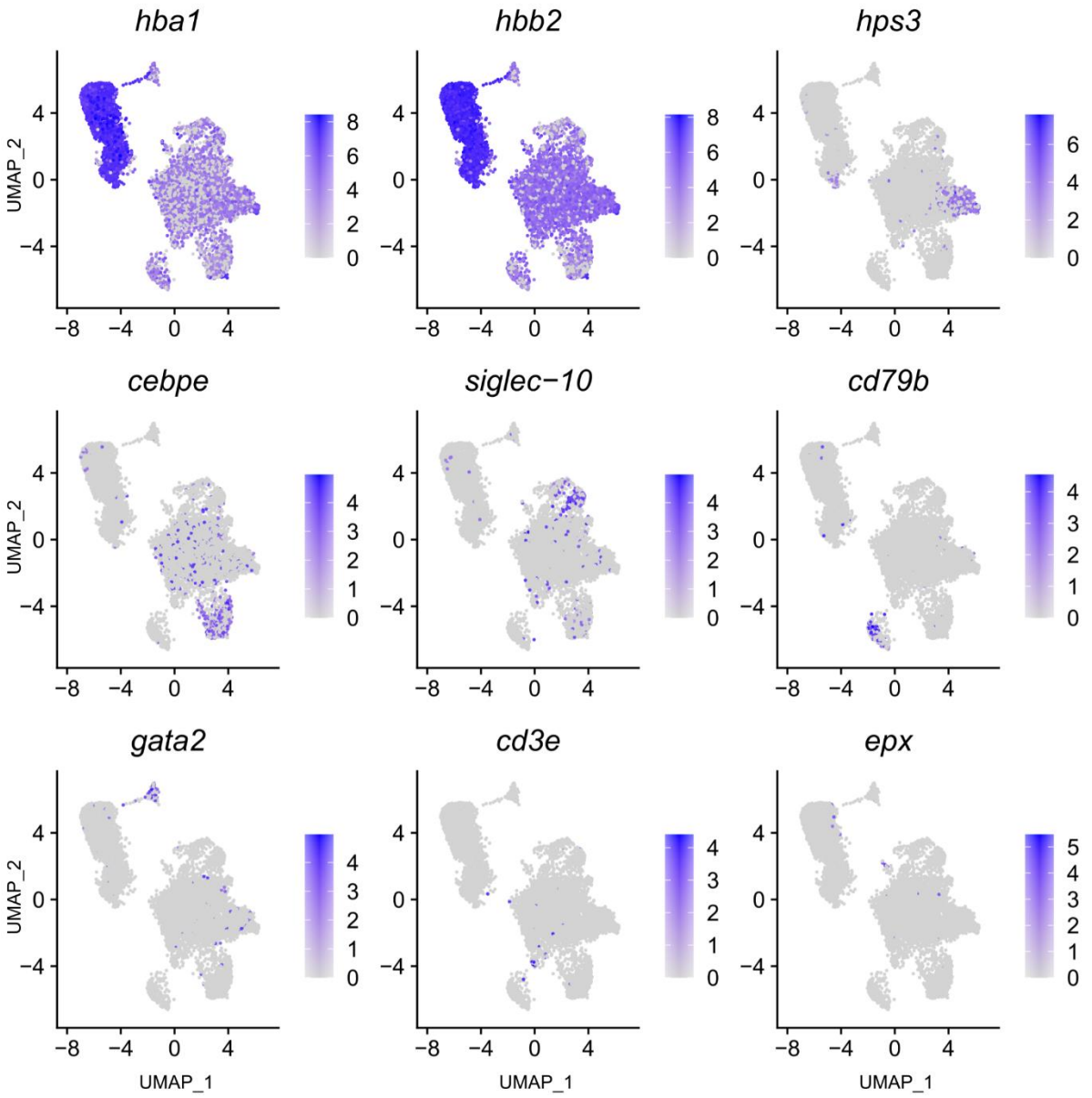


Figure 3. Feature plot highlighting *Syngnathus typhle* blood and head kidney cells expressing selected genes characterizing selected immune cell clusters within Uniform manifold approximation projection (UMAP). Increased cell colour intensity indicates increased gene expression.

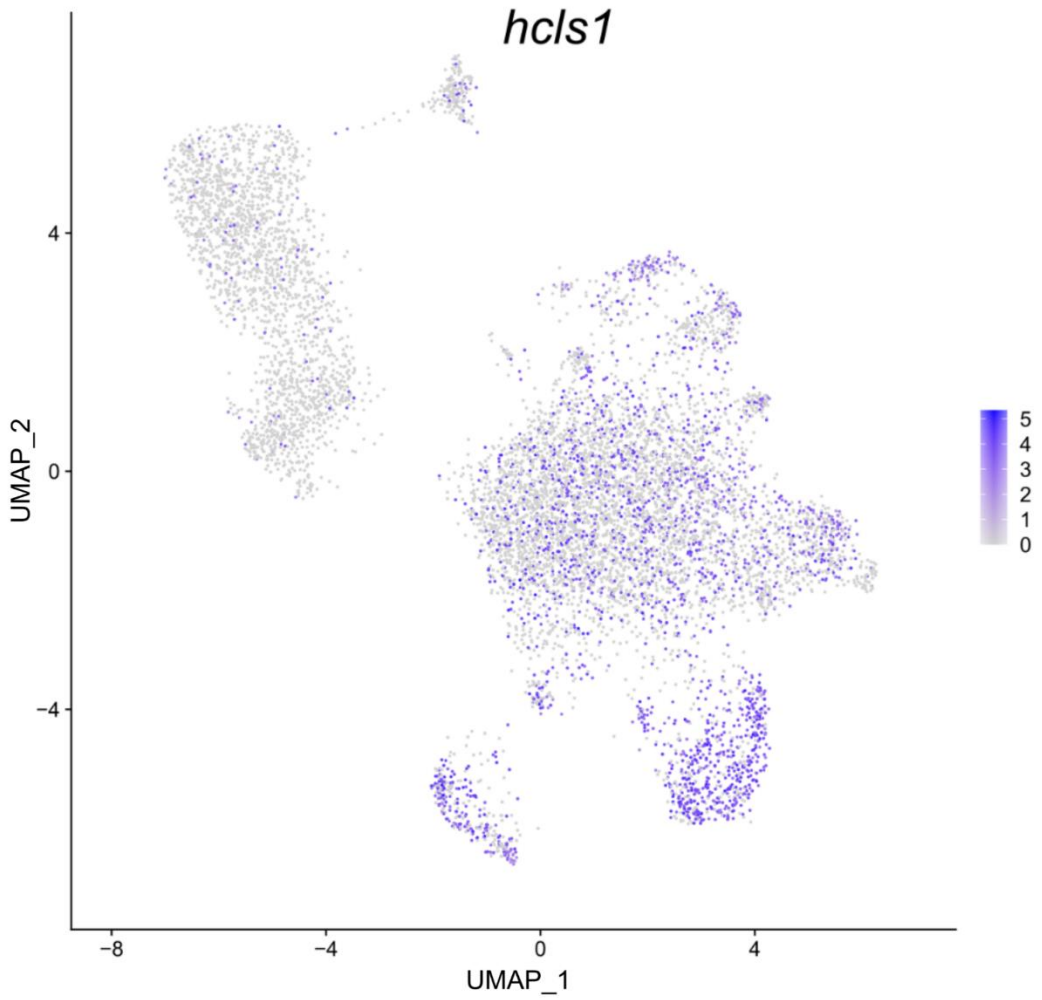


Figure 4. Feature plot highlighting *Syngnathus typhle* blood and head kidney cells expressing the *hcls1* gene within a Uniform manifold approximation projection (UMAP). Increased cell colour intensity indicates increased gene expression.

Thrombocytes

Thrombocytes (61 cells), or platelets, were also represented in *S. typhle* with differentially expressed genes including *ak9,* which can influence platelet and blood coagulation in humans (Studzińska et al., 2010). Further confirmation is provided by the upregulation of *thbs1*, a previously labelled thrombocyte marker in codfish (Guslund et al., 2020) and a prominently expressed gene in thrombocytes (Isenberg and Roberts, 2020). The vascular endothelial growth factor (*vegfa*) which shares a functional relation with platelets and tissue wound healing (Bao et al., 2009) was also highly expressed within the cluster.

Basophils and eosinophils

A selection of basophil related genes characterised cluster 8, including *tfpi* and *itgb3 (Pontén et al., 2008)* (174 cells). However, the most notable upregulation came from the gene coding for GATA2 (cell differentiation), a recognised cell marker for basophil granulocytes (Pontén et al., 2008; Monaco et al., 2019), distinguishing it from its close immune cell relatives.

The smallest cluster isolated in this study was attributed to eosinophils (12 cells). A large number of C-type lectins (*Galactose-specific lectin nattectin*) characterised the clusters expression profile, while the upregulated expression of eosinophil peroxidase (*epx*), a common marker and constituent of eosinophil intracellular granules in humans (Acharya and Ackerman, 2014), gave further support to a small eosinophil population presence in pipefish.

DISCUSSION

Previous work has described the evolutionary adaptations and genomic alterations that have shaped the peculiarities within Syngnathiformes immune system, including the loss of MHC II in *S. typhle* and an increased diversification of MHC I in the syngnathids that have evolved unique male pregnancy (Haase et al., 2013; Roth et al., 2020). The ramifications of these evolutionary changes on cellular expression level in *S. typhle* have been left unexplored up until this study, which was the first to characterise putative pipefish immune cell populations based on their individual gene expression profiles using single-cell RNA sequencing. Successful identification of a number of integral immune system constituents was achieved and the presence of relatable immune cell markers, congruent with those in other model organisms, provides a crucial baseline for future experimental and immune assessments in syngnathid fishes.

In *S. typhle* and other bony fishes, the head kidney is an important lymphoid organ and the epicentre for immune cell hematopoiesis (Uribe et al., 2011; Roth et al., 2012; Katzenback et al., 2016). Appropriately, the dominance of head kidney derived cells in the perceived hematopoietic progenitor cell cluster here supports the cell type classification. The size of the hematopoietic cluster shares similarities with previous single-cell transcriptome studies on zebrafish head kidney extracted cells (Baron et al., 2019). Research on zebrafish has also shown that the progressive maturation of thrombocytes is characterised by a shift towards thrombocyte functional genes and suppression of hematopoietic related genes relevant in cell proliferation and ribosomal biogenesis (Macaulay et al., 2016). This could go some way to explaining the lack of upregulated specific immune cell markers within the hematopoietic cell cluster, while the close relation with most of the other more distinguished cell groups could be

an indication of immature immune cells types yet to become immunologically active. Moreover, while lower in expressed significance, the upregulation of ribosomal transcripts within the hematopoietic cluster matches the ribosomal indications expressed by Macaulay et al. (2016) and Khajuria et al. (2018) with regards their role in hematopoiesis and cell differentiation. Interestingly, the presence of *hcls1*, a gene upregulated in the T and B cell clusters, was also shown to be expressed in many cell contingents of the hematopoietic cell cluster. The presence of these interspersed cells could indicate that many these cells are at different stages of maturity, while its strong expression in T- and B-lymphocytes could relate to *hcls1*'s antigen signalling function described previously (Yamanashi et al., 1997; Gomez et al., 2006).

Fish red blood cells are nucleated and have been shown to express immune related genes along with those related to gas exchange (Morera et al., 2011; Nombela and Ortega-Villaizan, 2018; Shen et al., 2018). This supports the expressed presence of *h2-d2* and *ifi27* here, suggesting erythrocytes could hold immunological relevance among *S. typhle*'s defenses. Indications suggest that the separation of the two perceived erythrocyte clusters could be due to a batch effect stemming from the blood extracted from two different individuals. It is therefore, challenging to determine if additional factors influence the cluster differences. Erythrocyte stage of maturity for example have been reported in fish previously, with expression differences existing between immature reticulocytes and mature red blood cells (Lund et al., 2000; Zexia et al., 2007; Tavares-Dias and Barcellos, 2017).

An encouraging number of immune related genes were found in *S. typhle*, especially when identifying, T-cell, B-cell, neutrophil and macrophage populations. Managing to identify these crucial immune system constituents is promising for future syngnathid immune studies, which will allow further sub-cell type distinctions within these white blood cell groups. Neutrophils constitute the largest circulating leukocyte population in humans (50-70%) (Welch et al., 1989; Mayadas et al., 2014), and while circulating neutrophil percentages in teleost fish are markedly reduced in comparison (~5%) (Vázquez and Guerrero, 2007; Havixbeck et al., 2016), head kidney neutrophil reserves have been shown to be extensive (Katzenback and Belosevic, 2009; Havixbeck et al., 2016), as was the case here in *S. typhle*. *Mmp9*, a neutrophil marker in cod and humans was suggested to drive neutrophil migration in mammals (Bradley et al., 2012), and its upregulation here could be an indication of a similar influence in syngnathid fishes. One of the most convincing markers extracted from the neutrophil cluster is the neutrophil development factor *cebpe*, which has been used as a neutrophil identifier in zebrafish (Tang et al., 2017).

The strong upregulation of the MHC II associated *cd209* in the macrophage cluster, an immune receptor expressed in antigen presenting cells, poses some questions. Previously, *Cd209* expression has been identified in the MHC II devoid Atlantic cod (Goetz et al., 2006; Solbakken

et al., 2019). This considered, it would appear that despite the loss of MHC II in both species *cd209* expression has been conserved. This could be an indication that it has adopted alternative antigen processing purpose or remains a phagocytic tool capable of facilitating viral and bacterial uptake. Macrophages are known to differentiate into pro-inflammatory (M1) and anti-inflammatory (M2) states (Murray and Wynn, 2011). *Cd209* has been shown to be highly upregulated in M2 macrophages compared with pro-inflammatory cells in mammals (Buchacher et al., 2015), while M2 macrophage activity has been linked to tissue remodelling and wound healing (Gordon, 2003; Xiao et al., 2008; Murray and Wynn, 2011). Therefore, the elevated presence of *cd209* here could be associated with anti-inflammatory M2 macrophages; however, determining the specific function of *cd209* in syngnathids requires further investigation.

A number of intriguing T-cell markers were featured in *S. typhle*. These included *gzma,* a protease constituent of cytotoxic T-cell granules in humans (Krähenbühl et al., 1988) and an important innate immunological component in fish (Chaves-Pozo et al., 2019). This granzyme was highlighted by Guslund et al. (2020) in association with a novel type of GATA-3 cytotoxic cell in codfish. Another marker, a subunit of the IL2R receptor associated with immune suppression, is an indicator for T_{regs} (Chinen et al., 2016) and $CD8^+$ cytotoxic T and NK-cell granulocytic defenses in fish (Fischer et al., 2013; Tang et al., 2017). Taken with the universal T-cell receptors represented within the cluster and the absence of additional markers, such as *gata-3* indicative of the cytotoxic cell lineage previously detected in codfish, make it difficult to conclusively allocate this group of cells to one specific T-cell subset. Nevertheless, in combination with additional immune cell isolation studies, these markers are vital when it comes to identifying T-cell subsets at a higher resolution in the future. Genomic assessments have concluded that *cd4* has been lost in *S. typhle*, bringing into question the functional or overall presence of $CD4^+$ T-cells (Roth et al., 2020). Appropriately, no markers specifically associated with $CD4^+$ T-cells were identified in this study, providing further support of their evolutionary disappearance in *S. typhle*. Whilst conclusively classifying the T-cell cluster presented here as $CD8^+$ cytotoxic T-cells was not possible, the unearthing of a number of MHC I related pathway constituents and cytotoxic related genes supports the presence of the $CD8^+$ T-cell subset in pipefish. However, determining whether the $CD8^+$ T-cell subset or alternative innate immune cell types are able to offset the loss of the MHC II pathway in *S. typhle* requires further investigation.

T-cell receptors and B-cell receptors in humans were thought to be exclusively expressed by their namesakes. This traditional concept has been challenged recently by Ahmed et al. (2019) who managed to isolate “dual expresser” lymphocytes capable of expressing both receptor types. This could explain the presence of a number of T-cell receptors that were found in both

the B- and T-cell clusters. Alternatively, a small T-cell subset may be imbedded with the larger B-cell cluster, grouping together based on upregulated genes shared between the lymphocyte lineages that transcend these receptors. Further work should attempt refine and explain the cell sub-types that exist within these two integral adaptive immune cell groups.

Eosinophils, like basophils and neutrophils, are granulocyte white blood cells charged with immune surveillance and inflammatory roles in humans (Hogan et al., 2008). Each are equipped with an array of cytoplasmic granules, of which some hold C-type lectins (Swaminathan et al., 2005). The upregulated expression of eosinophil peroxidase (*epx*), a common marker and constituent of eosinophil intracellular granules in humans (Acharya and Ackerman, 2014), gives convincing support to a small eosinophil population present in pipefish. Isolated initially from the venom of *Thalassophryne nattereri***,** *Galactose-specific lectin nattectin* (*nattectin*) is a C-type lectin with hemagglutination activity (Lopes-Ferreira et al., 2011). The prominent expression of *nattectin*, or a potentially similar C-type lectin within the cluster, could indicate that these granulocytes assist with coagulation or a similar immunomodulatory function that has been reported previously concerning *nattectin* (Saraiva et al., 2011; Lopes-Ferreira et al., 2011).

Deduced NK-cell-like cells in fish have been identified previously, however, clear transcript markers are still missing making their identities difficult to determine (Niu et al., 2020; Guslund et al., 2020). Characterising the putative NK-cell-like assigned cluster in this study was challenging, due to the restricted number of highly significant upregulated genes driving cluster differentiation. The tentative NK-cell-like assignment in this species would require additional clarification from additional single-cell sequencing assessments, in order to be able to confirm with confidence its involvement in the *S. typhle* immune repertoire.

Although many of the expected immune system constituents were identifiable in this study, there were also absentees, with dendritic cells being the most notable. Dendritic cells occupy a small percentage (~0.1%) of the total circulating cells in the blood, but are more prominent in mucosal areas in humans and are crucial for linking the innate and adaptive immune systems (Hart, 1997; Steinman et al., 2003). Despite their perceived absence in *S. typhle* in this study, their identification in other teleost fishes (Lugo-Villarino et al., 2010; Bassity and Clark, 2012; Guslund et al., 2020) suggests that the perhaps the tissues or resolution of analyses used here was not sufficient for their discovery. Due to their small population and shared expressed markers with other immune cell types, it is likely that this sub-population is being masked within another cell cluster. The data presented here was also insufficient to distinguish between macrophages and their monocyte progenitors.

This investigation succeeded in describing the first in-depth molecular characterisation of the broadnosed pipefish immune cell repertoire, utilizing single-cell transcriptomics. As with this study's predecessors which delved into the sparsely explored realm of teleost immune cell populations (Carmona et al., 2017; Hernández et al., 2018; Guslund et al., 2020; Niu et al., 2020; Perdiguero et al., 2021), a number of key immune cell sub-populations were identifiable providing some insight into the putative immune function of *S. typhle*. Establishing a baseline expression profile for each immune cell group identified here, along with corroborated gene markers, will be crucial for future experimental work such as those carrying out infection assessments on syngnathid fishes. This molecular assistance should extend to investigations concerning *S. typhle* relatives such as seahorses and other pipefishes, with potential scope for future comparative, multispecies single-cell assessments that can lay down a comprehensive immune cell overview of the Syngnathiforme lineage. Understanding the cellular repercussions and adaptations that may have evolved within these immunologically bizarre fishes could support future medical practices by shedding light on the way certain immune cell lineages evolve.

SYNTHESIS

This thesis consists of three chapters focused on understanding the evolution of male pregnancy in syngnathid fishes and its close relationship with the immune system by utilizing comparative transcriptomics. Multi-species analysis of **the syngnathid pregnancy transcriptome** was achieved, characterising brood types and pregnancy stages based on differential gene expression, highlighting trends shared between species and other forms of pregnancy. The **evolution of immunological tolerance** in syngnathids was explored by comparing gene expression data from extracted brooding tissue in pouched syngnathids with placenta-like structures and basal external egg-carrying gestation forms. In order to test if the loss of MHC II in the lineage influences the immunological tolerance measures, allograft and autograft fin-transplants were utilized to assess the allorecognition systems adopted in pipefish with and without MHC II. Lastly, this thesis explored the potential impacts, evolutionary adaptations and potential compensatory mechanisms that relate to the functional **loss of MHC II**, as well as establishing the first single-cell transcriptome characterisation of the syngnathid immune cell repertoire.

The syngnathid pregnancy transcriptome and stage dynamics

The spectrum of male pregnancy forms that have evolved among syngnathids range from basal external egg carriers to specialized sealed pouches with structures similar to that of eutherian placentas (Wilson et al., 2001; Carcupino et al., 2002; Stölting and Wilson, 2007; Ripley et al., 2010). Previous work has highlighted the benefits of examining gene expression at incremental stages during male pregnancy when attempting to disentangle the progressive physiological and immunological changes during gestation (Whittington et al., 2015; Roth et al., 2020). This was the first investigation to carry out a comprehensive transcriptome multi-species assessment of male pregnancy brooding tissue spanning the extremes of syngnathid pregnancy forms. **Chapter I** explored brood pouch gene expression of four syngnathid species at four different stages during pregnancy, highlighting a number of physiological characteristics associated with male pregnancy and similarities with female pregnancy. Despite independent evolutionary origins, this study suggests that similar mechanisms have evolved in syngnathids to adapt to the demands of internal gestation.

In **chapter I,** the greatest overall expression distinction was observed between the initial and concluding stages following the amalgamation of expression data from all species. This trend was retained following the removal of the pouchless *N. ophidion* and provided one of the first indications that in terms of gene expression the male pregnancy period is a highly dynamic process. The fundamental differences between early and late pregnancy stage expression

were highlighted further following the mFuzz soft-clustering analyses, as none of the four species exhibited an early/late combined cluster. These observations validate the need to include time as a variable when attempting to truly understand the mechanisms that drive pregnancy. Gestation is a non-linear process, therefore, research that aims to define its functionality and physiology must acknowledge its phase structure. Extracting information on biological processes that drive these stage expression differences was paramount in order to draw comparisons with other pregnancy forms which supports the understanding of its evolutionary path.

Mammalian mothers progress from a net anabolic to catabolic state as gestation advances, to support the changing demands of the developing embryo and maintaining the optimal environment that suits the offspring and the parent (Herrera, 2002; Lain and Catalano, 2007). These changes are also met with the tissue restructuring and modification processes to accommodate embryonic growth and vascularisation (Abrahams et al., 2004; Read et al., 2007; Osol and Mandala, 2009); a dynamic that has also been alluded to in male pregnancy (Carcupino et al., 1997; Laksanawimol et al., 2006; Dudley et al., 2021). The upregulation of catabolic processes during the concluding stages of male pregnancy in this study echo the fluctuations reported in mammals. While evidence of tissue remodeling supports the premise that, as with mammalian pregnancy, incubation tissue structure gradually changes during male pregnancy. The catabolic breakdown of internal pouch tissue, which likely minimises the interval between pregnancies, is exampled further by the active expulsion of the placenta-like connective tissue. This behavior is most notably observed in *Syngnathus* species during parturition and shares some resemblance to placental expulsion following labour in eutherian mammals (Ripley and Foran, 2006).

The role of inflammation during early pregnancy in mammals is acknowledged with tissue swelling assisting with the implantation of the fertilized egg into the wall of the uterus (Mor and Abrahams, 2002; Dekel et al., 2010; Chavan et al., 2017). From a visual standpoint, pouch tissue fleshiness in mature syngnathid males was seen to increase in preparation for the deposition of eggs, supporting previous descriptions (Harlin-Cognato et al., 2006; Whittington and Friesen, 2020). This visual observation was supported by the upregulation of inflammation genes during the early stages of pregnancy in syngnathids here, while previously it has also been recorded an upregulation during pouch development (Roth et al., 2020). It appears, therefore, that a similar inflammation affords implantation assistance in male pregnancy akin to in female pregnancy. As in mammals, pouched syngnathids possess blood rich internal pouch tissue, arranged into folds to maximize the contact with incubating eggs (Wilson et al., 2001; Carcupino et al., 2002; Kawaguchi et al., 2017; Whittington and Friesen, 2020). The induction of inflammatory pathways leads to immune cell recruitment, which in turn can release

cytokines which can stimulate tissue remodeling (Granot et al., 2012). Thus, it is conceivable that inflammation may lend to the extension of these tissue folds, benefiting the full immersion of eggs in pouched syngnathids.

The early inflammation trend was also exhibited in *N. ophidion,* which is intriguing considering it has a basal brooding form and reduced feto-paternal connection. Swelling of the small ventral strip of tissue integument possessed by *N. ophidion* is likely key to successful egg transfer and anchorage, and could be a plausible explanation for inflammation related expression found in the species. An interesting comparison can be drawn with the egg-carrying strategies of pelvic brooding ricefishes, which in congruence with *N. ophidion* did not evolve placenta-like structures capable of supplementing the progeny. Instead, following spawning a “plug” tissue develops within gonoduct which anchors filaments carrying eggs externally (Hart et al., 1984; Iwamatsu et al., 2007). In a recent study, the retention of these filaments was stipulated to have triggered inflammatory pathways that facilitated the evolution of the plug tissue (Hilgers et al., In press). A similar evolutionary occurrence may have arisen in the ancestors of *N. ophidion* males, with the stimulation of inflammatory processes upon contact with the donated eggs. Genes involved in inflammatory pathways are known to control angiogenesis, cell proliferation and related migration processes (Medzhitov, 2008). In turn, inflammatory signaling processes are conducive to facilitating the evolution and novel tissues. It appears that the co-option of pro-inflammatory processes for tissue remodeling purposes during mammalian embryo implantation (Chavan et al., 2017; Griffith et al., 2017; Stadtmauer and Wagner, 2020), are not limited to organisms with internal gestation. Findings here in *N. ophidion*, build on the emerging evidence of inflammation driving evolutionary innovation (Chavan et al., 2017; Wagner et al., 2019; Eckhart et al., 2019; Ehrlich et al., 2019; Hilgers et al., In press) which likely developed further to accommodate the increased feto-paternal endometrium utilized by pouched syngnathid species. Research in the future should attempt to strengthen the overall understanding of inflammation driven innovation in syngnathids, with potential differences between trunk and tail brooders serving as a compelling topic.

There have been various reports in gestating mammals pertaining to immunological participation during parturition (Joosten et al., 1991; Helguera et al., 2009; Gomez-Lopez et al., 2013). One example is the upregulation of placental MHC I in cows leading up labour, which is thought to assist with the separation of fetal membranes (Benedictus et al., 2012; Rapacz-Leonard et al., 2014; Benedictus et al., 2015). The MHC I upregulation found here during parturition compared with early pregnancy, renders the concept of parental immune mediated assistance during parturition highly plausible in pouched species. Offspring expulsion at the end of gestation in pouched syngnathids and especially in seahorses, is an intensive mechanical process (Garrick-Maidment, 1997; Frias-Torres, 2004). The upregulated

immune system during parturition could also be to protect the pregnant male from infection during labour. In the seahorse especially, the pouch is a heavily vascularized self-contained marsupium, conceptually optimal for colonization of harmful bacteria. Therefore, its prolonged exposure during labour would likely benefit from an activated immune system. This has been suggested previously in mammals, with stipulations suggesting that the downregulation of regulatory T-cells (T_{regs}) allows for upregulated activity of other immune processes in order to prevent infection during labour (Schober et al., 2012; Shah et al., 2020). The identification of T_{reg}-like cells and putative T_{reg} candidate genes in fish have been reported previously (Wen et al., 2011; Dijkstra, 2014). However, a comprehensive characterisation of T_{regs} in teleosts is still lacking and their function in relation to male pregnancy is still in need of further investigation (Nakanishi et al., 2015).

In the seahorse specifically, the upregulation of antigen presentation and processing genes in the late/parturition combined mFuzz cluster poses some interesting questions. Seahorses are associated with having a definitively 'sealed' brooding structure the duration of pregnancy, unlike the *Syngnathus* genus. The upregulation of the adaptive immune system not only during parturition but also late stage pregnancy could imply that the pouch is opened at some point prior to expulsion. This would be in line with the same reasons as the immune responses evoked during labour to protect against foreign antigen influx. Equally, it could be an indication that the feto-paternal connection that exists during pregnancy has reached its climax and the offspring are no longer under threat from the paternal immune system. In reality, an upregulation of the adaptive immune system in advanced syngnathid brooders during parturition could be carrying out both roles in offspring-paternal separation and infection prevention following the intense act of birth. Pregnant parents face the trade-off of preventing embryonic rejection through immune modulation processes, while also ensuring immunological alertness to external infection (La Rocca et al., 2014). This conundrum would appear to be resolved in the seahorse leading up to parturition, such that immunological tolerance requirements dissipate allowing for the upregulation of the adaptive immune system. Overall, these findings challenge the assumption that the seahorse pouch is fully sealed during the gestation period, but future work should aim to corroborate this and provide additional clarification of its function.

Upregulated inflammatory processes in reference to the conclusion of pregnancy are also significant in humans (Junqueira et al., 1980; Stjernholm-Vladic et al., 2004; Gomez-Lopez et al., 2013). Fewer indications of parturition involved inflammation are evident in the syngnathids investigated here apart from the downregulation of *il-10* (*H. erectus*), a strong anti-inflammation mediator. With that said, results here supporting a relationship between the onset of labour

and reactivation of the immune system following pregnancy, opens up an interesting avenue to explore further in male pregnancy related research.

The findings expressed here highlight the benefits of adopting a pregnancy stage specific approach to transcriptome-wide assessments. As having a temporal reference for gene expression data can provide a more nuanced understanding of function that is not possible with a single sampling point in time. Furthermore, by comparing four syngnathid species with different brooding types a more comprehensive assessment of syngnathid male pregnancy transcriptome was attainable, as well as additional insight into the evolutionary pathways that culminated in the advanced brooding forms. This could be an invaluable baseline for future studies which wishes to uncover additional oddities and evolutionary characteristics that exist within the male pregnancy spectrum.

Evolution of immunological tolerance in male pregnancy

The immune system's ability to maintain self-tolerance is just as integral as its defensive role against foreign pathogens. With that considered, the unusual functional loss of the key self-tolerance mediatory MHC II pathway in the syngnathid lineage proved a fascinating topic for investigation (Haase et al., 2013; Roth et al., 2020). The immunological capacity of these MHC II devoid fishes to mount an effective adaptive immune response and their ability to determine self from non-self were brought into question. Moreover, the implications regarding the link to male pregnancy was an intriguing concept to explore in more detail. To provide further insight into this immunological peculiarity, particular focus was assigned to the expression of immune related transcripts during pregnancy **(chapter I)**, while fin-transplants were utilized to directly assess allorecognition responses in fish with and without MHC II **(chapter II)**.

There is growing support that immune modulation occurs during male pregnancy and is important for maintaining immunological homeostasis between the offspring and parent (Roth et al., 2020). Transcriptome assessments here further support this deduction while also providing stage-specific data concerning the modulatory processes at work. The downregulation of MHC I related pathway expression during early pregnancy in pouched syngnathids could reflect the delicate feto-paternal connection at that time in development. The accented activity in the most advanced brooders (*H. erectus*) could be an indication that this evolutionary adaptation progressed further with increasing pouch specialization and parental investment. However, the nuances of adaptive immune functionality and the complexities associated with interlinked immune pathways can render interpreting expression data extremely challenging. It is therefore promising that a defined expression direction of antigen presenting and processing genes could be associated with a single pregnancy stage,

providing some detailed immunological tolerance characteristics that have not previously been published.

In **chapter I**, *N. ophidion* was the only species to not exhibit a trend of MHC related downregulation during early pregnancy, implying that immunological tolerance measures were not active in this basal form of male pregnancy. The deduced reduction in feto-paternal intimacy and physiological connection, rendered immunological tolerance processes redundant in species lacking pouches with placenta-like structures. It is interesting to note that in **chapter II** MHC I downregulation was exhibited in the *N. ophidion* autograft transplantees but not detected in *S. typhle*; a possible indication of immunological tolerance evocation stimulated by the recognition of self-tissue. While these findings would benefit from further investigation, it suggests that despite its absence during pregnancy, immunological tolerance measures still appear functional under different modes of stimulation in *N. ophidion*. It seems, therefore, that despite the differences between transplants and semi-allogeneic embryos, the fetal-paternal connection appears to be insufficient to stimulate an immunological reaction. This perhaps justifies further the assumptions made here that immunological tolerance in *N. ophidion* is not required during pregnancy as it is in pouch brooders. A number of brooding type variations exist between *N. ophidion* and the *Syngnathus* species investigated here. For example, *Doryrhamphus dactyliophorus* has evolved membranous egg compartments that retain deposited eggs, while *Stigmatopora* species have pouch extensions akin to *Syngnathus*, but without the complete egg envelopment (Wilson et al., 2001). Therefore, exploring the immune capabilities of similar basal brooders and those that have progressed towards fully formed pouches could provide some further insight into the evolution of immunological tolerance within the context of male pregnancy.

T_{regs} are recognised mediators of immunological tolerance during pregnancy in humans, suppressing Th1 immune responses upon activation (Somerset et al., 2004; Wing and Sakaguchi, 2010). Believed to have been derived from the same lineage as naive $CD4^+$ T-cells, T_{regs} could hold the key to fully understanding immune modulation in syngnathids, if in fact the cell type has remained conserved within the lineage. $CD4^+$ T-cells associate with MHC II, it therefore stands to reason that the evolutionary loss of MHC II could have facilitated a similar cellular disappearance. **Chapter III** aimed to shed some light on these questions and established the first putative immune cell characterisation of any syngnathid species. The absence of CD4 and MHC II related expression in *S. typhle* in this study supports the putative loss the $CD4^+$ T-cells. However, additional immune cell characterisations of syngnathids with functional MHC II components are required in order to conclusively determine if the cell type has been conserved or not in *S. typhle*. In addition to $CD4^+$ T-cells, the outcome of a successful pregnancy is dependent on a number of crucial immune cell types. For example, implantation

is associated with the increased proliferation of decidual NK-cells and antigen presenting cells, while immune modulation processes throughout pregnancy's duration are dictated by dendritic cells specifically (Kämmerer, 2005; Hanna et al., 2006; Laskarin et al., 2007). Dendritic cells play a pivotal role within the feto-maternal interface in humans, maintaining immunological tolerance during pregnancy and reactivating it leading up to parturition (Gardner and Moffett, 2003; Fest et al., 2007; Mor et al., 2011; Shah et al., 2020). Merging the approaches of **chapter I** and **chapter III** by assessing the brood pouch derived immune cell cohorts of syngnathids during pregnancy using single-cell transcriptomics, should be the next step to understanding immunological tolerance activity.

Based on the brood pouch expression data analysed here, immunological tolerance was likely important for the evolution of male pregnancy in pouched syngnathid species. Moreover, it can be speculated that without immunological assistance the evolution of advanced male pregnancy forms, such as those found in the seahorse, may not have been possible. In mammals, the evolution of a specialized trophoblast cells which do no not express MHC II, prevents the rejection of the implanted embryo through the avoidance of maternal T helper cells (Zheng et al., 1996; Erlebacher, 2001; Moffett and Loke, 2006). The facilitative role that MHC II loss may have had on the evolution of advanced male pregnancy in syngnathids, acting as a proxy for the tolerance roles afforded by trophoblast cells in mammals, remains difficult to conclude. It was predicted that the immunological tolerance activity would be greater in *S. typhle*, compared with *N. ophidion*, based on the fact that it is missing the MHC II pathway. However, transplant experiments were not able to support this hypothesis based on transcriptome data. So it appears, at least based on the transplant experiments carried out here that MHC II loss has not influenced the tolerance capacity of *S. typhle* and further research is required to determine if MHC II loss facilitated the evolution of male pregnancy.

The loss of MHC II

This thesis has strived to answer the potential reasons for and the immunological impact of MHC II functional loss in syngnathid fishes. Since the discovery of its transcriptomic and subsequent genomic absence, research has focused on *S. typhle*'s immunodeficiency (Haase et al., 2013; Small et al., 2016; Roth et al., 2020). This was largely supported by the fact that the vast majority of vertebrate species possess the MHC II pathway and for a long time it was believed to be an integral adaptive immune component (Litman et al., 2010). In order to understand how organisms can adapt to such a substantial immune loss, it is useful to look at similar evolutionary instances in the animal kingdom. Interestingly, almost every vertebrate with a sequenced genome can boast a fully intact adaptive immune system yet a number of marine fishes, namely Gadiformes, anglerfishes, elephant sharks and coelacanths have all

shown to be lacking important adaptive immune components (Star et al., 2011; Amemiya et al., 2013; Venkatesh et al., 2014; Malmstrøm et al., 2016; Dubin et al., 2019). More specifically, the genomic loss of MHC II has been recorded in Gadiformes (Star et al., 2011) and the anglerfish, *Lophius piscatorius* (Dubin et al., 2019), which perhaps emphasizes the dispensability of this adaptive immune pathway in teleost fishes. Incredibly, reduced MHC I diversity rather than MHC II in anglerfish has also been recorded in those adopting permanent sexual parasitism compared with those with temporary (Swann et al., 2020). As with the syngnathids, anglerfish researchers associate MHC loss with facilitating the evolution of their sexual processes (Dubin et al., 2019; Swann et al., 2020). Overall, these findings highlight the plasticity of the adaptive immune system in teleosts and its ability to adapt and rearrange its defenses to suit development.

It is known from previous reports that codfishes rely on an increased diversity of MHC I and species-specific innate immune receptors in the wake of MHC II pathway loss (Star et al., 2011; Tørresen et al., 2018). Similarly, in previous reports pipefish have also propounded that a switch to the MHC I cross-presentation pathway has coincided with MHC II loss (Roth et al., 2020). Analyses carried out here in this thesis provided further support of this immunological mechanistic switch. Firstly, no key MHC II pathway genes were uncovered in either *Syngnathus* species or in *H. erectus* in **chapter I**, while a number of the MHC I related branch were found in all species. Based on their association with allogenic rejection, MHC were expected to be prominent effectors in the transplant experiments carried out in **chapter II** on *S. typhle* and *N. ophidion*. Yet, MHC I antigens were only detected in an immunosuppressive fashion in *N. ophidion* autografts, with none detected in either allograft replicates. However, due to *N. ophidion* possessing a fully intact MHC II repertoire, these findings provide little explanation for the processes compensating for MHC loss. Alternatively, the upregulated expression of the gene coding for granzyme a (*gzma*) in *S. typhle* allografts gave a glimpse of the possible immune pathways at work. Owing to its association with $CD8^+$ cytotoxic T-lymphocytes (CTLs) which are activated through MHC I, *gzma*, could be an indicator that a MHC I related immune response has been activated through allograft instigation. CTLs are recognised effectors of allogenic rejection in mammals (Le Moine et al., 2002; Rocha et al., 2003) and fish (Tatner and Manning, 1983), and they could be chief mediators in the MHC II devoid *S. typhle*. Interestingly, *gzma* was also identified among the deduced T-cell cluster in the single-cell transcriptome assessments **(chapter III)** of the same species, supporting its importance within the immune cell repertoire. The receptor subunit, *il-2rb*, was also upregulated within the same cluster pertaining to CTL proliferatory function (Boyman and Sprent, 2012). While requiring further functional and single-cell assessments with greater

resolution, CTLs appear to be representatives in the *S. typhle* defense repertoire and it's not too far to speculate that they may compensate in some part to MHC II loss.

Future perspectives

The syngnathid fish lineage is perfectly suited for studies striving to answer questions on evolutionary adaptation and molecular changes, exemplified by the research project described here. The depth of scope for syngnathid research allows for an array of promising investigation ideas, especially when referring to its immune defenses and unique male pregnancy. This thesis merely scratched the surface when it comes to answering questions regarding the repercussions of MHC II loss within the lineage, but did assist with highlighting future experiment opportunities.

Gene expression indications here suggest MHC I may compensate to some degree for the absence of MHC II and that CTL function appears to at the forefront of allorecognition. For this reason, it would be intriguing to explore CTL function in syngnathids further. As of yet, the immune cell repertoires that occupy the brood pouch endometrium in syngnathids have only been discussed through bulk mRNA sequencing of pouch tissue. Combining the concepts taken from **chapter I** and **III** of this thesis, isolating immune cells within the seahorse brood pouch and then adopting single-cell mRNA profiling should be the next step. This should contribute to a better understanding of which immune cells mediate and patrol the feto-paternal interface, and do these populations vary between pouch types.

The impressive variation of adaptive immune components that exist within the syngnathid lineage provides an excellent opportunity to investigate immune cell evolution. Genomic adaptive immune rearrangements may have influenced the evolutionary survival of specific immune cell subtypes. Stipulations were made here that MHC II loss may have reduced the $CD4^+$ T-cell population numbers in *S. typhle*, while the reverse could be said for cell types operating through MHC I activation. To determine whether this is the case, the immune cell population dynamics of multiple syngnathids with differing adaptive immune repertoires need to be explored. This should be carried out in tandem with the visual identification of immune cells where possible to help clarify the gene expression driven deductions. Not only should this contextualize the findings described here in **chapter III** but it could provide priceless insight into immune cell evolution. If immune system rearrangements are found to influence immune cell evolution and population dynamics in syngnathids, it opens up the possibility that similar instances may have occurred in the animal kingdom. Shedding light on how immune cell lineages evolve could have huge implications for the field of evolutionary immunology and the progression of applied medical research in the future.

Author contributions

Chapter I

OR, **JP** and SJ conceived the study. **JP**, OR, RS. and KSW collected and processed samples at differing stages. **JP**, AD, RS and TB analysed the data. **JP** and OR wrote the manuscript with input from all co-authors.

Chapter II

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

Chapter III

OR, **JP** and SJ conceived the study. **JP** and NG processed fish cell samples and analysed the data. **JP** and OR wrote the manuscript with input from all co-authors.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit mit dem Titel:

MALE PREGNANCY AND THE EVOLUTIONARY IMPORTANCE OF IMMUNOLOGICAL TOLERANCE IN SYNGNATHID FISHES

mit Hilfe meiner Betreuer und Co-Autoren nur unter Zuhilfenahme der angegebenen Hilfsmittel und Quellen sowie unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft verfasst habe.

Die Arbeit wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt.
Dies ist mein einziges und bisher erstes Promotionsverfahren.
Teile dieser Arbeit wurden als Manuskripte in wissenschaftlichen Fachzeitschriften jeweilig mit Olivia Roth als Koautor eingereicht: Kapitel I in *Molecular Ecology;* Kapitel II in *Developmental & Comparative Immunology* und Kapitel III in *Frontiers in Immunology*.

.....

Jamie Parker

Kiel, 13.12.2021

List of publications

Publications accepted

Parker, J., Dubin, A., Schneider, R., Wagner, K., Jentoft, S., Böhne, A., Bayer, T., Roth O. 2021. Immunological tolerance in the evolution of male pregnancy. *Molecular Ecology*. Accepted.

Publications in review

Parker, J., Guslund, N., Jentoft, S., Roth, O. 2021. Characterisation of pipefish immune cell repertoire through single-cell transcriptomics. *Frontiers in Immunology*. In review.

Parker, J., Roth O. 2021 Comparative assessment of immunological tolerance in pipefish with and without the major histocompatibility complex class II. *Developmental and Comparative Immunology*. In review.

Acknowledgements

I would have been unable to complete this thesis without the support, advice and practical nous of many people involved around the project and its peripheries. Firstly, I would like to thank my supervisor Olivia Roth who gave me the chance to work on this fascinating research project. You have always been understanding and patient when there were times of difficulty and cannot thank you enough for allowing me the freedom to help shape this project from its beginning to what it has ultimately become. Despite research, related ups and downs I always found it easy to work with you and the group you have created over the years always instilled a sense of calmness. I could not have imagined doing my PhD anywhere else. These thanks are extended to all pipefish group members past and present for their all their assistance with aquaria maintenance, fish feeding and continuous scientific discussion. Without the help of Joe, Zora, Martin and Fabian, the fish needed for this study would have likely not have been available. I have huge respect for your ability to keep all things aquaria based from falling apart despite times of leaky tanks and drastic temperature changes. Similarly thank you to Franzi, Haqdil, Mirja, Paulina, Rebekka, Fabian, Denis and Fred for all of your fish feeding and group support throughout my time here. If I ever had any queries and problems around the institute and lab Mareike and Diana have always been on hand to help. Your ability to calm the nerves of people in stressful situations is something that I think everyone in the institute can attest to and your influence is always appreciated. I would like to thank all Ralf, Isa, Arseny, Jelena and Till for you continuous support in all things science, without your input my journey to this point would have been far less smooth. Further acknowledgement has to be given to Ralf, for his patience and willingness to help with challenging data problems and advice concerning analyses. Henry, Freya, Kim and Agnes, I have loved being part of the pipefish PhD contingent and it has been a pleasure sharing and overcoming the chaos associated with the experience. To Thorsten, FB3 and the wider GEOMAR community, thank you all for your support and being so welcoming during my time here. Aside from my work escapades, I could not have done without the pub, football, art, music and general conversation sessions involving Felix, Tadhg, Angela, Vero, Kwi, Nora, Lara, Vanessa, Melanie and Nico. All of you made my time at GEOMAR and integration into Baltic life that much more enjoyable!

To all my collaborative colleagues across the country and abroad I would like to give thanks. Thank you Sissel, Naomi and Siv for your welcoming presence and enthusiasm, you all made my trip to Oslo a warm experience despite endless, bucket loads of snow. I would also like to thank Sissel and Astrid for your priceless insights and advice during my times of analysis and writing. Further thanks are extended to all those associated with the IMPRS, and my thesis advisory committee who were crucial for my development as a scientist and helped me create a thesis that I am proud of.

Acknowledgements

I would like to thank my family for their unwavering unconditional support throughout my time here despite the geographic distance and continual confusion as to what I am actually studying. The lifelong encouragement that I have received from my parents, to do whatever I enjoy and choose to put my mind to, is the bedrock that my successes have been built upon during my academic journey. I would like to thank Julia for being by my side and for always providing me with inspiration in all facets of my life. The turbulence of the last year was negated by you and I will be forever grateful.

BIBLIOGRAPHY

Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G. 2004. Macrophages and apoptotic cell clearance during pregnancy. *American Journal of Reproductive Immunology*, **51:**275-282.

Acharya KR, Ackerman SJ. 2014. Eosinophil granule proteins: form and function. *Journal of Biological Chemistry*, **289:**17406-17415.

Ahmed R, Omidian Z, Giwa A, Cornwell B, Majety N, Bell DR, Lee S, Zhang H, Michels A, Desiderio S. 2019. A public BCR present in a unique dual-receptor-expressing lymphocyte from type 1 diabetes patients encodes a potent T cell autoantigen. *Cell*, **177:**1583-1599.

Ahnesjö I. 1989. Sex role reversal in two pipefish (Syngnathidae) species: Paternal care and male limitation of female reproductive success. Uppsala University, Uppsala.

Alemany A, Florescu M, Baron CS, Peterson-Maduro J, Van Oudenaarden A. 2018. Whole-organism clone tracing using single-cell sequencing. *Nature*, **556:**108-112.

Aluvihare VR, Kallikourdis M, Betz AG. 2004. Regulatory T cells mediate maternal tolerance to the fetus. *Nature Immunology*, **5:**266-271.

Alvarez J, Montelongo A, Iglesias A, Lasuncion M, Herrera E. 1996. Longitudinal study on lipoprotein profile, high density lipoprotein subclass, and postheparin lipases during gestation in women. *Journal of Lipid Research*, **37:**299-308.

Amemiya CT, Alföldi J, Lee AP, Fan S, Philippe H, MacCallum I, Braasch I, Manousaki T, Schneider I, Rohner N. 2013. The African coelacanth genome provides insights into tetrapod evolution. *Nature*, **496:**311-316.

Amoroso E. 1968. The evolution of viviparity. *Proceedings of the Royal Society of Medicine*, **61:**1188-1200.

Andrews S. 2010. FastQC: A quality control tool for high throughput sequence data., http://www.Bioinformatics.Babraham.Ac.Uk/Projects/Fastqc/

Apanius V, Penn D, Slev PR, Ruff LR, Potts WK. 1997. The nature of selection on the major histocompatibility complex. *Critical Reviews in Immunology*, **17:**179-224.

Auchincloss Jr H, Winn HJ. 2004. Clarence Cook Little (1888–1971): The Genetic Basis of Transplant Immunology. *American Journal of Transplantation*, **4:**155-159.

Ayala García MA, González Yebra B, López Flores AL, Guaní Guerra E. 2012. The major histocompatibility complex in transplantation. *Journal of transplantation*, **2012**.

Baas M, Besançon A, Goncalves T, Valette F, Yagita H, Sawitzki B, Volk H-D, Waeckel-Enée E, Rocha B, Chatenoud L. 2016. TGFβ-dependent expression of PD-1 and PD-L1 controls CD8+ T cell anergy in transplant tolerance. *elife*, **5:**e08133.

Bahr A, Wilson A. 2011. The impact of sex-role reversal on the diversity of the major histocompatibility complex: insights from the seahorse (Hippocampus abdominalis). *BMC Evolutionary Biology*, **11:**121.

Bai S, Thummel R, Godwin AR, Nagase H, Itoh Y, Li L, Evans R, McDermott J, Seiki M, Sarras Jr MP. 2005. Matrix metalloproteinase expression and function during fin regeneration in zebrafish: analysis of MT1-MMP, MMP2 and TIMP2. *Matrix Biology*, **24:**247-260.

Bainbridge DR. 2014. The evolution of pregnancy. *Early Human Development*, **90:**741-745.

Bao P, Kodra A, Tomic-Canic M, Golinko MS, Ehrlich HP, Brem H. 2009. The role of vascular endothelial growth factor in wound healing. *Journal of Surgical Research*, **153:**347-358.

Barash A, Dekel N, Fieldust S, Segal I, Schechtman E, Granot I. 2003. Local injury to the endometrium doubles the incidence of successful pregnancies in patients undergoing in vitro fertilization. *Fertility and Sterility*, **79:**1317-1322.

Baron CS, Barve A, Muraro MJ, van der Linden R, Dharmadhikari G, Lyubimova A, de Koning EJ, van Oudenaarden A. 2019. Cell type purification by single-cell transcriptome-trained sorting. *Cell*, **179:**527-542.

Bassity E, Clark TG. 2012. Functional identification of dendritic cells in the teleost model, rainbow trout (Oncorhynchus mykiss). *PloS One*, **7:**e33196.

Bauersachs S, Wolf E. 2012. Transcriptome analyses of bovine, porcine and equine endometrium during the pre-implantation phase. *Animal Reproduction Science*, **134:**84-94.

Baylis JR. 1981. The evolution of parental care in fishes, with reference to Darwin's rule of male sexual selection. *Environmental Biology of Fishes*, **6:**223-251.

Beemelmanns A, Poirier M, Bayer T, Kuenzel S, Roth O. 2019. Microbial embryonal colonization during pipefish male pregnancy. *Scientific Reports*, **9:**1-14.

Beemelmanns A, Roth O. 2016. Biparental immune priming in the pipefish Syngnathus typhle. *Zoology*, **119:**262-272.

Bell AW, Ehrhardt RA. 2002. Regulation of placental nutrient transport and implications for fetal growth. *Nutrition Research Reviews*, **15:**211-230.

Bellayr I, Mu X, Li Y. 2009. Biochemical insights into the role of matrix metalloproteinases in regeneration: challenges and recent developments. *Future Medicinal Chemistry*, **1:**1095-1111.

Benacerraf B. 1981. Role of MHC gene products in immune regulation. *Science*, **212:**1229-1238.

Benedictus L, Koets AP, Rutten VP. 2015. The role of placental MHC class I expression in immune-assisted separation of the fetal membranes in cattle. *Journal of Reproductive Immunology*, **112:**11-19.

Benedictus L, Thomas AJ, Jorritsma R, Davies CJ, Koets AP. 2012. Two-Way Calf to Dam Major Histocompatibility Class I Compatibility Increases Risk for Retained Placenta in Cattle. *American Journal of Reproductive Immunology*, **67:**224-230.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B Methodology*, **57:**289-300.

Berglund A, Rosenqvist G, Svensson I. 1986. Reversed sex roles and parental energy investment in zygotes of two pipefish (Syngnathidae) species. *Marine Ecology Progress Series*, **29:**209-215.

Beuchat CA, Vleck D. 1990. Metabolic consequences of viviparity in a lizard, Sceloporus jarrovi. *Physiological Zoology*, **63:**555-570.

Billingham RE, Brent L, Medawar PB. 1953. 'Actively Acquired Tolerance' of Foreign Cells. *Nature*, **172:**603-606.

Bird S, Zou J, Savan R, Kono T, Sakai M, Woo J, Secombes C. 2005. Characterisation and expression analysis of an interleukin 6 homologue in the Japanese pufferfish, Fugu rubripes. *Developmental & Comparative Immunology*, **29:**775-789.

Blackburn DG. 1982. Evolutionary origins of viviparity in the Reptilia. I. Sauria. *Amphibia-Reptilia*, **3:**185-205.

Blackburn DG. 1992. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. *American Zoologist*, **32:**313-321.

Blackburn DG. 1999. Viviparity and oviparity: evolution and reproductive strategies. In: Encyclopedia of Reproduction. New York, USA, Academic Press, p. 994-1003.

Blackburn DG. 2015. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. *Journal of Morphology*, **276:**961-990.

Blumer LS. 1979. Male parental care in the bony fishes. *The Quarterly Review of Biology*, **54:**149-161.

Blumer LS. 1982. A bibliography and categorization of bony fishes exhibiting parental care. *Zoological Journal of the Linnean Society*, **75:**1-22.

Boehm T. 2011. Design principles of adaptive immune systems. *Nature Reviews Immunology*, **11:**307-317.

Borghans JA, Beltman JB, De Boer RJ. 2004. MHC polymorphism under host-pathogen coevolution. *Immunogenetics*, **55:**732-739.

Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM, Sherlock G. 2004. GO:: TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinformatics*, **20:**3710-3715.

Boyman O, Sprent J. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. *Nature Reviews Immunology*, **12:**180-190.

Bradley LM, Douglass MF, Chatterjee D, Akira S, Baaten BJ. 2012. Matrix metalloprotease 9 mediates neutrophil migration into the airways in response to influenza virus-induced toll-like receptor signaling. *PLoS Pathogens*, **8:**e1002641.

Brandley MC, Young RL, Warren DL, Thompson MB, Wagner GP. 2012. Uterine gene expression in the live-bearing lizard, Chalcides ocellatus, reveals convergence of squamate reptile and mammalian pregnancy mechanisms. *Genome Biology and Evolution*, **4:**394-411.

Braza F, Brouard S, Chadban S, Goldstein DR. 2016. Role of TLRs and DAMPs in allograft inflammation and transplant outcomes. *Nature Reviews Nephrology*, **12:**281-290.

Breder C, Rosen D. 1966. Modes of reproduction in fishes. New York, New York, USA, Natural History Press.

Brown C, Mullins LJ, Wesencraft K, McConnell G, Beltran M, Henderson NC, Conway B, Hoffmann S, Rider S, Mullins JJ. 2021. ScRNA transcription profile of adult zebrafish podocytes using a novel reporter strain. *Cellular Physiology and Biochemistry*, **55:**35-47.

Bryant DM, Johnson K, DiTommaso T, Tickle T, Couger MB, Payzin-Dogru D, Lee TJ, Leigh ND, Kuo T-H, Davis FG. 2017. A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. *Cell reports*, **18:**762-776.

Buchacher T, Ohradanova-Repic A, Stockinger H, Fischer MB, Weber V. 2015. M2 polarization of human macrophages favors survival of the intracellular pathogen Chlamydia pneumoniae. *PloS One*, **10:**e0143593.

Buchfink B, Xie C, Huson DH. 2015. Fast and sensitive protein alignment using DIAMOND. *Nature Methods*, **12:**59-60.

Butler A, Hoffman P, Smibert P, Papalexi E, Satija R. 2018. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nature Biotechnology*, **36:**411.

Carcupino M, Baldacci A, Mazzini M, Franzoi P. 1997. Morphological organization of the male brood pouch epithelium of Syngnathus abaster Risso (Teleostea, Syngnathidae) before, during, and after egg incubation. *Tissue and Cell*, **29:**21-30.

Carcupino M, Baldacci A, Mazzini M, Franzoi P. 2002. Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a morphological and ultrastructural comparative study on three anatomically different pouches. *Journal of Fish Biology*, **61:**1465-1480.

Cardwell T, Sheffer R, Hedrick P. 2001. MHC variation and tissue transplantation in fish. *Journal of Heredity*, **92:**305-308.

Carmona SJ, Teichmann SA, Ferreira L, Macaulay IC, Stubbington MJ, Cvejic A, Gfeller D. 2017. Single-cell transcriptome analysis of fish immune cells provides insight into the evolution of vertebrate immune cell types. *Genome Research*, **27:**451-461.

Carpenter KE, De Angelis N. 2002. The living marine resources of the Western Central Atlantic. Rome, Italy, Food and agriculture organization of the United Nations.

Celli S, Albert ML, Bousso P. 2011. Visualizing the innate and adaptive immune responses underlying allograft rejection by two-photon microscopy. *Nature Medicine*, **17:**744-749.

Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss JF, Petraglia F. 2009. Inflammation and pregnancy. *Reproductive Sciences*, **16:**206-215.

Chavan AR, Griffith OW, Wagner GP. 2017. The inflammation paradox in the evolution of mammalian pregnancy: turning a foe into a friend. *Current Opinion in Genetics & Development*, **47:**24-32.

Chaves-Pozo E, Valero Y, Lozano MT, Rodríguez-Cerezo P, Miao L, Campo V, Esteban MA, Cuesta A. 2019. Fish Granzyme A shows a greater role than Granzyme B in fish innate cell-mediated cytotoxicity. *Frontiers in Immunology*, **10:**2579.

Chen Q, Lu X-J, Chen J. 2015. Identification and functional characterization of the CSF1R gene from grass carp Ctenopharyngodon idellus and its use as a marker of monocytes/macrophages. *Fish & Shellfish Immunology*, **45:**386-398.

Chen S, Zhou Y, Chen Y, Gu J. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, **34:**i884-i890.

Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, Gasteiger G, Feng Y, Fontenot JD, Rudensky AY. 2016. An essential role for the IL-2 receptor in T reg cell function. *Nature Immunology*, **17:**1322-1333.

Choy J. 2010. Granzymes and perforin in solid organ transplant rejection. *Cell Death and Differentiation*, **17:**567-576.

Clement M, Haddad P, Soulie A, Benvenuti C, Lichtenheld MG, Podack E, Sigaux N, Sasportes M. 1991. Perforin and granzyme B as markers for acute rejection in heart transplantation. *International Immunology*, **3:**1175-1181.

Clutton-Brock TH. 1991. The evolution of parental care. Princeton University Press.

Consortium TU. 2021. UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Research*, **49:**D480-D489.

Cooper MD, Alder MN. 2006. The evolution of adaptive immune systems. *Cell*, **124:**815-822.

Covacu R, Philip H, Jaronen M, Almeida J, Kenison JE, Darko S, Chao C-C, Yaari G, Louzoun Y, Carmel L. 2016. System-wide analysis of the T cell response. *Cell reports*, **14:**2733-2744.

Cuellar O, Smart C. 1977. Analysis of histoincompatibility in a natural population of the bisexual whiptail lizard Cnemidophorus tigris. *Transplantation*, **24:**127-133.

Cuesta A, Esteban MÁ, Meseguer J. 2003. In vitro effect of chitin particles on the innate cellular immune system of gilthead seabream (Sparus aurata L.). *Fish & Shellfish Immunology*, **15:**1-11.

Dausset J. 1981. The major histocompatibility complex in man. *Science*, **213:**1469-1474.

Dawson CE. 1985. Indo-Pacific Pipefishes. Mississippi, Gulf Coast Research Laboratory.

Dawson CE. 1986. Syngnathidae. In: Fishes of the North-eastern Atlantic and Mediterranean II. Paris, UNESCO, p. 628–639.

Dee CT, Nagaraju RT, Athanasiadis EI, Gray C, Del Ama LF, Johnston SA, Secombes CJ, Cvejic A, Hurlstone AF. 2016. CD4-Transgenic zebrafish reveal tissue-resident Th2- and regulatory T cell–like populations and diverse mononuclear phagocytes. *The Journal of Immunology*, **197:**3520-3530.

Dekel N, Gnainsky Y, Granot I, Mor G. 2010. Inflammation and implantation. *American Journal of Reproductive Immunology*, **63:**17-21.

DeMarco V. 1993. Metabolic rates of female viviparous lizards (Sceloporus jarrovi) throughout the reproductive cycle: do pregnant lizards adhere to standard allometry? *Physiological Zoology*, **66:**166-180.

Denne SC, Patel D, Kalhan SC. 1991. Leucine kinetics and fuel utilization during a brief fast in human pregnancy. *Metabolism*, **40:**1249-1256.

Dijkstra JM. 2014. TH 2 and T reg candidate genes in elephant shark. *Nature*, **511:**E7-E9.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, **29:**15-21.

Dubin A, Jørgensen TE, Moum T, Johansen SD, Jakt LM. 2019. Complete loss of the MHC II pathway in an anglerfish, Lophius piscatorius. *Biology Letters*, **15:**20190594.

Dudley J, Hannaford P, Dowland S, Lindsay L, Thompson M, Murphy C, Van Dyke J, Whittington C. 2021. Structural changes to the brood pouch of male pregnant seahorses (Hippocampus abdominalis) facilitate exchange between father and embryos. *Placenta*, **114:**115-123.

Eckhart L, Ehrlich F, Tschachler E. 2019. A stress response program at the origin of evolutionary innovation in the skin. *Evolutionary Bioinformatics*, **15:**1176934319862246.

Edwards SV, Hedrick PW. 1998. Evolution and ecology of MHC molecules: from genomics to sexual selection. *Trends in Ecology & Evolution*, **13:**305-311.

Ehrlich F, Fischer H, Langbein L, Praetzel-Wunder S, Ebner B, Figlak K, Weissenbacher A, Sipos W, Tschachler E, Eckhart L. 2019. Differential evolution of the epidermal keratin cytoskeleton in terrestrial and aquatic mammals. *Molecular Biology and Evolution*, **36:**328-340.

Emms DM, Kelly S. 2015. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. *Genome Biology*, **16:**157.

Emms DM, Kelly S. 2019. OrthoFinder: phylogenetic orthology inference for comparative genomics. *Genome Biology*, **20:**1-14.

Erlebacher A. 2001. Why isn't the fetus rejected? *Current Opinion in Immunology*, **13:**590-593.

Ernerudh J, Berg G, Mjösberg J. 2011. Regulatory T helper cells in pregnancy and their roles in systemic versus local immune tolerance. *American Journal of Reproductive Immunology*, **66:**31-43.

Ewels P, Magnusson M, Lundin S, Käller M. 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, **32:**3047-3048.

Farber DL, Netea MG, Radbruch A, Rajewsky K, Zinkernagel RM. 2016. Immunological memory: lessons from the past and a look to the future. *Nature Reviews Immunology*, **16:**124-128.

Farrell JA, Wang Y, Riesenfeld SJ, Shekhar K, Regev A, Schier AF. 2018. Single-cell reconstruction of developmental trajectories during zebrafish embryogenesis. *Science*, **360:**6392.

Fernando MM, Stevens CR, Walsh EC, De Jager PL, Goyette P, Plenge RM, Vyse TJ, Rioux JD. 2008. Defining the role of the MHC in autoimmunity: a review and pooled analysis. *PLoS Genetics*, **4:**e1000024.

Ferrara N. 2000. Vascular endothelial growth factor and the regulation of angiogenesis. *Recent Progress in Hormone Research*, **55:**15-35.

Fest S, Aldo PB, Abrahams V, Visintin I, Alvero A, Chen R, Chavez S, Romero R, Mor G. 2007. Trophoblast–macrophage interactions: a regulatory network for the protection of pregnancy. *American Journal of Reproductive Immunology*, **57:**55-66.

Fischer U, Koppang EO, Nakanishi T. 2013. Teleost T and NK cell immunity. *Fish & Shellfish Immunology*, **35:**197-206.

Flajnik MF, Kasahara M. 2010. Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nature Reviews Genetics*, **11:**47.

Folkman J. 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Medicine*, **1:**27-30.

Förster R, Davalos-Misslitz AC, Rot A. 2008. CCR7 and its ligands: balancing immunity and tolerance. *Nature Reviews: Immunology*, **8:**362-371.

Frias-Torres S. 2004. Notes on aquarium brood release and feeding of the opossum pipefish, Microphis brachyurus lineatus. *Gulf and Caribbean Research*, **16:**73-75.

Futosi K, Fodor S, Mócsai A. 2013. Reprint of Neutrophil cell surface receptors and their intracellular signal transduction pathways. *International Immunopharmacology*, **17:**1185-1197.

Futschik ME, Carlisle B. 2005. Noise-robust soft clustering of gene expression time-course data. *Journal of Bioinformatics and Computational Biology*, **3:**965-988.

Gao J, Senthil M, Ren B, Yan J, Xing Q, Yu J, Zhang L, Yim J. 2010. IRF-1 transcriptionally upregulates PUMA, which mediates the mitochondrial apoptotic pathway in IRF-1-induced apoptosis in cancer cells. *Cell Death and Differentiation*, **17:**699-709.

Gardner L, Moffett A. 2003. Dendritic cells in the human decidua. *Biology of Reproduction*, **69:**1438-1446.

Garnica AD, Chan W-Y. 1996. The role of the placenta in fetal nutrition and growth. *Journal of the American College of Nutrition*, **15:**206-222.

Garrick-Maidment N. 1997. Seahorses: Conservation and care. Tfh Publications Incorporated.

Gil-Krzewska A, Murakami Y, Peruzzi G, O'Brien KJ, Merideth MA, Cullinane AR, Gahl WA, Coligan JE, Gochuico BR, Krzewski K. 2017. Natural killer cell activity and dysfunction in Hermansky-Pudlak syndrome. *British Journal of Haematology*, **176:**118-123.

Gilpin SE, Li Q, Evangelista-Leite D, Ren X, Reinhardt DP, Frey BL, Ott HC. 2017. Fibrillin-2 and Tenascin-C bridge the age gap in lung epithelial regeneration. *Biomaterials*, **140:**212-219.

Gittleman JL. 1981. The phylogeny of parental care in fishes. *Animal Behaviour*, **29:**936-941.

Goetz FW, McCauley L, Goetz GW, Norberg B. 2006. Using global genome approaches to address problems in cod mariculture. *ICES Journal of Marine Science*, **63:**393-399.

Gomez-Lopez N, Vega-Sanchez R, Castillo-Castrejon M, Romero R, Cubeiro-Arreola K, Vadillo-Ortega F. 2013. Evidence for a role for the adaptive immune response in human term parturition. *American Journal of Reproductive Immunology*, **69:**212-230.

Gomez TS, McCarney SD, Carrizosa E, Labno CM, Comiskey EO, Nolz JC, Zhu P, Freedman BD, Clark MR, Rawlings DJ. 2006. HS1 functions as an essential actin-regulatory adaptor protein at the immune synapse. *Immunity*, **24:**741-752.

Goncalves IB, Ahnesjö I, Kvarnemo C. 2015. Embryo oxygenation in pipefish brood pouches: novel insights. *Journal of Experimental Biology*, **218:**1639-1646.

Goodnow CC, Sprent J, de St Groth BF, Vinuesa CG. 2005. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature*, **435:**590-597.

Goodwin NB, Balshine-Earn S, Reynolds JD. 1998. Evolutionary transitions in parental care in cichlid fish. *Proceedings of the Royal Society B: Biological Sciences*, **265:**2265-2272.

Gordon S. 2003. Alternative activation of macrophages. *Nature reviews immunology*, **3:**23-35.

Granot I, Gnainsky Y, Dekel N. 2012. Endometrial inflammation and effect on implantation improvement and pregnancy outcome. *Reproduction*, **144:**661-668.

Griffith OW, Chavan AR, Protopapas S, Maziarz J, Romero R, Wagner GP. 2017. Embryo implantation evolved from an ancestral inflammatory attachment reaction. *Proceedings of the National Academy of Science*, **114:**E6566-E6575.

Griffith OW, Ujvari B, Belov K, Thompson MB. 2013. Placental lipoprotein lipase (LPL) gene expression in a placentotrophic lizard, Pseudemoia entrecasteauxii. *Journal of Experimental Zoology, Part B: Molecular and Developmental Evolution*, **320:**465-470.

Griffiths GM, Namikawa R, Billingham M, Weissman I, Mueller C, Liu CC, Young JDE. 1991. Granzyme A and perforin as markers for rejection in cardiac transplantation. *European Journal of Immunology*, **21:**687-692.

Grimholt U. 2016. MHC and evolution in teleosts. *Biology*, **5:**6.

Gross MR, Shine R. 1981. Parental care and mode of fertilization in ectothermic vertebrates. *Evolution*, **35:**775-793.

Guleria I, Khosroshahi A, Ansari MJ, Habicht A, Azuma M, Yagita H, Noelle RJ, Coyle A, Mellor AL, Khoury SJ. 2005. A critical role for the programmed death ligand 1 in fetomaternal tolerance. *Journal of Experimental Medicine*, **202:**231-237.

Guslund NC, Solbakken MH, Brieuc MS, Jentoft S, Jakobsen KS, Qiao S-W. 2020. Single-cell transcriptome profiling of immune cell repertoire of the Atlantic cod which naturally lacks the major histocompatibility class II system. *Frontiers in Immunology*, **11:**2602.

Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, Couger MB, Eccles D, Li B, Lieber M. 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols*, **8:**1494-1512.

Haase D, Roth O, Kalbe M, Schmiedeskamp G, Scharsack JP, Rosenstiel P, Reusch TB. 2013. Absence of major histocompatibility complex class II mediated immunity in pipefish, Syngnathus typhle: evidence from deep transcriptome sequencing. *Biology Letters*, **9:**20130044.

Hamai Y, Fujii T, Miki A, Geraghty DE, Harada I, Takai Y, Kozuma S, Tsutsumi O, Taketani Y. 1999. Quantitative Assessment of Human Leukocyte Antigen-G Protein in Amniotic Fluid by a Double-Determinant Enzyme-Linked Immunosorbent Assay Using Anti-Human Leukocyte Antigen-G-Specific Antibody '87G'. *American Journal of Reproductive Immunology*, **41:**293-295.

Hamilton H, Saarman N, Short G, Sellas AB, Moore B, Hoang T, Grace CL, Gomon M, Crow K, Simison WB. 2017. Molecular phylogeny and patterns of diversification in Syngnathid fishes. *Molecular Phylogenetics and Evolution*, **107:**388-403.

Han X, Wang R, Zhou Y, Fei L, Sun H, Lai S, Saadatpour A, Zhou Z, Chen H, Ye F. 2018. Mapping the mouse cell atlas by microwell-seq. *Cell*, **172:**1091-1107.

Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, Prus D, Cohen-Daniel L, Arnon TI, Manaster I. 2006. Decidual NK cells regulate key

developmental processes at the human fetal-maternal interface. *Nature Medicine*, **12:**1065-1074.

Hansen VL, Faber LS, Salehpoor AA, Miller RD. 2017. A pronounced uterine pro-inflammatory response at parturition is an ancient feature in mammals. *Proceedings: Biological Sciences*, **284:**20171694.

Hanson C, Bolling S, Stoolman L, Schlegelmilch J, Abrams G, Miska P, Deeb G. 1988. Cytoimmunologic monitoring and heart transplantation. *The Journal of heart transplantation*, **7:**424-429.

Haresign TW, Shumway SE. 1981. Permeability of the marsupium of the pipefish Syngnathus fuscus to [14C]-alpha amino isobutyric acid. *Comparative Biochemistry and Physiology. Part A: Physiology*, **69:**603-604.

Harlin-Cognato A, Hoffman EA, Jones AG. 2006. Gene cooption without duplication during the evolution of a male-pregnancy gene in pipefish. **103:**19407-19412.

Hart DN. 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood, The Journal of the American Society of Hematology*, **90:**3245-3287.

Hart NH, Pietri R, Donovan M. 1984. The structure of the chorion and associated surface filaments in Oryzias—evidence for the presence of extracellular tubules. *Journal of Experimental Zoology*, **230:**273-296.

Hartl D, Lee CG, Da Silva CA, Chupp GL, Elias JA. 2009. Novel biomarkers in asthma: chemokines and chitinase-like proteins. *Current Opinion in Allergy and Clinical Immunology*, **9:**60-66.

Hashimoto K, Nakanishi T, Kurosawa Y. 1990. Isolation of carp genes encoding major histocompatibility complex antigens. *Proceedings of the National Academy of Sciences*, **87:**6863-6867.

Havixbeck JJ, Rieger AM, Wong ME, Hodgkinson JW, Barreda DR. 2016. Neutrophil contributions to the induction and regulation of the acute inflammatory response in teleost fish. *Journal of Leukocyte Biology*, **99:**241-252.

Hayes MP, Berrebi GA, Henkart PA. 1989. Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. *The Journal of experimental medicine*, **170:**933-946.

Hedlund M, Stenqvist A-C, Nagaeva O, Kjellberg L, Wulff M, Baranov V, Mincheva-Nilsson L. 2009. Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function. *The Journal of Immunology*, **183:**340-351.

Helguera G, Eghbali M, Sforza D, Minosyan TY, Toro L, Stefani E. 2009. Changes in global gene expression in rat myometrium in transition from late pregnancy to parturition. *Physiological Genomics*, **36:**89-97.

Herald ES. 1959. From pipefish to seahorse — a study of phylogenetic relationships. *Proceedings of the California Academy of Sciences*, **29:**465-473.

Hernández PP, Strzelecka PM, Athanasiadis EI, Hall D, Robalo AF, Collins CM, Boudinot P, Levraud J-P, Cvejic A. 2018. Single-cell transcriptional analysis reveals ILC-like cells in zebrafish. *Science immunology*, **3:**29.

Herrera E. 2002. Lipid metabolism in pregnancy and its consequences in the fetus and newborn. *Endocrine*, **19:**43-55.

Hiby SE, Walker JJ, O'Shaughnessy KM, Redman CW, Carrington M, Trowsdale J, Moffett A. 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *Journal of Experimental Medicine*, **200:**957-965.

Hildemann W. 1972. Transplantation reactions of two species of Osteichthyes (Teleostei) from South Pacific coral reefs. *Transplantation*, **14:**261-267.

Hildemann W, Haas R. 1960. Comparative studies of homotransplanatation in fishes. *Journal of Cellular and Comparative Physiology*, **55:**227-233.

Hilgers L, Roth O, Nolte AW, Schüller A, Spanke T, Flury JM, Utama IV, Altmüller J, Wowor D, Misof B, et al. In press. Inflammation and mammalian placenta gene co-option contributed to the evolution of a novel egg-anchoring tissue in pelvic brooding ricefishes. *Current Biology*,

Hogan SP, Rosenberg HF, Moqbel R, Phipps S, Foster PS, Lacy P, Kay AB, Rothenberg ME. 2008. Eosinophils: biological properties and role in health and disease. *Clinical and Experimental Allergy*, **38:**709-750.

Hogarth PJ. 1976. Institute of Biology Studies in Biology No. 75. Viviparity. Southampton, UK, Camelot Press.

Holets LM, Hunt JS, Petroff MG. 2006. Trophoblast CD274 (B7-H1) is differentially expressed across gestation: influence of oxygen concentration. *Biology of Reproduction*, **74:**352-358.

Isenberg JS, Roberts DD. 2020. THBS1 (thrombospondin-1). *Atlas of genetics and cytogenetics in oncology and haematology*, **24:**291.

Iwamatsu T, Kobayashi H, Shibata Y, Sato M, Tsuji N, Takakura K-i. 2007. Oviposition cycle in the oviparous fish Xenopoecilus sarasinorum. *Zoological Science*, **24:**1122-1127.

Jablonka E, Lamb MJ. 2014. Evolution in four dimensions, revised edition: Genetic, epigenetic, behavioral, and symbolic variation in the history of life. MIT press.

Jones AG, Rosenqvist G, Berglund A, Avise JC. 1999. The genetic mating system of a sex-role-reversed pipefish (Syngnathus typhle): a molecular inquiry. *Behavioral Ecology Sociobiology*, **46:**357-365.

Joosten I, Sanders M, Hensen E. 1991. Involvement of major histocompatibility complex class I compatibility between dam and calf in the aetiology of bovine retained placenta. *Animal Genetics*, **22:**455-463.

Junqueira L, Zugaib M, Montes G, Toledo O, Krisztan R, Shigihara K. 1980. Morphologic and histochemical evidence for the occurrence of collagenolysis and for the role of neutrophilic polymorphonuclear leukocytes during cervical dilation. *American Journal of Obstetrics and Gynecology*, **138:**273-281.

Kaastrup P, Stet RJ, Tigchelaar AJ, Egberts E, van Muiswinkel WB. 1989. A major histocompatibility locus in fish: serological identification and segregation of transplantation antigens in the common carp (Cyprinus carpio L.). *Immunogenetics*, **30:**284-290.

Kallman K. 1970. Genetics of tissue transplantation in Teleostei. *Transplant. Proceed.*, **2:**263-271.

Kallman KD, Gordon M. 1957. Transplantation of fins in Xiphophorin fishes. *Annals of the New York Academy of Sciences*, **71:**307-318.

Kallman KD, Gordon M. 1958. Genetics of fin transplantation in xiphophorin fishes. *Annals of the New York Academy of Sciences*, **73:**599-610.

Kämmerer U. 2005. Antigen-presenting cells in the decidua. *Immunology of Pregnancy*, **89:**96-104.

Kasahara M. 2013. Major histocompatibility complex: evolution, structure, and function. Springer Science & Business Media.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, **30:**772-780.

Katzenback BA, Belosevic M. 2009. Isolation and functional characterization of neutrophil-like cells, from goldfish (Carassius auratus L.) kidney. *Developmental & Comparative Immunology*, **33:**601-611.

Katzenback BA, Belosevic M. 2012. Colony-stimulating factor-1 receptor protein expression is a specific marker for goldfish (Carassius auratus L.) macrophage progenitors and their differentiated cell types. *Fish & Shellfish Immunology*, **32:**434-445.

Katzenback BA, Katakura F, Belosevic M. 2016. Goldfish (Carassius auratus L.) as a model system to study the growth factors, receptors and transcription factors that govern myelopoiesis in fish. *Developmental & Comparative Immunology*, **58:**68-85.

Kawaguchi M, Okubo R, Harada A, Miyasaka K, Takada K, Hiroi J, Yasumasu S. 2017. Morphology of brood pouch formation in the pot-bellied seahorse Hippocampus abdominalis. *Zoological letters*, **3:**1-16.

Keller IS, Roth O. 2020. Parental investment and immune dynamics in sex-role reversed pipefishes. *PloS One*, **15:**e0228974.

Kelly RW. 1994. Pregnancy maintenance and parturition: the role of prostaglandin in manipulating the immune and inflammatory response. *Endocrine Reviews*, **15:**684-706.

Kennedy T. 1977. Evidence for a role for prostaglandins in the initiation of blastocyst implantation in the rat. *Biology of Reproduction*, **16:**286-291.

Ketterson ED, Nolan Jr V. 1994. Male parental behavior in birds. *Annual Review of Ecology Systematics*, **25:**601-628.

Khajuria RK, Munschauer M, Ulirsch JC, Fiorini C, Ludwig LS, McFarland SK, Abdulhay NJ, Specht H, Keshishian H, Mani DR. 2018. Ribosome levels selectively regulate translation and lineage commitment in human hematopoiesis. *Cell*, **173:**90-103.

Kim M, Seo H, Choi Y, Yoo I, Seo M, Lee C-K, Kim H, Ka H. 2015. Analysis of stage-specific gene expression profiles in the uterine endometrium during pregnancy in pigs. *PloS One*, **10:**e0143436.

Kim PK, Armstrong M, Liu Y, Yan P, Bucher B, Zuckerbraun BS, Gambotto A, Billiar TR, Yim JH. 2004. IRF-1 expression induces apoptosis and inhibits tumor growth in mouse mammary cancer cells in vitro and in vivo. *Oncogene*, **23:**1125-1135.

King JC. 2000. Physiology of pregnancy and nutrient metabolism. *American Journal of Clinical Nutrition*, **71:**1218-1225.

Kleiman DG, Malcolm JR. 1981. The evolution of male parental investment in mammals. In: Parental care in mammals. Springer, p. 347-387.

Knox K, Baker JC. 2008. Genomic evolution of the placenta using co-option and duplication and divergence. *Genome Research*, **18:**695-705.

Kolm N. 2019. Parental care. *Reproductive Biology and Phylogeny of fishes (Agnathans and Body Fishes)*, 351-371.

Komi DEA, Kazemi T, Bussink AP. 2016. New insights into the relationship between chitinase-3-like-1 and asthma. *Current Allergy and Asthma Reports*, **16:**1-10.

Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. *Science*, **248:**220-223.

Krähenbühl O, Rey C, Jenne D, Lanzavecchia A, Groscurth P, Carrel S, Tschopp J. 1988. Characterization of granzymes A and B isolated from granules of cloned human cytotoxic T lymphocytes. *The Journal of Immunology*, **141:**3471-3477.

Kröger A, Köster M, Schroeder K, Hauser H, Mueller PP. 2002. Activities of IRF-1. *Journal of Interferon & Cytokine Research*, **22:**5-14.

Kronester-Frei A. 1975. Licht-und elektronenmikroskopische Untersuchungen am Brutepithel des Mannchens von Nerophis lumbriciformis (Pennant 1776), Syngnathidae, unter spezieller Beriicksichtigung der strukturellen Verinderung der Eihiille. *Forma Functio*, **8:**419-462.

Krueger F.2015. Trim galore!: A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files. https://www.bioinformatics.babraham.ac.uk/projects/trim_galore// (Date of access: (01/04/2018).

Kummer JA, Wever PC, Kamp AM, ten Berge IJ, Hack CE, Weening JJ. 1995. Expression of granzyme A and B proteins by cytotoxic lymphocytes involved in acute renal allograft rejection. *Kidney International*, **47:**70-77.

Kvarnemo C, Mobley KB, Partridge C, Jones A, Ahnesjö I. 2011. Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish Syngnathus typhle. *Journal of Fish Biology*, **78:**1725-1737.

La Rocca C, Carbone F, Longobardi S, Matarese G. 2014. The immunology of pregnancy: regulatory T cells control maternal immune tolerance toward the fetus. *Immunology Letters*, **162:**41-48.

Lain KY, Catalano PM. 2007. Metabolic changes in pregnancy. *Clinical Obstetrics and Gynecology*, **50:**938-948.

Laksanawimol P, Damrongphol P, Kruatrachue M. 2006. Alteration of the brood pouch morphology during gestation of male seahorses, Hippocampus kuda. *Marine & Freshwater Research*, **57:**497-502.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods*, **9:**357.

Laskarin G, Kämmerer U, Rukavina D, Thomson AW, Fernandez N, Blois SM. 2007. Antigen-presenting cells and materno-fetal tolerance: an emerging role for dendritic cells. *American Journal of Reproductive Immunology*, **58:**255-267.

Le Moine A, Goldman M, Abramowicz D. 2002. Multiple pathways to allograft rejection. *Transplantation*, **73:**1373-1381.

Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, Kang M-J, He C-H, Takyar S, Elias JA. 2011. Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annual Review of Physiology*, **73:**479-501.

Lee CG, Da Silva CA, Lee J-Y, Hartl D, Elias JA. 2008. Chitin regulation of immune responses: an old molecule with new roles. *Current Opinion in Immunology*, **20:**684-689.

Lei H, Leong D, Smith LR, Barton ER. 2013. Matrix metalloproteinase 13 is a new contributor to skeletal muscle regeneration and critical for myoblast migration. *American Journal of Physiology-Cell Physiology*, **305:**529-538.

Lekstrom-Himes J, Xanthopoulos KG. 1998. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *Journal of Biological Chemistry*, **273:**28545-28548.

Lemström KB, Krebs R, Nykänen AI, Tikkanen JM, Sihvola RK, Aaltola EM, Häyry PJ, Wood J, Alitalo K, Ylä-Herttuala S. 2002. Vascular endothelial growth factor enhances cardiac allograft arteriosclerosis. *Circulation*, **105:**2524-2530.

Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, **12:**323.

Li C, Li Y, Qin G, Chen Z, Qu M, Zhang B, Han X, Wang X, Qian P-y, Lin Q. 2020. Regulatory Role of Retinoic Acid in Male Pregnancy of the Seahorse. *The Innovation*, **1:**100052.

Li S, Li B, Jiang H, Wang Y, Qu M, Duan H, Zhou Q, Shi W. 2013. Macrophage depletion impairs corneal wound healing after autologous transplantation in mice. *PloS One*, **8:**e61799.

Lin Q, Fan S, Zhang Y, Xu M, Zhang H, Yang Y, Lee AP, Woltering JM, Ravi V, Gunter HM. 2016. The seahorse genome and the evolution of its specialized morphology. *Nature*, **540:**395.

Lin T, Liu X, Xiao D, Zhang D. 2017. Plasma levels of immune factors and sex steroids in the male seahorse Hippocampus erectus during a breeding cycle. *Fish Physiology and Biochemistry*, **43:**889-899.

Lin T, Zhang D, Liu X, Xiao D. 2016. Parental care improves immunity in the seahorse (Hippocampus erectus). *Fish & Shellfish Immunology*, **58:**554-562.

Linton J, Soloff B. 1964. The physiology of the brood pouch of the male sea horse Hippocampus erectus. *Bulletin of Marine Science*, **14:**45-61.

Litman GW, Rast JP, Fugmann SD. 2010. The origins of vertebrate adaptive immunity. *Nature Reviews Immunology*, **10:**543-553.

Ljunggren H-G, Kärre K. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology Today*, **11:**237-244.

Long JA, Trinajstic K, Johanson Z. 2009. Devonian arthrodire embryos and the origin of internal fertilization in vertebrates. *Nature*, **457:**1124.

Lopes-Ferreira M, Magalhães GS, Fernandez JH, Inácio de Loiola M, Le Ho P, Lima C, Valente RH, Moura-da-Silva AM. 2011. Structural and biological characterization of Nattectin, a new C-type lectin from the venomous fish Thalassophryne nattereri. *Biochimie*, **93:**971-980.

Lourie SA, Vincent AC, Hall HJ. 1999. Seahorses: an identification guide to the world's species and their conservation. Project Seahorse.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, **15:**550.

Lugo-Villarino G, Balla KM, Stachura DL, Bañuelos K, Werneck MB, Traver D. 2010. Identification of dendritic antigen-presenting cells in the zebrafish. *Proceedings of the National Academy of Sciences*, **107:**15850-15855.

Lund SG, Phillips M, Moyes CD, Tufts BL. 2000. The effects of cell ageing on protein synthesis in rainbow trout (Oncorhynchus mykiss) red blood cells. *Journal of Experimental Biology*, **203:**2219-2228.

Luo W, Wang X, Qu H, Qin G, Zhang H, Lin Q. 2016. Genomic structure and expression pattern of MHC IIα and IIβ genes reveal an unusual immune trait in lined seahorse Hippocampus erectus. *Fish & Shellfish Immunology*, **58:**521-529.

Macaulay IC, Svensson V, Labalette C, Ferreira L, Hamey F, Voet T, Teichmann SA, Cvejic A. 2016. Single-cell RNA-sequencing reveals a continuous spectrum of differentiation in hematopoietic cells. *Cell reports*, **14:**966-977.

Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, Tirosh I, Bialas AR, Kamitaki N, Martersteck EM, et al. 2015. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell*, **161:**1202-1214.

Malmstrøm M, Matschiner M, Tørresen OK, Star B, Snipen LG, Hansen TF, Baalsrud HT, Nederbragt AJ, Hanel R, Salzburger W. 2016. Evolution of the immune system influences speciation rates in teleost fishes. *Nature Genetics*, **48:**1204-1210.

Manríquez-Morán NL, Méndez-de la Cruz FR. 2008. Genetic homogeneity between two populations of the parthenogenetic lizard Aspidoscelis cozumela. *Revista mexicana de biodiversidad*, **79:**421-426.

Marzi M, Vigano A, Trabattoni D, Villa M, Salvaggio A, Clerici E, Clerici M. 1996. Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clinical and Experimental Immunology*, **106:**127-133.

Masonjones HD. 2001. The effect of social context and reproductive status on the metabolic rates of dwarf seahorses (Hippocampus zosterae). *Comparative Biochemistry and Physiology. Part A: Molecular and Integrative Physiology*, **129:**541-555.

Matsunaga T, Rahman A. 1998. What brought the adaptive immune system to vertebrates?-The jaw hypothesis and the seahorse. *Immunological Reviews*, **166:**177-186.

Mayadas TN, Cullere X, Lowell CA. 2014. The multifaceted functions of neutrophils. *Annual Review of Pathology: Mechanisms of Disease*, **9:**181-218.

McInnes L, Healy J, Melville J.2018. Umap: Uniform manifold approximation and projection for dimension reduction. Preprint at https://arxiv.org/abs/1802.03426.

McKinney E, McLeod T, Sigel M. 1981. Allograft rejection in a holostean fish, Lepisosteus platyrhincus. *Developmental & Comparative Immunology*, **5:**65-74.

Medawar PB. 1953. Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. *Symposia of the Society for Experimental Biology*, **7:**320–338.

Medzhitov R. 2008. Origin and physiological roles of inflammation. *Nature*, **454:**428-435.

Miyake Y, Toyonaga K, Mori D, Kakuta S, Hoshino Y, Oyamada A, Yamada H, Ono K-i, Suyama M, Iwakura Y. 2013. C-type lectin MCL is an FcRγ-coupled receptor that mediates the adjuvanticity of mycobacterial cord factor. *Immunity*, **38:**1050-1062.

Mobley KB, Small, C. M., & Jones, A. G. . 2011. The genetics and genomics of Syngnathidae: pipefishes, seahorses and seadragons. *Journal of Fish Biology*, **78(6):**1624-1646.

Moffett-King A. 2002. Natural killer cells and pregnancy. *Nature Reviews Immunology*, **2:**656-663.

Moffett A, Loke C. 2006. Immunology of placentation in eutherian mammals. *Nature Reviews Immunology*, **6:**584-594.

Mohammadabadi M, Bordbar F, Jensen J, Du M, Guo W. 2021. Key Genes Regulating Skeletal Muscle Development and Growth in Farm Animals. *Animals*, **11:**835.

Monaco G, Lee B, Xu W, Mustafah S, Hwang YY, Carre C, Burdin N, Visan L, Ceccarelli M, Poidinger M. 2019. RNA-Seq signatures normalized by mRNA abundance allow absolute deconvolution of human immune cell types. *Cell reports*, **26:**1627-1640.

Mor G. 2007. Pregnancy reconceived. *Natural History*, **116:**36.

Mor G, Abrahams VM. 2002. Immunology of implantation. *Immunology and allergy clinics*, **22:**545-565.

Mor G, Cardenas I. 2010. The immune system in pregnancy: a unique complexity. *American Journal of Reproductive Immunology*, **63:**425-433.

Mor G, Cardenas I, Abrahams V, Guller S. 2011. Inflammation and pregnancy: the role of the immune system at the implantation site. *Annals of the New York Academy of Sciences*, **1221:**80.

Morera D, Roher N, Ribas L, Balasch JC, Doñate C, Callol A, Boltaña S, Roberts S, Goetz G, Goetz FW. 2011. RNA-Seq reveals an integrated immune response in nucleated erythrocytes. *PloS One*, **6:**e26998.

Mori DN, Kreisel D, Fullerton JN, Gilroy DW, Goldstein DR. 2014. Inflammatory triggers of acute rejection of organ allografts. *Immunological Reviews*, **258:**132-144.

Mungal C.2003. "map2slim". URL: http://search.cpan.org/~cmungall/goperl/scripts/map2slim, Last updated: March 20, 2007.

Murphy B, Thompson M, Belov K. 2009. Evolution of viviparity and the maternal immune system: major histocompatibility complex (MHC) class I genes in skinks. *Orbit: University of Sydney Undergraduate Research Journal*, **1**.

Murphy S, Tomasi T. 1998. Absence of MHC class II antigen expression in trophoblast cells results from a lack of class II transactivator (CIITA) gene expression. *Molecular Reproduction and Development*, **51:**1-12.

Murray PJ, Wynn TA. 2011. Protective and pathogenic functions of macrophage subsets. *Nature reviews immunology*, **11:**723-737.

Naismith D, Morgan B. 1976. The biphasic nature of protein metabolism during pregnancy in the rat. *British Journal of Nutrition*, **36:**563-566.

Nakanishi T. 1987. Histocompatibility analyses in tetraploids induced from clonal triploid crucian carp and in gynogenetic diploid goldfish. *Journal of Fish Biology*, **31:**35-40.

Nakanishi T, Shibasaki Y, Matsuura Y. 2015. T cells in fish. *Biology*, **4:**640-663.

Nardi F. 1935. Das Verhalten der Schuppen erwachsener Fische bei regenerations-und Transplantationsversuchen. *Development Genes and Evolution*, **133:**621-663.

Neefjes J, Jongsma ML, Paul P, Bakke O. 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature reviews immunology*, **11:**823-836.

Neiffer DL, Stamper MA. 2009. Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. *ILAR journal*, **50:**343-360.

Nemesh J.2016. Drop-seq core computational protocol. McCarroll Laboratory http://mccarrolllab. com/wp-content/uploads/2016/03/Drop-seqAlignmentCookbookv1. 2Jan2016.

Nepom GT, Erlich H. 1991. MHC class-II molecules and autoimmunity. *Annual Review of Immunology*, **9:**493-525.

Niu J, Huang Y, Liu X, Zhang Z, Tang J, Wang B, Lu Y, Cai J, Jian J. 2020. Single-cell RNA-seq reveals different subsets of non-specific cytotoxic cells in teleost. *Genomics*, **112:**5170-5179.

Nombela I, Ortega-Villaizan MdM. 2018. Nucleated red blood cells: Immune cell mediators of the antiviral response. *PLoS Pathogens*, **14:**e1006910.

Oppenheimer JR. 1970. Mouthbreeding in fishes. *Animal Behaviour*, **18:**493-503.

Orsi N, Tribe R. 2008. Cytokine networks and the regulation of uterine function in pregnancy and parturition. *Journal of Neuroendocrinology*, **20:**462-469.

Osol G, Mandala M. 2009. Maternal uterine vascular remodeling during pregnancy. *Physiology*, **24:**58-71.

Parker J, Dubin A, Schneider R, Wagner K, Jentoft S, Böhne A, Bayer T, Roth O. Immunological tolerance in the evolution of male pregnancy. *Molecular Ecology*, In press.

Parker J, Guslund N, S J, Roth O. Characterisation of pipefish immune cell repertoire through single-cell transcriptomics. In revision.

Parker J, Roth O. Comparative assessment of immunological tolerance in pipefish with and without the major histocompatibility complex class II. In revision.

Partridge C, Shardo J, Boettcher A. 2007. Osmoregulatory role of the brood pouch in the euryhaline Gulf pipefish, Syngnathus scovelli. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **147:**556-561.

Penn DJ, Potts WK. 1999. The evolution of mating preferences and major histocompatibility complex genes. *The American Naturalist*, **153:**145-164.

Perdiguero P, Morel E, Tafalla C. 2021. Diversity of Rainbow Trout Blood B Cells Revealed by Single Cell RNA Sequencing. *Biology*, **10:**511.

Perfetto SP, Chattopadhyay PK, Roederer M. 2004. Seventeen-colour flow cytometry: unravelling the immune system. *Nature Reviews Immunology*, **4:**648-655.

Peters PJ, Borst J, Oorschot V, Fukuda M, Krähenbühl O, Tschopp J, Slot JW, Geuze HJ. 1991. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *The Journal of experimental medicine*, **173:**1099-1109.

Pitsillides CM, Runnels JM, Spencer JA, Zhi L, Wu MX, Lin CP. 2011. Cell labeling approaches for fluorescence-based in vivo flow cytometry. *Cytometry Part A*, **79:**758-765.

Pontén F, Jirström K, Uhlen M. 2008. The Human Protein Atlas—a tool for pathology. *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland*, **216:**387-393.

Poyser N. 1995. The control of prostaglandin production by the endometrium in relation to luteolysis and menstruation. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, **53:**147-195.

Pressley PH. 1981. Parental Effort and the Evolution of Nest-Guarding Tactics in the Threespine Stickleback, Gasterosteus Aculeatus L. *Evolution*, **35:**282-295.

Price MN, Dehal PS, Arkin AP. 2010. FastTree 2–approximately maximum-likelihood trees for large alignments. *PloS One*, **5:**e9490.

Qin G, Zhang Y, Zhang B, Zhang Y, Liu Y, Lin Q. 2020. Environmental estrogens and progestins disturb testis and brood pouch development with modifying transcriptomes in male-pregnancy lined seahorse Hippocampus erectus. *Science of the Total Environment*, **715:**136840.

R Development Core Team. 2013. R: A language and environment for statistical computing.

Raghupathy R, Makhseed M, Azizieh F, Omu A, Gupta M, Farhat R. 2000. Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. *Human Reproduction*, **15:**713-718.

Raj B, Wagner DE, McKenna A, Pandey S, Klein AM, Shendure J, Gagnon JA, Schier AF. 2018. Simultaneous single-cell profiling of lineages and cell types in the vertebrate brain. *Nature Biotechnology*, **36:**442-450.

Rajnoch J, Viklický O. 2004. Angiogenesis and organ transplantation. *Folia Microbiologica*, **49:**499.

Ramoni C, Luciani F, Spadaro F, Lugini L, Lozupone F, Fais S. 2002. Differential expression and distribution of ezrin, radixin and moesin in human natural killer cells. *European Journal of Immunology*, **32:**3059-3065.

Rapacz-Leonard A, Dąbrowska M, Janowski T. 2014. Major histocompatibility complex I mediates immunological tolerance of the trophoblast during pregnancy and may mediate rejection during parturition. *Mediators of Inflammation*, **2014:**11.

Ravi V, Venkatesh B. 2018. The divergent genomes of teleosts. *Annual review of animal biosciences*, **6:**47-68.

Read CP, Word RA, Ruscheinsky MA, Timmons BC, Mahendroo MS. 2007. Cervical remodeling during pregnancy and parturition: molecular characterization of the softening phase in mice. *Reproduction*, **134:**327-340.

Reynolds L, Ferrell C, Robertson DA, Ford S. 1986. Metabolism of the gravid uterus, foetus and utero-placenta at several stages of gestation in cows. *The Journal of Agricultural Science*, **106:**437-444.

Reznick DN, Mateos M, Springer MS. 2002. Independent origins and rapid evolution of the placenta in the fish genus Poeciliopsis. *Science*, **298:**1018-1020.

Riese II DJ, Cullum RL. 2014. Epiregulin: roles in normal physiology and cancer. *Seminars in Cell & Developmental Biology*, **28:** 49-56.

Ripley J, Williams P, Foran C. 2010. Morphological and quantitative changes in paternal brood-pouch vasculature during embryonic development in two Syngnathus pipefishes. *Journal of Fish Biology*, **77:**67-79.

Ripley JL. 2009. Osmoregulatory role of the paternal brood pouch for two Syngnathus species. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **154:**98-104.

Ripley JL, Foran CM. 2006. Differential parental nutrient allocation in two congeneric pipefish species (Syngnathidae: Syngnathus spp.). *Journal of Experimental Biology*, **209:**1112-1121.

Ripley JL, Foran CM. 2009. Direct evidence for embryonic uptake of paternally-derived nutrients in two pipefishes (Syngnathidae: Syngnathus spp.). *Journal of Comparative Physiology B*, **179:**325-333.

Roca FJ, Sepulcre MP, López-Castejón G, Meseguer J, Mulero V. 2006. The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. *Molecular Immunology*, **43:**1418-1423.

Rocha PN, Plumb TJ, Crowley SD, Coffman TM. 2003. Effector mechanisms in transplant rejection. *Immunological Reviews*, **196:**51-64.

Rosenqvist G, Berglund A. 2011. Sexual signals and mating patterns in Syngnathidae. *Journal of Fish Biology*, **78:**1647-1661.

Rőszer T. 2015. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediators of Inflammation*, **2015:**816460.

Roth O, Klein V, Beemelmanns A, Scharsack JP, Reusch TB. 2012. Male pregnancy and biparental immune priming. *The American Naturalist*, **180:**802-814.

Roth O, Scharsack J, Keller I, Reusch TB. 2011. Bateman's principle and immunity in a sex-role reversed pipefish. *Journal of Evolutionary Biology*, **24:**1410-1420.

Roth O, Solbakken MH, Tørresen OK, Bayer T, Matschiner M, Baalsrud HT, Hoff SNK, Brieuc MSO, Haase D, Hanel R. 2020. Evolution of male pregnancy associated with remodeling of canonical vertebrate immunity in seahorses and pipefishes. *Proceedings of the National Academy of Sciences*, **117:**9431-9439.

Roth O, Sundin J, Berglund A, Rosenqvist G, Wegner KM. 2014. Male mate choice relies on major histocompatibility complex class I in a sex-role-reversed pipefish. *Journal of Evolutionary Biology*, **27:**929-938.

Rothchild I. 1983. Role of progesterone in initiating and maintaining pregnancy. In: Progesterone and Progestins. New York, Raven Press, p. 219-229.

Sakai M, Tsuda H, Tanebe K, Sasaki Y, Saito S. 2002. Interleukin-12 secretion by peripheral blood mononuclear cells is decreased in normal pregnant subjects and increased in preeclamptic patients. *American Journal of Reproductive Immunology*, **47:**91-97.

Salcedo T, Azzoni L, Wolf SF, Perussia B. 1993. Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. *The Journal of Immunology*, **151:**2511-2520.

Salvagiotto G, Zhao Y, Vodyanik M, Ruotti V, Stewart R, Marra M, Thomson J, Eaves C, Slukvin I. 2008. Molecular profiling reveals similarities and differences between primitive subsets of hematopoietic cells generated in vitro from human embryonic stem cells and in vivo during embryogenesis. *Experimental Hematology*, **36:**1377-1389.

Saraiva TC, Grund LZ, Komegae EN, Ramos AD, Conceição K, Orii NM, Lopes-Ferreira M, Lima C. 2011. Nattectin a fish C-type lectin drives Th1 responses in vivo: licenses macrophages to differentiate into cells exhibiting typical DC function. *International Immunopharmacology*, **11:**1546-1556.

Sargent RC, Gross MR. 1986. Williams' principle: an explanation of parental care in teleost fishes. In: The behaviour of teleost fishes. Springer, p. 275-293.

Sauter V. 1934. Regeneration und Transplantation bei erwachsenen Fischen. *Development Genes and Evolution*, **132:**1-41.

Schluter SF, Bernstein RM, Bernstein H, Marchalonis JJ. 1999. 'Big Bang'emergence of the combinatorial immune system. *Developmental and Comparative Immunology*, **23:**107-111.

Schober L, Radnai D, Schmitt E, Mahnke K, Sohn C, Steinborn A. 2012. Term and preterm labor: decreased suppressive activity and changes in composition of the regulatory T-cell pool. *Immunology and Cell Biology*, **90:**935-944.

Šećerov S. 1912. Weitere Farbwechsel-und Hauttransplantationsversuche an der Bartgrundel (Nemachilus barbatula L.). *Archiv für Entwicklungsmechanik der Organismen*, **33:**716-722.

Shah NM, Edey LF, Imami N, Johnson MR. 2020. Human labour is associated with altered regulatory T cell function and maternal immune activation. *Clinical and Experimental Immunology*, **199:**182-200.

Shen Y, Wang D, Zhao J, Chen X. 2018. Fish red blood cells express immune genes and responses. *Aquaculture and Fisheries*, **3:**14-21.

Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, **4:**44.

Shiina T, Hosomichi K, Inoko H, Kulski JK. 2009. The HLA genomic loci map: expression, interaction, diversity and disease. *Journal of Human Genetics*, **54:**15-39.

Sitras V, Fenton C, Paulssen R, Vårtun Å, Acharya G. 2012. Differences in gene expression between first and third trimester human placenta: a microarray study. *PloS One*, **7:**e33294.

Skalkos ZM, Van Dyke JU, Whittington CM. 2020. Paternal nutrient provisioning during male pregnancy in the seahorse Hippocampus abdominalis. *Journal of Comparative Physiology B*, **190:**1-10.

Small CM, Bassham S, Catchen J, Amores A, Fuiten AM, Brown RS, Jones AG, Cresko WA. 2016. The genome of the Gulf pipefish enables understanding of evolutionary innovations. *Genome Biology*, **17:**258.

Small CM, Harlin-Cognato AD, Jones AG. 2013. Functional similarity and molecular divergence of a novel reproductive transcriptome in two male-pregnant Syngnathus pipefish species. *Ecology and Evolution*, **3:**4092-4108.

Snell GD. 1948. Methods for the study of histocompatibility genes. *Journal of genetics*, **49:**87-108.

Snell GD. 1981. Studies in histocompatibility. *Science*, **213:**172-178.

Snell GD, Higgins GF. 1951. Alleles at the histocompatibility-2 locus in the mouse as determined by tumor transplantation. *Genetics*, **36:**306.

Snoek M, Albertella MR, van Kooij M, Wixon J, van Vugt H, de Groot K, Campbell RD. 2000. G7c, a novel gene in the mouse and human major histocompatibility complex class III region, possibly controlling lung tumor susceptibility. *Immunogenetics*, **51:**383-386.

Solbakken MH, Jentoft S, Reitan T, Mikkelsen H, Jakobsen KS, Seppola M. 2019. Whole transcriptome analysis of the Atlantic cod vaccine response reveals subtle changes in adaptive immunity. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, **31:**100597.

Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT. 2004. Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. *Immunology*, **112:**38-43.

Soncin F, Khater M, To C, Pizzo D, Farah O, Wakeland A, Rajan KAN, Nelson KK, Chang C-W, Moretto-Zita M. 2018. Comparative analysis of mouse and human placentae across gestation reveals species-specific regulators of placental development. *Development*, **145:**dev.156273.

Soneson C, Love MI, Robinson MD. 2015. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research*, **4:**1521.

Song L, Florea L. 2015. Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. *GigaScience*, **4:**s13742-13015-10089-y.

Spencer TE, Johnson GA, Burghardt RC, Bazer FW. 2004. Progesterone and Placental Hormone Actions on the Uterus: Insights from Domestic Animals1. *Biology of Reproduction*, **71:**2-10.

Stadtmauer DJ, Wagner GP. 2020. Cooperative inflammation: the recruitment of inflammatory signaling in marsupial and eutherian pregnancy. *Journal of Reproductive Immunology*, **137:**102626.

Star B, Nederbragt AJ, Jentoft S, Grimholt U, Malmstrøm M, Gregers TF, Rounge TB, Paulsen J, Solbakken MH, Sharma A. 2011. The genome sequence of Atlantic cod reveals a unique immune system. *Nature*, **477:**207.

Star B, Nederbragt AJ, Jentoft S, Grimholt U, Malmstrøm M, Gregers TF, Rounge TB, Paulsen J, Solbakken MH, Sharma A. 2011. The genome sequence of Atlantic cod reveals a unique immune system. *Nature*, **477:**207-210.

Stastny P, Nunez G, Pettaway C. 1986. Class II MHC antigens on human monocytes, endothelial cells, and dendritic cells. In: HLA class II antigens. Springer, p. 356-373.

Stearns SC, Nesse RM, Govindaraju DR, Ellison PT. 2010. Evolutionary perspectives on health and medicine. *Proceedings of the National Academy of Sciences*, **107:**1691-1695.

Steinman RM, Hawiger D, Liu K, Bonifaz L, Bonnyay D, Mahnke K, Iyoda T, Ravetch J, Dhodapkar M, Inaba K. 2003. Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. *Annals of the New York Academy of Sciences*, **987:**15-25.

Stjernholm-Vladic Y, Stygar D, Mansson C, Masironi B, Akerberg S, Wang H, Ekman-Ordeberg G, Sahlin L. 2004. Factors involved in the inflammatory events of cervical ripening in humans. *Reproductive Biology and Endocrinology*, **2:**1-17.

Stölting KN, Wilson AB. 2007. Male pregnancy in seahorses and pipefish: beyond the mammalian model. *Bioessays*, **29:**884-896.

Studzińska B, Seroka A, Lepicka M, Roszek K, Komoszyński M. 2010. The increase of adenylate kinase activity in the blood can control aggregation of platelets in coronary or peripheral arterial ischemia. *Health*, **2:**246.

Svensson-Arvelund J, Mehta RB, Lindau R, Mirrasekhian E, Rodriguez-Martinez H, Berg G, Lash GE, Jenmalm MC, Ernerudh J. 2015. The human fetal placenta promotes tolerance against the semiallogeneic fetus by inducing regulatory T cells and homeostatic M2 macrophages. *Journal of Immunology*, **194:**1534-1544.

Swaminathan GJ, Myszka DG, Katsamba PS, Ohnuki LE, Gleich GJ, Acharya KR. 2005. Eosinophil-granule major basic protein, a C-type lectin, binds heparin. *Biochemistry*, **44:**14152-14158.

Swann JB, Holland SJ, Petersen M, Pietsch TW, Boehm T. 2020. The immunogenetics of sexual parasitism. *Science*, **369:**1608-1615.

Takahata N, Nei M. 1990. Allelic genealogy under overdominant and frequency-dependent selection and polymorphism of major histocompatibility complex loci. *Genetics*, **124:**967-978.

Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, Wang X, Bodeau J, Tuch BB, Siddiqui A. 2009. mRNA-Seq whole-transcriptome analysis of a single cell. *Nature Methods*, **6:**377-382.

Tang Q, Iyer S, Lobbardi R, Moore JC, Chen H, Lareau C, Hebert C, Shaw ML, Neftel C, Suva ML. 2017. Dissecting hematopoietic and renal cell heterogeneity in adult zebrafish at single-cell resolution using RNA sequencing. *Journal of Experimental Medicine*, **214:**2875-2887.

Tatner MF, Manning MJ. 1983. The ontogeny of cellular immunity in the rainbow trout, Salmogairdneri Richardson, in relation to the stage of development of the lymphoid organs. *Developmental & Comparative Immunology*, **7:**69-75.

Tavares-Dias M, Barcellos JFM. 2017. Peripheral blood cells of the armored catfish Hoplosternum littorale Hancock, 1828: a morphological and cytochemical study. *Journal of Morphological Sciences*, **22:**0-0.

Tayade C, Esadeg S, Fang Y, Croy BA. 2005. Functions of alpha 2 macroglobulins in pregnancy. *Molecular and Cellular Endocrinology*, **245:**60-66.

Teles A, Zenclussen A. 2013. Pregnancy establishment is influenced by CCR7 and CCR9 (P6360). *Journal of Immunology*, **190:**1999.1996.

Thibault RE, Schultz RJ. 1978. Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution*, 320-333.

Thomas L. 1959. Cellular and Humoral Aspects of the Hypersensitive States. London, Cassell.

Tinkle DW, Gibbons JW. 1977. The distribution and evolution of viviparity in reptiles.

Tonnesen MG, Feng X, Clark RA. 2000. Angiogenesis in wound healing. *Journal of Investigative Dermatology Symposium Proceedings*, **5:** 40-46.

Tørresen OK, Brieuc MS, Solbakken MH, Sørhus E, Nederbragt AJ, Jakobsen KS, Meier S, Edvardsen RB, Jentoft S. 2018. Genomic architecture of haddock (Melanogrammus aeglefinus) shows expansions of innate immune genes and short tandem repeats. *BMC Genomics*, **19:**1-17.

Trachtenberg E, Korber B, Sollars C, Kepler TB, Hraber PT, Hayes E, Funkhouser R, Fugate M, Theiler J, Hsu YS. 2003. Advantage of rare HLA supertype in HIV disease progression. *Nature Medicine*, **9:**928-935.

Trentin L, Zambello R, Faggian G, Livi U, Thiene G, Gasparotto G, Agostini C. 1992. Phenotypic and functional characterization of cytotoxic cells derived from endomyocardial biopsies in human cardiac allografts. *Cellular Immunology*, **141:**332-341.

Trivers R. 1972. Parental investment and sexual selection. Biological Laboratories, Harvard University Cambridge.

Tronco JA, Bruna RdA, Bastos NM, Alcântara SA, da Silveira JC, da Silva MG. 2020. Alpha-2-macroglobulin from circulating exosome-like vesicles is increased in women with preterm pregnancies. *Scientific Reports*, **10:**1-7.

Turner C. 1947. Viviparity in teleost fishes. *The Scientific Monthly*, **65:**508-518.

Umans L, Serneels L, Overbergh L, Stas L, Van Leuven F. 1999. alpha2-macroglobulin- and murinoglobulin-1- deficient mice. A mouse model for acute pancreatitis. *American Journal of Pathology*, **155:**983-993.

Umehara H, Inoue H, Huang J, Kono T, Minami Y, Tanaka Y, Okazaki T, Mimori T, Bloom ET, Domae N. 2002. Role for adapter proteins in costimulatory signals of CD2 and IL-2 on NK cell activation. *Molecular Immunology*, **38:**587-596.

Uribe C, Folch H, Enríquez R, Moran G. 2011. Innate and adaptive immunity in teleost fish: a review. *Veterinarni Medicina*, **56:**486.

Van Dyke JU, Brandley MC, Thompson MB. 2014. The evolution of viviparity: molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes. *Reproduction*, **147:**R15-R26.

van Eijk M, Scheij SS, van Roomen CP, Speijer D, Boot RG, Aerts JM. 2007. TLR-and NOD2-dependent regulation of human phagocyte-specific chitotriosidase. *FEBS Letters*, **581:**5389-5395.

van Eijk M, van Roomen CP, Renkema GH, Bussink AP, Andrews L, Blommaart EF, Sugar A, Verhoeven AJ, Boot RG, Aerts JM. 2005. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. *International Immunology*, **17:**1505-1512.

Van Ham SM, Heutinck KM, Jorritsma T, Bemelman FJ, Strik MC, Vos W, Muris JJ, Florquin S, Ten Berge IJ, Rowshani AT. 2010. Urinary granzyme A mRNA is a biomarker to diagnose subclinical and acute cellular rejection in kidney transplant recipients. *Kidney International*, **78:**1033-1040.

Vázquez GR, Guerrero G. 2007. Characterization of blood cells and hematological parameters in Cichlasoma dimerus (Teleostei, Perciformes). *Tissue and Cell*, **39:**151-160.

Venkatesh B, Lee AP, Ravi V, Maurya AK, Lian MM, Swann JB, Ohta Y, Flajnik MF, Sutoh Y, Kasahara M. 2014. Elephant shark genome provides unique insights into gnathostome evolution. *Nature*, **505:**174-179.

Vincent A. 1990. A seahorse father makes a good mother. *Natural History*, **12:**34-42.

Vincent ACJ, Berglund A, Ahnesjö I. 1995. Reproductive ecology of five pipefish species in one eelgrass meadow. *Environmental Biology of Fishes*, **44:**347-361.

Von Boehmer H, Kisielow P. 1990. Self-nonself discrimination by T cells. *Science*, **248:**1369-1373.

Wagner DE, Weinreb C, Collins ZM, Briggs JA, Megason SG, Klein AM. 2018. Single-cell mapping of gene expression landscapes and lineage in the zebrafish embryo. *Science*, **360:**981-987.

Wagner GP, Erkenbrack EM, Love AC. 2019. Stress-induced evolutionary innovation: a mechanism for the origin of cell types. *Bioessays*, **41:**1800188.

Wang J, Li H, Xue B, Deng R, Huang X, Xu Y, Chen S, Tian R, Wang X, Xun Z. 2020. IRF1 promotes the innate immune response to viral infection by enhancing the activation of IRF3. *Journal of Virology*, **94:**e01231-01220.

Wang X, Zhang Y, Zhang H, Qin G, Lin Q. 2019. Complete mitochondrial genomes of eight seahorses and pipefishes (Syngnathiformes: Syngnathidae): insight into the adaptive radiation of syngnathid fishes. *BMC Evolutionary Biology*, **19:**1-11.

Watanabe S. 1999. The role of male brood pouch in the reproduction of the seaweed pipefish, Syngnathus schlegeli. University of Tokyo, Tokyo.

Watanabe S, Kaneko T, Watanabe Y. 1999. Immunocytochemical detection of mitochondria-rich cells in the brood pouch epithelium of the pipefish, Syngnathus schlegeli : structural comparison with mitochondria-rich cells in the gills and larval epidermis. *Cell and Tissue Research*, **295:**141-149.

Wedemeyer A, Kliemann L, Srivastav A, Schielke C, Reusch TB, Rosenstiel P. 2017. An improved filtering algorithm for big read datasets and its application to single-cell assembly. *BMC Bioinformatics*, **18:**1-11.

Weekes HC. 1935. A Review of Placentation among Reptiles with, particular regard to the Function and Evolution of the Placenta. *Journal of Zoology*, **105:**625-645.

Wegmann TG, Lin H, Guilbert L, Mosmann TR. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunology Today*, **14:**353-356.

Wei Y, Yang C-R, Wei Y-P, Zhao Z-A, Hou Y, Schatten H, Sun Q-Y. 2014. Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals. *Proceedings of the National Academy of Sciences*, **111:**1873-1878.

Weissgerber TL, Wolfe LA. 2006. Physiological adaptation in early human pregnancy: adaptation to balance maternal-fetal demands. *Applied Physiology, Nutrition, and Metabolism*, **31:**1-11.

Welch DR, Schissel DJ, Howrey RP, Aeed PA. 1989. Tumor-elicited polymorphonuclear cells, in contrast to" normal" circulating polymorphonuclear cells, stimulate invasive and metastatic potentials of rat mammary adenocarcinoma cells. *Proceedings of the National Academy of Sciences*, **86:**5859-5863.

Wen J, Mercado GP, Volland A, Doden HL, Lickwar CR, Crooks T, Kakiyama G, Kelly C, Cocchiaro JL, Ridlon JM. 2021. Fxr signaling and microbial metabolism of bile salts in the zebrafish intestine. *Science advances*, **7:**eabg1371.

Wen Y, Fang W, Xiang L-X, Pan R-L, Shao J-Z. 2011. Identification of Treg-like cells in Tetraodon: insight into the origin of regulatory T subsets during early vertebrate evolution. *Cellular and Molecular Life Sciences*, **68:**2615-2626.

Whittington CM, Friesen CR. 2020. The evolution and physiology of male pregnancy in syngnathid fishes. *Biological Reviews*, **95:**1252-1272.

Whittington CM, Griffith OW, Qi W, Thompson MB, Wilson AB. 2015. Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Molecular Biology and Evolution*, **32:**3114-3131.

Williams CJ, Chu A, Jefferson WN, Casero D, Sudhakar D, Khurana N, Hogue CP, Aryasomayajula C, Patel P, Sullivan P. 2017. Epithelial membrane protein 2 (EMP2) deficiency alters placental angiogenesis, mimicking features of human placental insufficiency. *The Journal of pathology*, **242:**246-259.

Wilson AB. 2017. MHC and adaptive immunity in teleost fishes. *Immunogenetics*, **69:**521-528.

Wilson AB, Ahnesjo I, Vincent ACJ, Meyer A. 2003. The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family Syngnathidae). *Evolution*, **57:**1374-1386.

Wilson AB, Vincent A, Ahnesjö I, Meyer A. 2001. Male pregnancy in seahorses and pipefishes (family Syngnathidae): rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny. *Journal of Heredity*, **92:**159-166.

Wing K, Sakaguchi S. 2010. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nature Immunology*, **11:**7-13.

Wourms JP. 1981. Viviparity: the maternal-fetal relationship in fishes. *American Zoologist*, **21:**473-515.

Wourms JP. 1991. Reproduction and development ofSebastes in the context of the evolution of piscine viviparity. *Environmental Biology of Fishes*, **30:**111-126.

Wourms JP, Atz JW, Stribling MD. 1991. Viviparity and the maternal-embryonic relationship in the coelacanth Latimeria chalumnae. In: The biology of Latimeria chalumnae and evolution of coelacanths. Springer, p. 225-248.

Wourms JP, Demski LS. 1993. The reproduction and development of sharks, skates, rays and ratfishes: introduction, history, overview, and future prospects. In: The reproduction and development of sharks, skates, rays and ratfishes. Springer, p. 7-21.

Wourms JP, Lombardi J. 1992. Reflections on the evolution of piscine viviparity. *American Zoologist*, **32:**276-293.

Wu M, Chen H, Chen S, Chao K, Yang Y, Ho H. 2001. Increase in the production of interleukin-10 early after implantation is related to the success of pregnancy. *American Journal of Reproductive Immunology*, **46:**386-392.

Wu Y-S, Chen S-N. 2014. Apoptotic cell: linkage of inflammation and wound healing. *Frontiers in Pharmacology*, **5:**1.

Wu Y, Liu Y, Zhang H, Wang X, Lin Q. 2020. Expression patterns of alpha-2 macroglobulin reveal potential immune functions in brood pouch of the lined seahorse Hippocampus erectus. *Aquaculture*, **533:**736064.

Wu Y, Zhang H, Zhang B, Lin Q, Liu Y. 2021. Molecular characterization of TLR22 and its role in immunological modification of the brood pouch of the lined seahorse, Hippocampus erectus. *Aquaculture*, **539:**736628.

Xiao W, Hong H, Kawakami Y, Lowell CA, Kawakami T. 2008. Regulation of myeloproliferation and M2 macrophage programming in mice by Lyn/Hck, SHIP, and Stat5. *The Journal of clinical investigation*, **118:**924-934.

Yamanashi Y, Fukuda T, Nishizumi H, Inazu T, Higashi K-i, Kitamura D, Ishida T, Yamamura H, Watanabe T, Yamamoto T. 1997. Role of tyrosine phosphorylation of HS1 in B cell antigen receptor-mediated apoptosis. *The Journal of experimental medicine*, **185:**1387-1392.

Zenclussen AC, Gerlof K, Zenclussen ML, Ritschel S, Zambon Bertoja A, Fest S, Hontsu S, Ueha S, Matsushima K, Leber J. 2006. Regulatory T cells induce a privileged tolerant microenvironment at the fetal-maternal interface. *European Journal of Immunology*, **36:**82-94.

Zeng Z, Liu F, Li S. 2017. Metabolic adaptations in pregnancy: a review. *Annals of Nutrition and Metabolism*, **70:**59-65.

Zexia G, Weimin W, Yi Y, Abbas K, Dapeng L, Guiwei Z, Diana JS. 2007. Morphological studies of peripheral blood cells of the Chinese sturgeon, Acipenser sinensis. *Fish Physiology and Biochemistry*, **33:**213-222.

Zhang B, Zhang H, Qin G, Liu Y, Han X, Yin J, Lin Q. 2019. TLR2 gene in seahorse brood pouch plays key functional roles in LPS-induced antibacterial responses. *Journal of Fish Diseases*, **42:**1085-1089.

Zhang H, Qin G, Zhang Y, Li S, Lin Q. 2016. The leptin system and its expression at different nutritional and pregnant stages in lined seahorse (Hippocampus erectus). *Biology open*, **5:**1508-1515.

Zhang N, Xu B, Mou C, Yang W, Wei J, Lu L, Zhu J, Du J, Wu X, Ye L. 2003. Molecular profile of the unique species of traditional Chinese medicine, Chinese seahorse (Hippocampus kuda Bleeker). *FEBS Letters*, **550:**124-134.

Zhao W-J, Zhu M. 2010. Siluro-Devonian vertebrate biostratigraphy and biogeography of China. *Palaeoworld*, **19:**4-26.

Zheng J, Johnson ML, Redmer DA, Reynolds LP. 1996. Estrogen and progesterone receptors, cell proliferation, and c-fos expression in the ovine uterus during early pregnancy. *Endocrinology*, **137:**340-348.

Zhu L-y, Nie L, Zhu G, Xiang L-x, Shao J-z. 2013. Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. *Developmental & Comparative Immunology*, **39:**39-62.

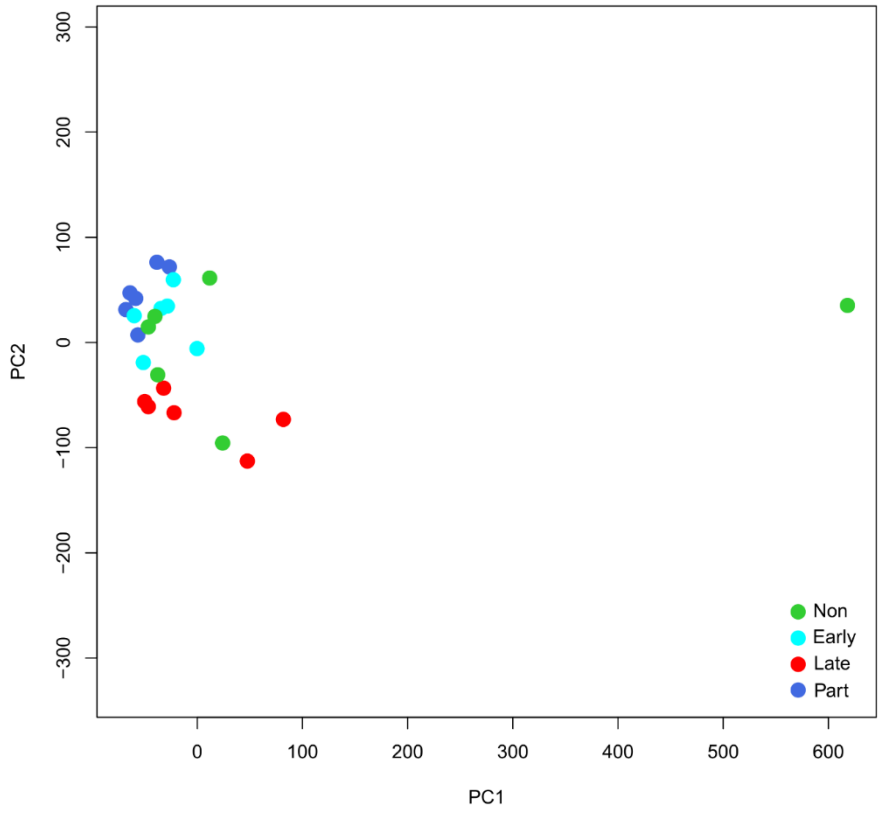
APPENDICES

Appendix – Chapter I

Supplementary table 1. Fish species proteomes used for orthofinder references

Species	Common name	Proteome ref.
Danio rerio	Zebrafish	GRCz11
Gasterosteus aculeatus	Three-spined stickleback	BROADS1
Oryzias latipes	Medaka	ASM223467v1
Takifugu rubripes	Japanese pufferfish	fTakRub1.2
Tetraodon nigroviridis	Green spotted puffer	TETRAODON8
Lepisosteus oculatus	Spotted gar	LepOcu1
Hippocampus comes	Tiger tail seahorse	QL1_v1
Syngnathus acus	Greater pipefish	fSynAcu1.2
Hippocampus erectus	Lined seahorse	
Syngnathus rostellatus	Nilsson's pipefish	
Syngnathus typhle	Broadnosed pipefish	
Nerophis ophidion	Straightnose pipefish	

All proteomes accessed from ensemble database (release 101), except S. Acus proteome (NCBI GenBanc acc.number: GCA_901709675.2



Supplementary figure 1. Principal component analysis plot for *S. rostellatus* raw counts indicating distinct outlier.

Supplementary table 2. MANOVA results from PC loadings (PC 1:8) for individual species. Increasing number of asterisks (*) relates to increased significance.

Species	PC	D.f.	Res	Pillai	F values	*P*	Sig
Hippocampus erectus	1-8	3	20	2.41	7.58	3.62E-09	***
Syngnathus rostellatus	1-8	3	19	2.14	4.32	1.73E-05	***
Syngnathus typhle	1-8	3	20	2.41	7.70	2.84E-09	***
Nerophis ophidion	1-8	3	20	2.44	8.17	1.07E-09	***

Significance: * < .05, ** < .01, *** < 0.001

Supplementary table 3. ANOVA results from PC loadings (PC 1:8) for individual species. Increasing number of asterisks (*) relates to increased significance.

Species	PC	D.f.	Res	SumSq	F values	P	Sig
Hippocampus erectus	1	3	20	1898.23	0.33	8.06E-01	
	2	3	20	12816.63	9.48	4.20E-04	***
	3	3	20	8901.19	7.47	1.52E-03	**
	4	3	20	7330.16	12.36	8.53E-05	***
	5	3	20	1327.39	1.50	2.45E-01	
	6	3	20	209.70	0.24	8.69E-01	
	7	3	20	1200.20	1.86	1.68E-01	
	8	3	20	734.01	1.24	3.23E-01	
Syngnathus rostellatus	1	3	19	13120.58	4.60	1.39E-02	*
	2	3	19	7686.32	3.17	4.80E-02	*
	3	3	19	5565.08	3.91	2.49E-02	*
	4	3	19	3290.73	3.39	3.94E-02	*
	5	3	19	769.02	0.80	5.09E-01	
	6	3	19	1998.66	3.34	4.11E-02	*
	7	3	19	231.81	0.27	8.46E-01	
	8	3	19	742.14	1.14	3.60E-01	
Syngnathus typhle	1	3	20	1626.39	0.39	7.61E-01	
	2	3	20	11596.62	5.52	6.27E-03	**
	3	3	20	6126.75	9.06	5.44E-04	***
	4	3	20	4464.61	5.40	6.92E-03	
	5	3	20	2501.52	2.52	8.69E-02	
	6	3	20	2912.35	4.00	2.20E-02	*
	7	3	20	1111.32	1.40	2.73E-01	
	8	3	20	285.94	0.41	7.50E-01	
Nerophis ophidion	1	3	20	44409.13	105.41	2.00E-12	***
	2	3	20	2515.26	0.68	5.73E-01	
	3	3	20	5224.19	3.51	3.42E-02	*
	4	3	20	3295.86	3.18	4.62E-02	*
	5	3	20	1358.54	2.13	1.29E-01	
	6	3	20	842.84	1.49	2.49E-01	
	7	3	20	1164.52	2.54	8.56E-02	
	8	3	20	148.01	0.27	8.48E-01	

Significance: * < .05, ** < .01, *** < 0.001

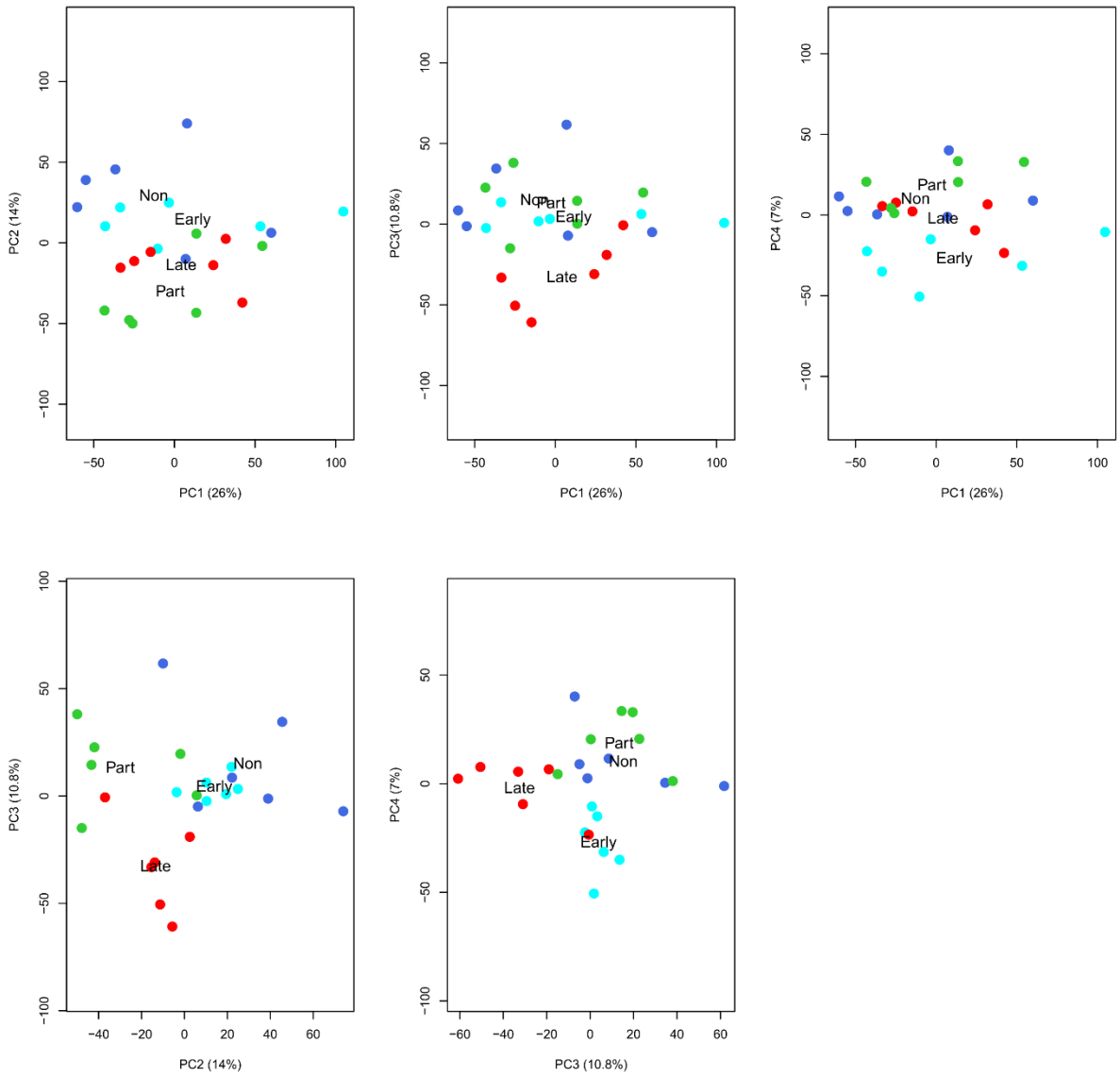
Supplementary table 4. Post-hoc Tukey-test results from PC loadings for individual species. Tests only carried out on PCs exhibiting significant stage differences. Increasing number of asterisks (*) increased significance.

Species	PC	Stage	diff	lwr	upr	*p*	Sig
Hippocampus erectus	2	L-E	-27.32	-61.62	6.98	1.49E-01	
		N-E	15.64	-18.66	49.94	5.88E-01	
		P-E	-43.74	-78.04	-9.43	9.55E-03	**
		N-L	42.96	8.66	77.26	1.10E-02	*
		P-L	-16.42	-50.72	17.88	5.50E-01	
		P-N	-59.38	-93.68	-25.08	5.27E-04	***
	3	L-E	-36.40	-68.60	-4.20	2.32E-02	*
		N-E	11.37	-20.83	43.57	7.57E-01	
		P-E	9.48	-22.72	41.68	8.42E-01	
		N-L	47.77	15.57	79.97	2.56E-03	**
		P-L	45.88	13.68	78.07	3.72E-03	**
		P-N	-1.89	-34.09	30.30	9.98E-01	
	4	L-E	25.69	2.96	48.41	2.32E-02	*
		N-E	37.90	15.18	60.63	7.87E-04	***
		P-E	46.31	23.59	69.04	7.66E-05	***
		N-L	12.22	-10.51	34.94	4.53E-01	
		P-L	20.63	-2.10	43.35	8.37E-02	*
		P-N	8.41	-14.31	31.13	7.31E-01	
Syngnathus rostellatus	1	L-E	50.06	0.00	100.12	5.00E-02	*
		N-E	24.71	-27.79	77.21	5.60E-01	
		P-E	60.48	10.42	110.54	1.47E-02	*
		N-L	-25.34	-77.85	27.16	5.40E-01	
		P-L	10.42	-39.64	60.48	9.35E-01	
		P-N	35.77	-16.73	88.27	2.55E-01	
	2	L-E	0.34	-45.80	46.48	1.00E+00	
		N-E	44.08	-4.31	92.47	8.19E-02	
		P-E	27.17	-18.97	73.31	3.73E-01	
		N-L	43.74	-4.65	92.13	8.50E-02	
		P-L	26.83	-19.30	72.97	3.84E-01	
		P-N	-16.91	-65.30	31.48	7.61E-01	
	3	L-E	3.77	-31.59	39.14	9.90E-01	
		N-E	-30.03	-67.13	7.06	1.39E-01	
		P-E	-28.34	-63.71	7.03	1.45E-01	
		N-L	-33.81	-70.90	3.28	8.17E-02	
		P-L	-32.12	-67.48	3.25	8.32E-02	
		P-N	1.69	-35.40	38.79	9.99E-01	
	4	L-E	-32.43	-61.64	-3.22	2.64E-02	*
		N-E	-10.29	-40.93	20.34	7.81E-01	
		P-E	-16.10	-45.31	13.11	4.29E-01	
		N-L	22.14	-8.50	52.77	2.12E-01	
		P-L	16.33	-12.88	45.54	4.17E-01	
		P-N	-5.81	-36.44	24.83	9.50E-01	
	6	L-E	-5.87	-28.79	17.05	8.88E-01	

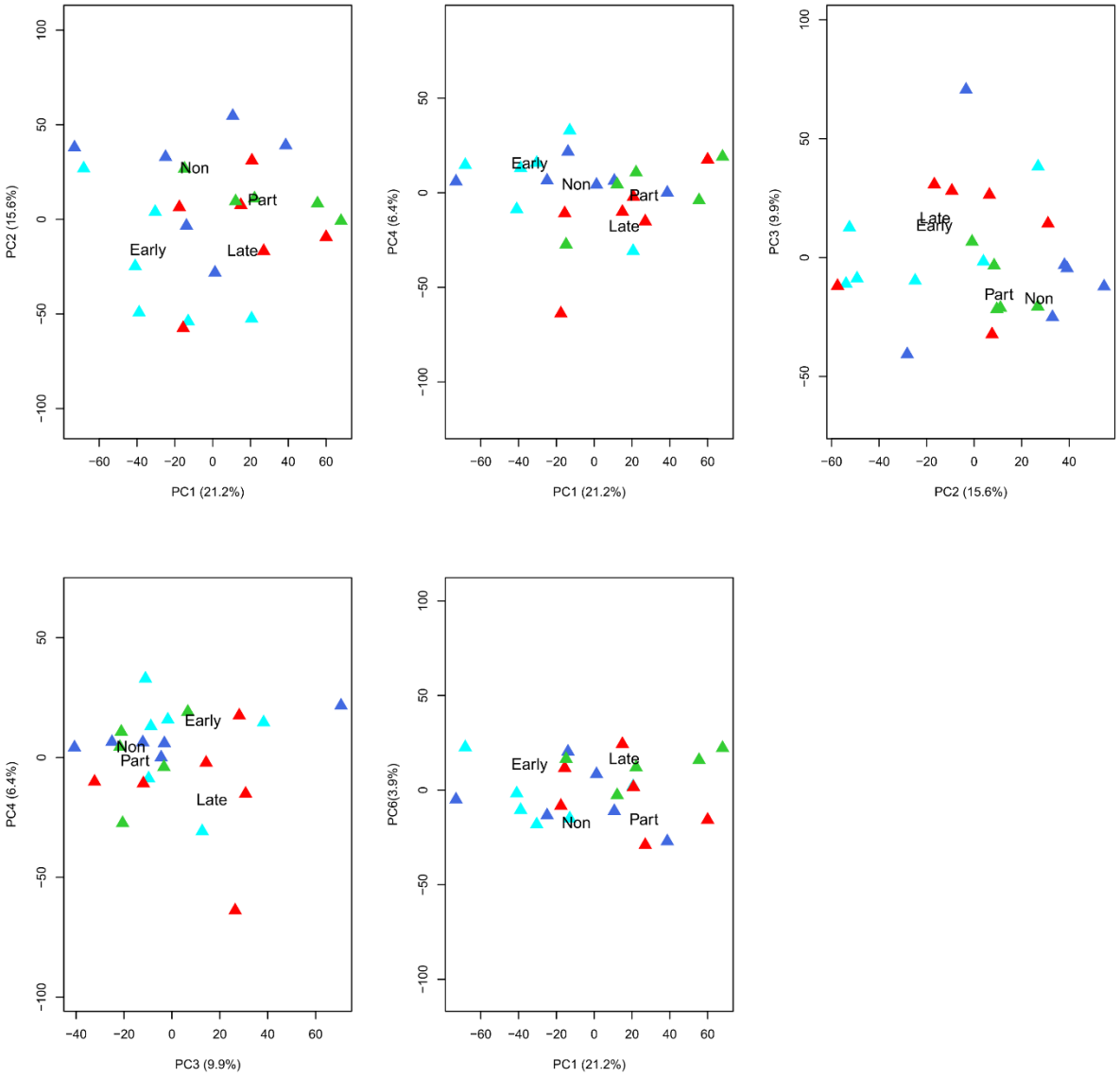
		N-E	-9.21	-33.25	14.83	7.07E-01	
		P-E	15.12	-7.80	38.04	2.80E-01	
		N-L	-3.34	-27.38	20.70	9.79E-01	
		P-L	20.99	-1.93	43.91	7.98E-02	*
		P-N	24.33	0.29	48.37	4.67E-02	*
Syngnathus typhle	2	L-E	39.83	-2.92	82.57	7.34E-02	*
		N-E	32.79	-9.96	75.54	1.73E-01	
		P-E	-12.84	-55.59	29.91	8.34E-01	
		N-L	-7.04	-49.79	35.71	9.67E-01	
		P-L	-52.67	-95.42	-9.92	1.25E-02	*
		P-N	-45.63	-88.38	-2.88	3.38E-02	*
	3	L-E	-13.37	-37.63	10.90	4.33E-01	
		N-E	-32.27	-56.53	-8.00	6.78E-03	**
		P-E	-40.91	-65.18	-16.65	7.01E-04	***
		N-L	-18.90	-43.17	5.37	1.63E-01	
		P-L	-27.54	-51.81	-3.28	2.26E-02	*
		P-N	-8.64	-32.91	15.62	7.53E-01	
	6	L-E	-1.08	-26.25	24.09	9.99E-01	
		N-E	12.60	-12.56	37.77	5.13E-01	
		P-E	-18.36	-43.53	6.81	2.06E-01	
		N-L	13.68	-11.48	38.85	4.44E-01	
		P-L	-17.28	-42.45	7.89	2.51E-01	
		P-N	-30.96	-56.13	-5.79	1.26E-02	*
Nerophis ophidion	1	L-E	71.42	52.27	90.57	0.00E+00	
		N-E	-21.54	-40.69	-2.39	2.40E-02	*
		P-E	76.25	57.10	95.40	0.00E+00	
		N-L	-92.96	-112.11	-73.81	0.00E+00	
		P-L	4.83	-14.32	23.98	8.94E-01	
		P-N	97.79	78.64	116.94	0.00E+00	
	3	L-E	-17.43	-53.42	18.57	5.40E-01	
		N-E	-36.84	-72.83	-0.84	4.37E-02	*
		P-E	-34.07	-70.06	1.93	6.76E-02	*
		N-L	-19.41	-55.41	16.58	4.51E-01	
		P-L	-16.64	-52.64	19.35	5.77E-01	
		P-N	2.77	-33.22	38.77	9.96E-01	
	4	L-E	-20.74	-50.76	9.28	2.46E-01	
		N-E	-32.62	-62.64	-2.60	3.02E-02	*
		P-E	-20.16	-50.18	9.86	2.68E-01	
		N-L	-11.87	-41.89	18.15	6.89E-01	
		P-L	0.58	-29.44	30.60	1.00E+00	
		P-N	12.46	-17.56	42.48	6.57E-01	

Significance: * < .05, ** < .01, *** < 0.001

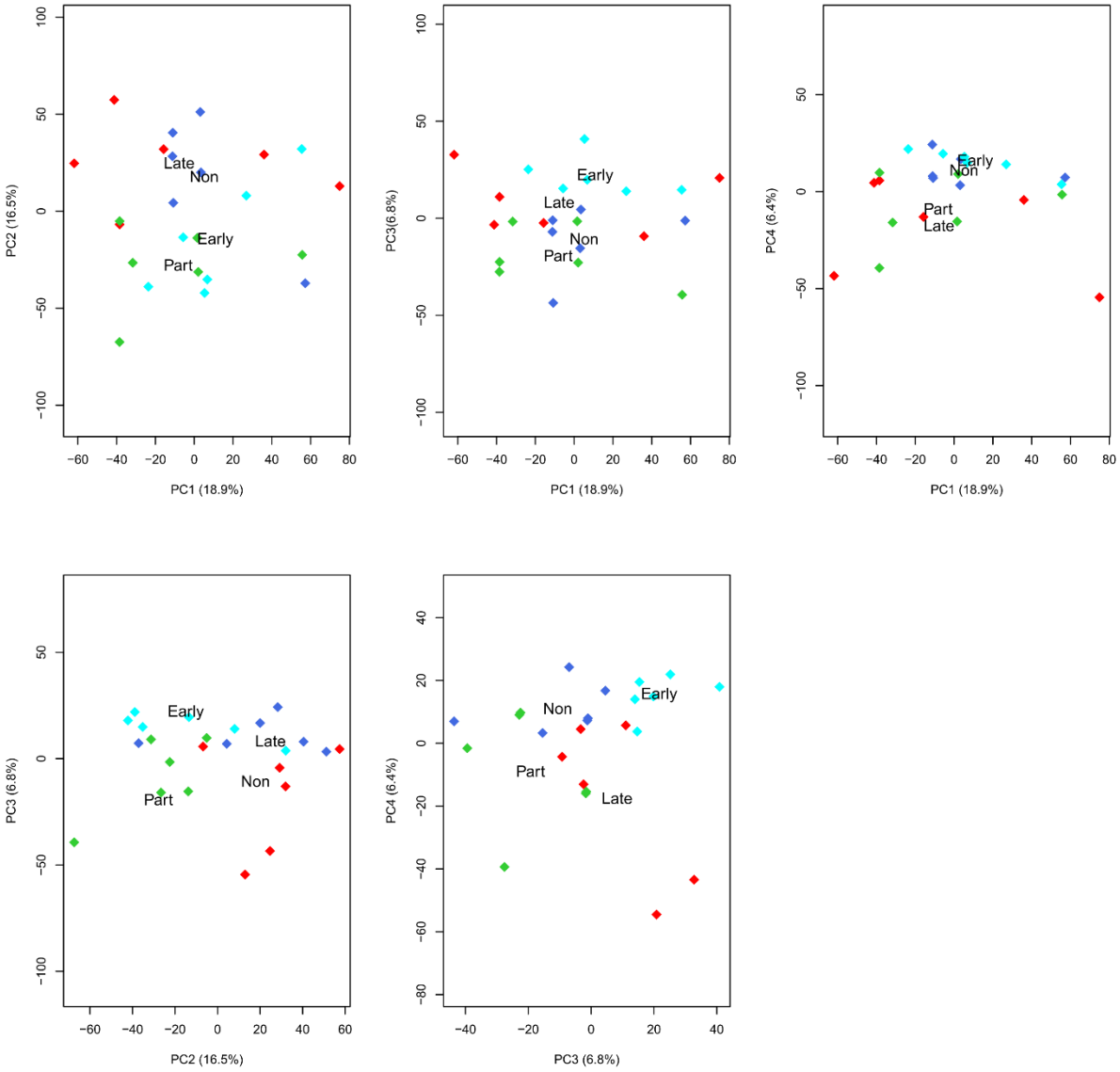
N = Non-pregnant, E = Early, L = Late, P = Parturition



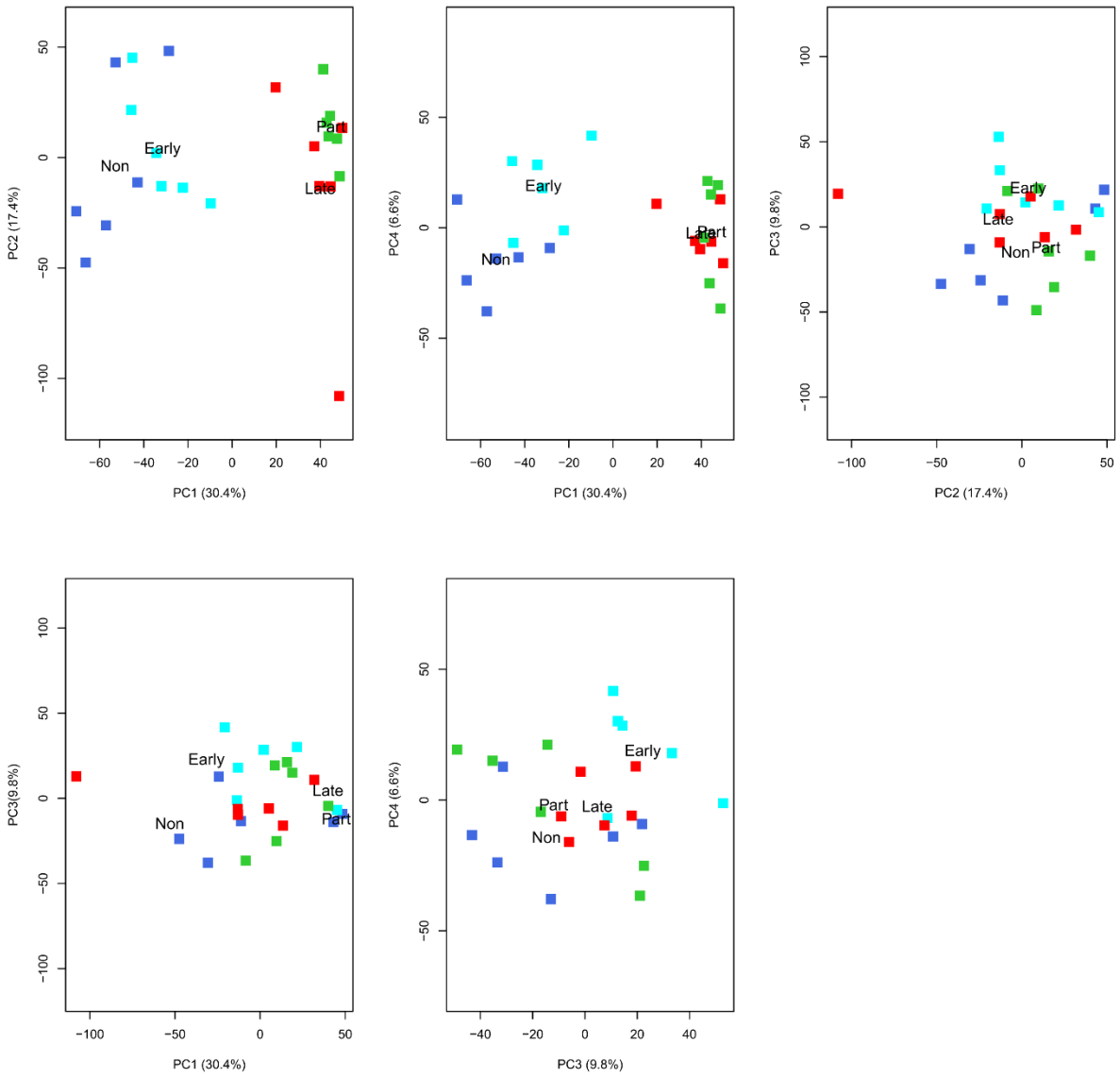
Supplementary figure 2. PCA component visualizations for *Hippocampus erectus*. Navy blue, cyan, red and green represent non-pregnant, early pregnancy, late pregnancy and parturition stage replicates, respectively. Stage text labels represent stage means.



Supplementary figure 3. PCA component visualizations for *Syngnathus rostellatus* combined. Navy blue, cyan, red and green represent non-pregnant, early pregnancy, late pregnancy and parturition stage replicates, respectively. Stage text labels represent stage means.



Supplementary figure 4. PCA component visualizations for *Syngnathus typhle*. Navy blue, cyan, red and green represent non-pregnant, early pregnancy, late pregnancy and parturition stage replicates, respectively. Stage text labels represent stage means.



Supplementary figure 5. PCA component visualizations for *Nerophis ophidion*. Navy blue, cyan, red and green represent non-pregnant, early pregnancy, late pregnancy and parturition stage replicates, respectively. Stage text labels represent stage means.

Supplementary table 5. MANOVA results from PC loadings (PC 1:8) for all species combined and pouched species only. Increasing number of asterisks (*) relates to increased significance.

Species	PC	D.f.	Res	Pillai	F values	*P*	Sig
All	1-8	3	91	1.10	6.22	3.27E-15	***
Pouched	1-8	3	67	1.33	6.20	6.29E-14	***

Significance: * < .05, ** < .01, *** < 0.001

Supplementary table 6. ANOVA results from PC loadings (PC 1:8) for all species combined and pouched species only. Increasing number of asterisks (*) relates to increased significance.

Species	PC	D.f.	Res	SumSq	F values	*P*	Sig
All	1	3	91	6241	2.16	9.85E-02	
	2	3	91	21746	10.87	3.59E-06	***
	3	3	91	3313	2.03	1.16E-01	
	4	3	91	6318	8.30	6.11E-05	***
	5	3	91	1420	1.89	1.37E-01	
	6	3	91	1330	2.53	6.23E-02	
	7	3	91	3802	10.17	7.66E-06	***
	8	3	91	1431	3.58	1.69E-02	*
Pouched	1	3	67	6552	2.04	0.117	
	2	3	67	2995	1.47	0.229	
	3	3	67	12524	14.95	1.49E-07	***
	4	3	67	5139	5.55	0.00183	**
	5	3	67	2953	4.49	0.00626	**
	6	3	67	1822	2.92	0.0404	*
	7	3	67	1874	4.22	0.0086	**
	8	3	67	1627	3.82	0.0138	*

Significance: * < .05, ** < .01, *** < 0.001

Supplementary table 7. Post-hoc Tukey-test results from PC loadings for all species combined and pouched species only. Tests only carried out on PCs exhibiting significant stage differences. Increasing number of asterisks (*) relates to increased significance.

Species	PC	Comparison	diff	lwr	upr	*p*	Sig
All	2	L-E	22.09	2.59	41.60	1.99E-02	*
		N-E	-0.59	-20.30	19.13	1.00E+00	
		P-E	35.00	15.49	54.51	5.47E-05	***
		N-L	-22.68	-42.40	-2.96	1.75E-02	*
		P-L	12.91	-6.60	32.41	3.13E-01	
		P-N	35.59	15.87	55.30	4.91E-05	***
	4	L-E	15.12	3.09	27.15	7.68E-03	**
		N-E	-6.09	-18.25	6.08	5.59E-01	
		P-E	9.29	-2.74	21.32	1.88E-01	
		N-L	-21.21	-33.37	-9.04	9.15E-05	***
		P-L	-5.83	-17.86	6.21	5.86E-01	
		P-N	15.38	3.22	27.54	7.21E-03	**
	7	L-E	-0.44	-8.87	8.00	9.99E-01	
		N-E	9.37	0.85	17.89	2.53E-02	*
		P-E	14.38	5.95	22.82	1.34E-04	***
		N-L	9.81	1.28	18.33	1.74E-02	*
		P-L	14.82	6.39	23.25	7.97E-05	***
		P-N	5.01	-3.51	13.54	4.19E-01	
Pouched	3	L-E	-22.57	-37.24	-7.89	7.61E-04	***
		N-E	8.80	-6.09	23.69	4.10E-01	

	P-E	-20.47	-35.14	-5.79	2.62E-03	**
	N-L	31.37	16.48	46.26	3.10E-06	***
	P-L	2.10	-12.57	16.78	9.82E-01	
	P-N	-29.27	-44.16	-14.38	1.30E-05	***
4	L-E	-3.78	-19.21	11.64	9.17E-01	
	N-E	-19.07	-34.72	-3.42	1.07E-02	*
	P-E	-18.32	-33.74	-2.89	1.35E-02	*
	N-L	-15.29	-30.94	0.36	5.79E-02	*
	P-L	-14.54	-29.96	0.89	7.18E-02	*
	P-N	0.76	-14.89	16.40	9.99E-01	
5	L-E	-4.97	-17.98	8.03	7.46E-01	
	N-E	7.28	-5.92	20.48	4.71E-01	
	P-E	11.66	-1.35	24.66	9.47E-02	*
	N-L	12.25	-0.95	25.45	7.82E-02	*
	P-L	16.63	3.62	29.64	6.73E-03	**
	P-N	4.38	-8.82	17.58	8.18E-01	
7	L-E	-0.82	-11.51	9.87	9.97E-01	
	N-E	9.08	-1.77	19.92	1.32E-01	
	P-E	10.49	-0.20	21.18	5.64E-02	*
	N-L	9.89	-0.95	20.74	8.62E-02	*
	P-L	11.31	0.62	22.00	3.41E-02	*
	P-N	1.41	-9.43	12.26	9.86E-01	

Significance: * < .05, ** < .01, *** < 0.001
N = Non-pregnant, E = Early, L = Late, P = Parturition

Supplementary table 8. The top 675 most influential orthologs, defined by residual loadings, for principal components used to display pregnancy stage expression differences in all species (PC2, PC4) and pouched species only (PC3, PC4) data sets. Loading values: positive and negative values indicate their association to the PCs shown here in reference to PCA plots.

Data set	Principal component	No. of orthologs (+ve)	No. of orthologs (-ve)
All species	PC2	445	230
	PC4	383	292
	PC7	275	400
Pouched species	PC3	427	248
	PC4	536	139
	PC5	345	330
	PC7	375	299

Supplementary tables 9-36 – full table can be accessed online.

Supplementary table 37. Differentially expressed genes with functional role in MHC processing and presentation in four syngnathid species. Positive values indicate upregulation and negative values represent downregulation.

Species	*Stage*	*Gene*	*log2fc*	*Function*
H. erectus	Non	*il10*	**1.90**	MHC II, anti-inflammation
H. erectus	Non	*psmd1*	**-1.23**	MHC I, antigen presentation
H. erectus	Non	*mfsd6b*	**-1.03**	MHC I, antigen presentation
H. erectus	Non	*h2-k1*	**-1.31**	MHC I, antigen presentation
H. erectus	Non	*ap1m1*	**-1.10**	MHC II, antigen presentation
H. erectus	Non	*kifap3*	**-1.02**	MHC II, antigen presentation
H. erectus	Non	*kif23*	**-1.42**	MHC II, antigen presentation
H. erectus	Non	*racgap1*	**-1.85**	MHC II, antigen presentation
H. erectus	Non	*racgap1(2)*	**-1.91**	MHC II, antigen presentation
H. erectus	Early	*h2-k1*	**1.41**	MHC I, antigen presentation
H. erectus	Early	*il10*	**2.28**	MHC II, anti-inflammation
H. erectus	Early	*psmd1*	**-1.14**	MHC I, antigen presentation
H. erectus	Early	*mfsd6b*	**-1.50**	MHC I, antigen presentation
H. erectus	Early	*mfsd6a*	**-1.84**	MHC I, antigen presentation
H. erectus	Early	*mfsd6*	**-1.94**	MHC I, antigen presentation
H. erectus	Early	*kifap3*	**-1.16**	MHC II, antigen presentation
H. erectus	Early	*was*	**-1.15**	T-cell antigen processing and presentation
H. erectus	Late	*mfsd6b*	**-1.21**	MHC I, antigen presentation
H. erectus	Late	*mfsd6a*	**-1.29**	MHC I, antigen presentation
H. erectus	Late	*ctsl*	**-1.49**	MHC II, antigen presentation
S. typhle	Non	*mr1*	**3.29**	MHC I, antigen presentation
S. typhle	Non	*fgl2*	**2.00**	Negative and positive, antigen presentation
S. typhle	Non	*fgl2*	**1.38**	Negative and positive, antigen presentation
S. typhle	Non	*ccr7*	**1.58**	antigen presentation
S. typhle	Non	*b12*	**1.13**	MHC I, antigen presentation
S. typhle	Non	*cd209*	**1.03**	MHC II, antigen presentation
S. typhle	Non	*cd209*	**1.37**	MHC II, antigen presentation
S. typhle	Early	*fgl2*	**1.86**	Negative and positive, antigen presentation
S. typhle	Early	*klrk1*	**1.56**	MHC I, antigen presentation
S. typhle	Early	*ccr7*	**1.11**	antigen presentation
S. typhle	Early	*lgals3*	**-2.80**	MHC, antigen presentation
S. typhle	Early	*ap1b1*	**-21.51**	MHC II, antigen presentation
S. rostellatus	Non	*tapbpl*	**-1.40**	MHC I, antigen presentation
S. rostellatus	Non	*b12*	**-1.79**	MHC I, antigen presentation
S. rostellatus	Non	*b12*	**-1.87**	MHC I, antigen presentation
S. rostellatus	Non	*thbs1*	**-2.12**	MHC II, antigen presentation
S. rostellatus	Early	*rag1*	**2.58**	V(D)J recombination
S. rostellatus	Early	*racgap1*	**1.58**	MHC II, antigen presentation
S. rostellatus	Early	*erap1*	**-1.07**	MHC I, antigen presentation
S. rostellatus	Early	*mfsd6*	**-1.16**	MHC I, antigen presentation

S. rostellatus	Early	*mr1*	**-1.25**	MHC I, antigen presentation
S. rostellatus	Early	*marchf8*	**-1.27**	MHC II, antigen presentation
S. rostellatus	Early	*h2-k1*	**-1.30**	MHC I, antigen presentation
S. rostellatus	Early	*pycard*	**-2.18**	MHC II, antigen presentation
S. rostellatus	Early	*pycard*	**-2.72**	MHC II, antigen presentation
S. rostellatus	Late	*ctsl*	**-2.76**	MHC II, antigen presentation
S. rostellatus	Late	*marchf8*	**-1.42**	MHC II, antigen presentation
N. ophidion	Non	*fgl1*	**7.20**	MHC II, immune suppression
N. ophidion	Non	*klrk1*	**5.78**	MHC I, antigen presentation
N. ophidion	Non	*cd81*	**4.91**	MHC II, antigen presentation
N. ophidion	Non	*hivep2*	**4.80**	MHC I, antigen presentation
N. ophidion	Non	*pkm*	**4.40**	MHC II, antigen presentation
N. ophidion	Non	*fgl1*	**4.17**	MHC II, immune suppression
N. ophidion	Non	*thbs1*	**3.46**	MHC II, antigen presentation
N. ophidion	Non	*racgap1*	**3.44**	MHC II, antigen presentation
N. ophidion	Non	*fgl2*	**3.44**	Negative and positive, antigen presentation
N. ophidion	Non	*kif23*	**3.43**	MHC II, antigen presentation
N. ophidion	Non	*kif18a*	**3.18**	MHC II, antigen presentation
N. ophidion	Non	*kif3b*	**3.15**	MHC II, antigen presentation
N. ophidion	Non	*kif3b*	**3.15**	MHC II, antigen presentation
N. ophidion	Non	*racgap1*	**3.02**	MHC II, antigen presentation
N. ophidion	Non	*cd209*	**2.91**	MHC II, antigen presentation
N. ophidion	Non	*cd81*	**2.84**	MHC II, antigen presentation
N. ophidion	Non	*klc2*	**2.79**	MHC II, antigen presentation
N. ophidion	Non	*cd209*	**2.72**	MHC II, antigen presentation
N. ophidion	Non	*fgl2*	**2.51**	Negative and positive, antigen presentation
N. ophidion	Non	*cd209*	**2.48**	MHC II, antigen presentation
N. ophidion	Non	*fgl2*	**2.26**	Negative and positive, antigen presentation
N. ophidion	Non	*kif23 (1)*	**2.17**	MHC II, antigen presentation
N. ophidion	Non	*kif15*	**2.10**	MHC II, antigen presentation
N. ophidion	Non	*kif3c*	**1.70**	MHC II, antigen presentation
N. ophidion	Non	*kif3c (1)*	**1.30**	MHC II, antigen presentation
N. ophidion	Non	*tapbp*	**-1.09**	MHC I, antigen presentation
N. ophidion	Non	*tapbp*	**-1.09**	MHC I, antigen presentation
N. ophidion	Non	*hm13*	**-1.10**	MHC I, antigen presentation
N. ophidion	Non	*sec24a*	**-1.11**	MHC, antigen presentation
N. ophidion	Non	*mr1*	**-1.21**	MHC I, antigen presentation
N. ophidion	Non	*tap2*	**-1.25**	MHC I, antigen presentation
N. ophidion	Non	*mr1*	**-1.35**	MHC I, antigen presentation
N. ophidion	Non	*tap2*	**-1.39**	MHC I, antigen presentation
N. ophidion	Non	*rfxank*	**-1.42**	MHC II, antigen presentation
N. ophidion	Non	*mr1*	**-1.64**	MHC I, antigen presentation
N. ophidion	Non	*h2-q9*	**-1.66**	MHC I, antigen presentation
N. ophidion	Non	*irf8*	**-1.70**	MHC I & II, antigen presentation
N. ophidion	Non	*b2m*	**-1.70**	MHC I, antigen presentation

N. ophidion	Non	*sec24c*	**-1.82**	MHC, antigen presentation
N. ophidion	Non	*ap1m1*	**-2.12**	MHC II, antigen presentation
N. ophidion	Non	*irf4*	**-2.46**	MHC I, antigen presentation
N. ophidion	Non	*mfsd6*	**-2.49**	MHC I, antigen presentation
N. ophidion	Non	*ctsl*	**-23.52**	MHC II, antigen presentation
N. ophidion	Early	*fgl1*	**5.31**	MHC II, immune suppression
N. ophidion	Early	*fgl2*	**4.39**	Negative and positive, antigen presentation
N. ophidion	Early	*cd81*	**3.81**	MHC II, antigen presentation
N. ophidion	Early	*hivep2*	**3.78**	MHC I, antigen presentation
N. ophidion	Early	*ctsh*	**3.75**	MHC I, antigen presentation
N. ophidion	Early	*fgl1*	**3.59**	MHC II, immune suppression
N. ophidion	Early	*mr1*	**3.49**	MHC I, antigen presentation
N. ophidion	Early	*cd209*	**3.33**	MHC II, antigen presentation
N. ophidion	Early	*thbs1*	**3.32**	MHC II, antigen presentation
N. ophidion	Early	*pkm*	**2.84**	MHC II, antigen presentation
N. ophidion	Early	*racgap1*	**2.66**	MHC II, antigen presentation
N. ophidion	Early	*kif18a*	**2.43**	MHC II, antigen presentation
N. ophidion	Early	*kif3b*	**2.36**	MHC II, antigen presentation
N. ophidion	Early	*cd209*	**2.34**	MHC II, antigen presentation
N. ophidion	Early	*kif23*	**2.33**	MHC II, antigen presentation
N. ophidion	Early	*racgap1*	**2.31**	MHC II, antigen presentation
N. ophidion	Early	*klc2*	**2.30**	MHC II, antigen presentation
N. ophidion	Early	*spi1*	**2.26**	MHC II, negative regulation of biosynthesis
N. ophidion	Early	*fgl2*	**2.23**	Negative and positive, antigen presentation
N. ophidion	Early	*fgl2*	**2.22**	Negative and positive, antigen presentation
N. ophidion	Early	*cd81 (2)*	**2.16**	MHC II, antigen presentation
N. ophidion	Early	*cd209*	**2.07**	MHC II, antigen presentation
N. ophidion	Early	*kif23*	**1.80**	MHC II, antigen presentation
N. ophidion	Early	*flt3*	**1.56**	Antigen presentation
N. ophidion	Early	*spi1*	**1.36**	MHC II, negative regulation of biosynthesis
N. ophidion	Early	*kif15*	**1.29**	MHC II, antigen presentation
N. ophidion	Early	*hm13*	**-1.06**	MHC I, antigen presentation
N. ophidion	Early	*mr1*	**-1.09**	MHC I, antigen presentation
N. ophidion	Early	*sec24a*	**-1.13**	MHC, antigen presentation
N. ophidion	Early	*ap1m1*	**-1.16**	MHC II, antigen presentation
N. ophidion	Early	*nfx1*	**-1.18**	MHC II, negative regulation of biosynthesis
N. ophidion	Early	*tap2*	**-1.29**	MHC I, antigen presentation
N. ophidion	Early	*mr1*	**-1.36**	MHC I, antigen presentation
N. ophidion	Early	*h2-q9*	**-1.37**	MHC I, antigen presentation
N. ophidion	Early	*rfxank*	**-1.47**	MHC II, antigen presentation
N. ophidion	Early	*mfsd6*	**-1.51**	MHC I, antigen presentation
N. ophidion	Early	*irf4*	**-1.74**	MHC I, antigen presentation

N. ophidion	Early	*sec24c*	**-1.82**	MHC, antigen presentation
N. ophidion	Early	*ctsl*	**-23.66**	MHC II, antigen presentation

Supplementary table 38 – full table can be accessed online.

Supplementary table 39 - Broad GO slim term mapper annotations for genes assigned to mFuzz pregancy stage clusters in *Nerophis ophidion* , *Hippocampus erectus* and *Syngnathus rostellatus* . Percentages represent the the proportion of genes (upregulated and downregulated combined) present in the total number of submitted genes and red highlights indicate the top three representations each respective cluster within each species

	Nerophis ophidion		***Hippocampus erectus***		***Syngnathus rostellatus***		***Syngnathus typhle***	
Non-pregnant	*Nerophis ophidion* (90 genes)		*Hippocampus erectus* (100 genes)		*Syngnathus rostellatus* (15 genes)		*Syngnathus typhle* (8 genes)	
	signal transduction	**42.22%**	signal transduction	**31.00%**	anatomical structure development	**53.33%**	signal transduction	**62.50%**
	anatomical structure development	**33.33%**	anatomical structure development	**34.00%**	signal transduction	**40.00%**	anatomical structure development	**37.50%**
	response to stress	**31.11%**	biosynthetic process	**33.00%**	transport	**40.00%**	cell motility	25.00%
	biosynthetic process	**31.11%**	cellular nitrogen compound metabolism	29.00%	cell differentiation	33.33%	biosynthetic process	**37.50%**
	cellular nitrogen compound metabolism	30.00%	transport	27.00%	catabolic process	33.33%	cellular nitrogen compound metabolism	37.50%
	transport	30.00%	cell differentiation	27.00%	response to stress	33.33%	immune system process	12.50%
	immune system process	15.56%	immune system process	13.00%	immune system process	6.67%	cell differentiation	50.00%
Early	*Nerophis ophidion* (639 genes)		*Hippocampus erectus* (63 genes)		*Syngnathus rostellatus* (71 genes)		*Syngnathus typhle* (9 genes)	
	cellular nitrogen compound metabolism	**40.38%**	signal transduction	**36.51%**	anatomical structure development	**52.11%**	signal transduction	**55.56%**
	biosynthetic process	**37.72%**	anatomical structure development	28.57%	cellular nitrogen compound metabolism	**45.07%**	transport	44.44%
	signal transduction	**31.77%**	transport	**38.10%**	signal transduction	**40.85%**	anatomical structure development	**33.33%**
	transport	31.14%	biosynthetic process	**31.75%**	biosynthetic process	38.03%	response to stress	22.22%
	anatomical structure development	30.20%	cell differentiation	26.98%	cell differentiation	35.21%	biosynthetic process	**22.22%**
	immune system process	15.65%	immune system process	25.40%	cellular protein mod process	23.94%	cellular nitrogen compound metabolism	22.22%
	cellular protein mod process	23.94%	response to stress	26.98%	transport	22.54%	cell differentiation	22.22%
			cellular nitrogen compound metabolism	25.40%	immune system process	14.08%	immune system process	0.00%
Late			*Hippocampus erectus* (182 genes)		*Syngnathus rostellatus* (16 genes)			
			signal transduction	34.07%	signal transduction	**56.25%**		
			anatomical structure development	**38.46%**	anatomical structure development	**56.25%**		
			biosynthetic process	**35.71%**	cellular nitrogen compound metabolism	**56.25%**		
			transport	31.87%	cell differentiation	**37.50%**		
			cellular nitrogen compound metabolism	**43.41%**	transport	37.50%		
			immune system process	16.48%	immune system process	31.25%		
			cell differentiation	30.22%	cell motility	25.00%		

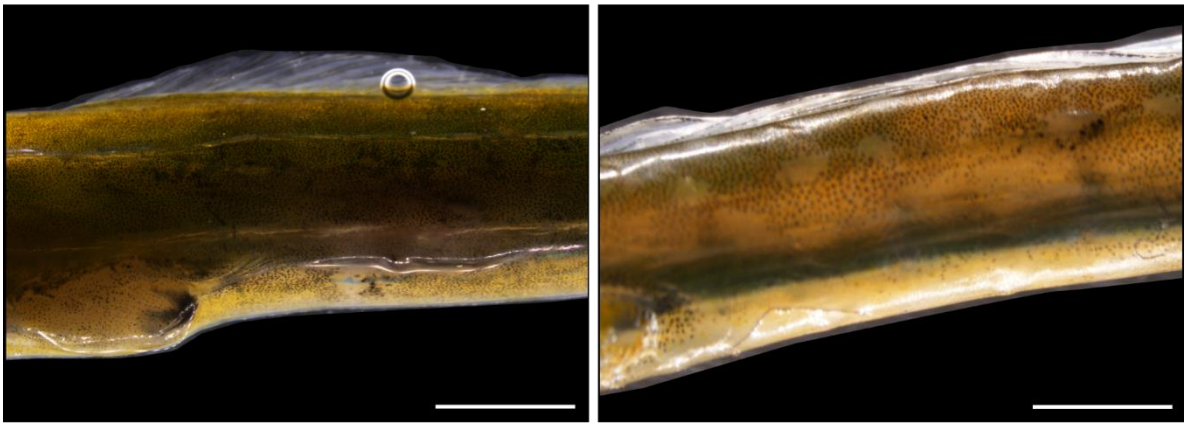
Part	*Hippocampus erectus* (205 genes)		*Syngnathus rostellatus* (72 genes)		*Syngnathus typhle* (12 genes)	
	signal transduction	**42.93%**	signal transduction	**31.94%**	signal transduction	**66.67%**
	transport	**43.90%**	anatomical structure development	**33.33%**	anatomical structure development	**58.33%**
	cellular protein mod process	**37.56%**	transport	**33.33%**	response to stress	41.67%
	anatomical structure development	36.59%	biosynthetic process	29.17%	biosynthetic process	**50.00%**
	biosynthetic process	35.12%	cell differentiation	22.22%	cellular nitrogen compound metabolism	50.00%
	cellular nitrogen compound metabolism	30.73%	cellular nitrogen compound metabolism	20.83%	immune system process	25.00%
	immune system process	24.39%	immune system process	16.67%	cellular protein mod process	41.67%
Part					*Syngnathus typhle* (20 genes)	
					signal transduction	**25.00%**
					anatomical structure development	**50.00%**
					cell component assembly	20.00%
					biosynthetic process	**25.00%**
					cellular nitrogen compound metabolism	25.00%
					immune system process	5.00%
					cell differentiation	30.00%
Non and part	*Hippocampus erectus* (130 genes)		*Syngnathus rostellatus* (54 genes)			
	signal transduction	**46.92%**	anatomical structure development	**38.89%**		
	anatomical structure development	**46.15%**	transport	**38.89%**		
	transport	**39.23%**	signal transduction	**33.33%**		
	biosynthetic process	33.85%	biosynthetic process	**33.33%**		
	cell differentiation	31.54%	cellular nitrogen compound metabolism	37.04%		
	cellular protein mod process	29.23%	vesicle-mediated transport	25.93%		
	immune system process	15.08%	immune system process	14.81%		
Late and Part	*Nerophis ophidion* (536 genes)		*Hippocampus erectus* (187 genes)			
	signal transduction	31.48%	signal transduction	**30.48%**		
	anatomical structure development	**35.60%**	anatomical structure development	29.95%		
	transport	26.75%	transport	29.41%		
	biosynthetic process	**36.42%**	biosynthetic process	**39.57%**		
	cellular nitrogen compound metabolism	**38.68%**	cellular nitrogen compound metabolism	**46.52%**		
	cellular protein mod process	27.57%	cellular protein mod process	22.99%		
	immune system process	12.14%	immune system process	11.76%		

Non and Early				
Nerophis ophidion (364 genes)				
biosynthetic process	36.26%			
cellular nitrogen compound metabolism	35.44%			
signal transduction	34.62%			
anatomical structure development	34.34%			
transport	33.24%			
cell differentiation	25.00%			
immune system process	17.86%			
Nerophis ophidion (2) (472 genes)				
signal transduction	35.38%			
anatomical structure development	39.41%			
transport	29.24%			
biosynthetic process	32.84%			
cellular nitrogen compound metabolism	33.05%			
cellular protein mod process	26.91%			
immune system process	15.89%			

Supplementary tables 40-42 – full tables can be accessed online.

Appendix – Chapter II

Nerophis ophidion



Syngnathus typhle

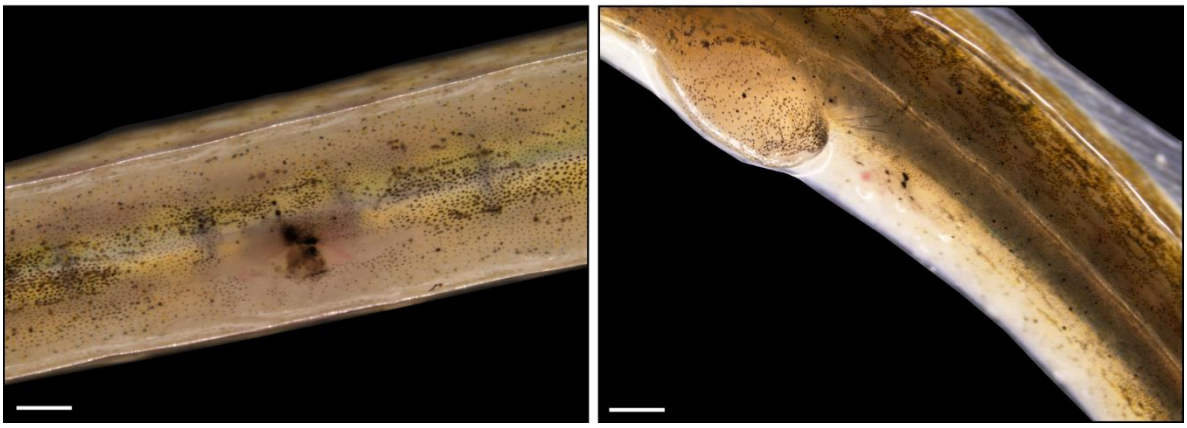


Figure 1. Images depicting examples of fin-transplant “sloughing” or shedding following transplant surgery in *S. typhle* and *N. ophidion*. Scale bar represents 1mm

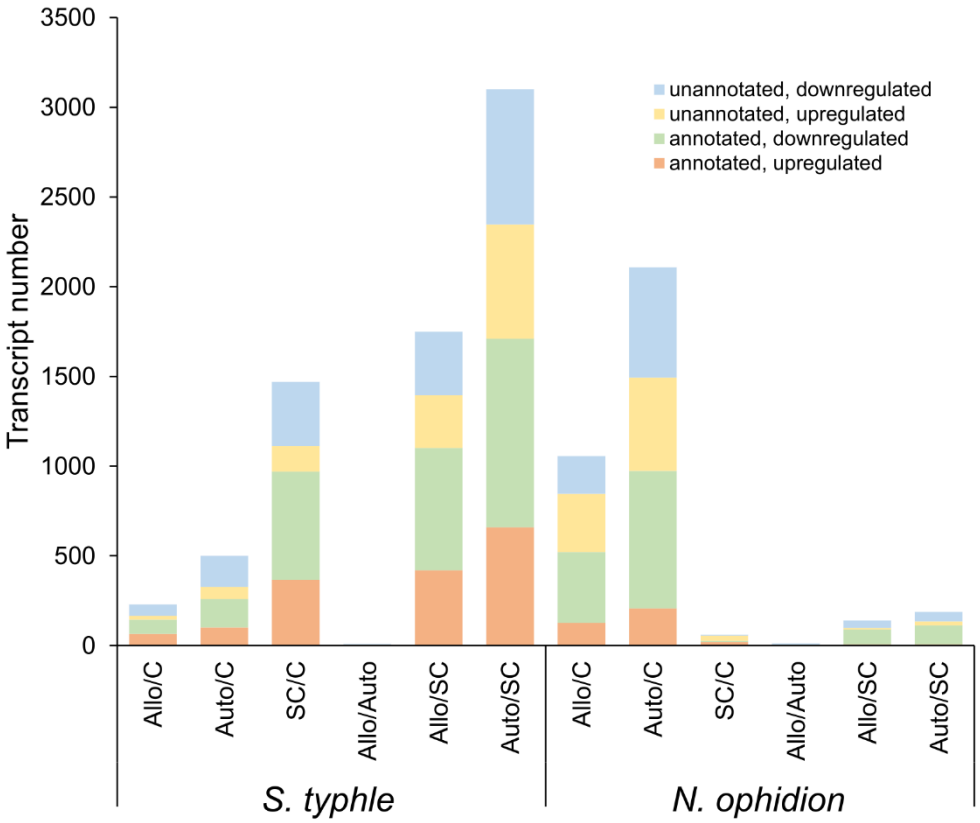


Figure 2. Differentially expressed transcript numbers for surgical type pairwise comparisons (upregulated/downregulated) of pipefish species, *Syngnathus typhle* and *Nerophis ophidion*.

Supplementary tables 1-2 – full tables can be accessed online.

Appendix – Chapter III

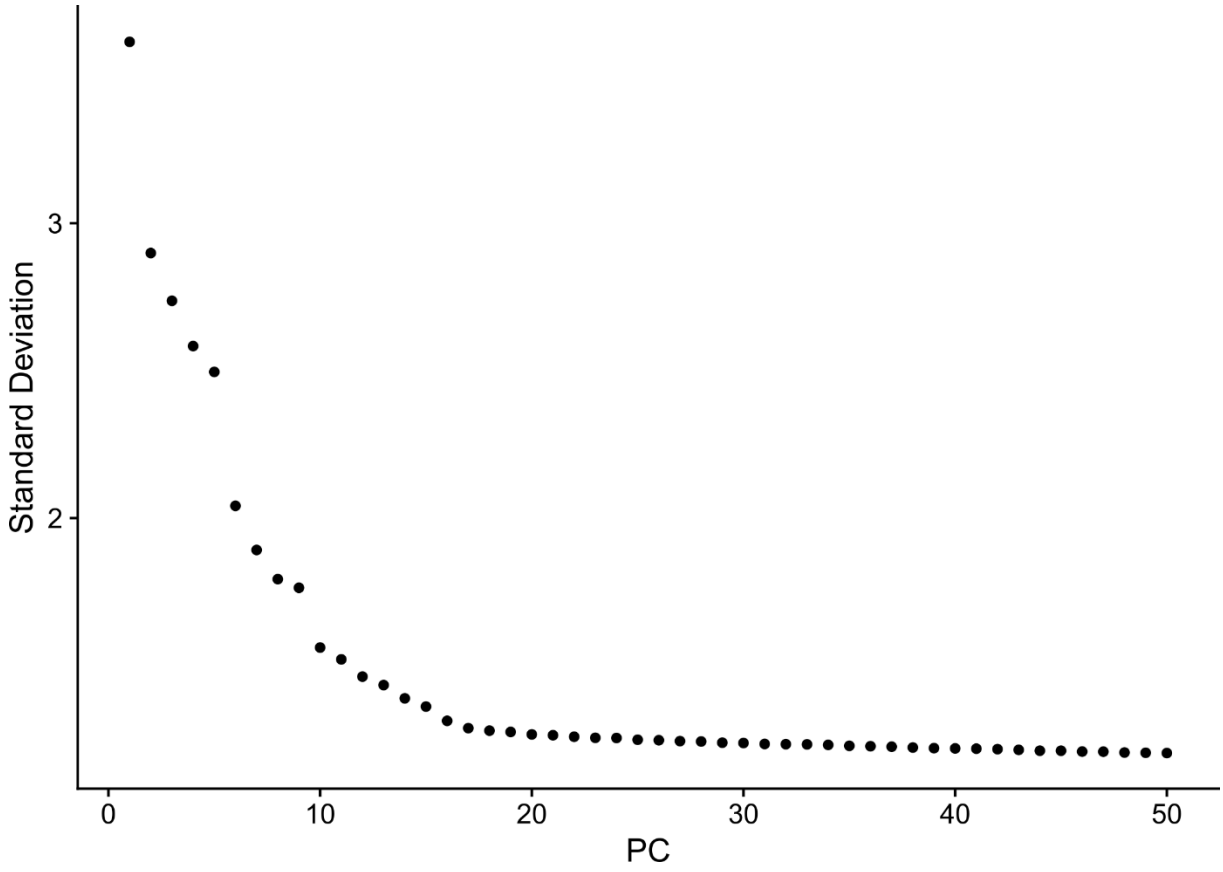


Figure 1. Elbow plot exhibiting principal component rankings based on associated explained variation.

Supplementary tables 1-2 – full tables can be accessed online.

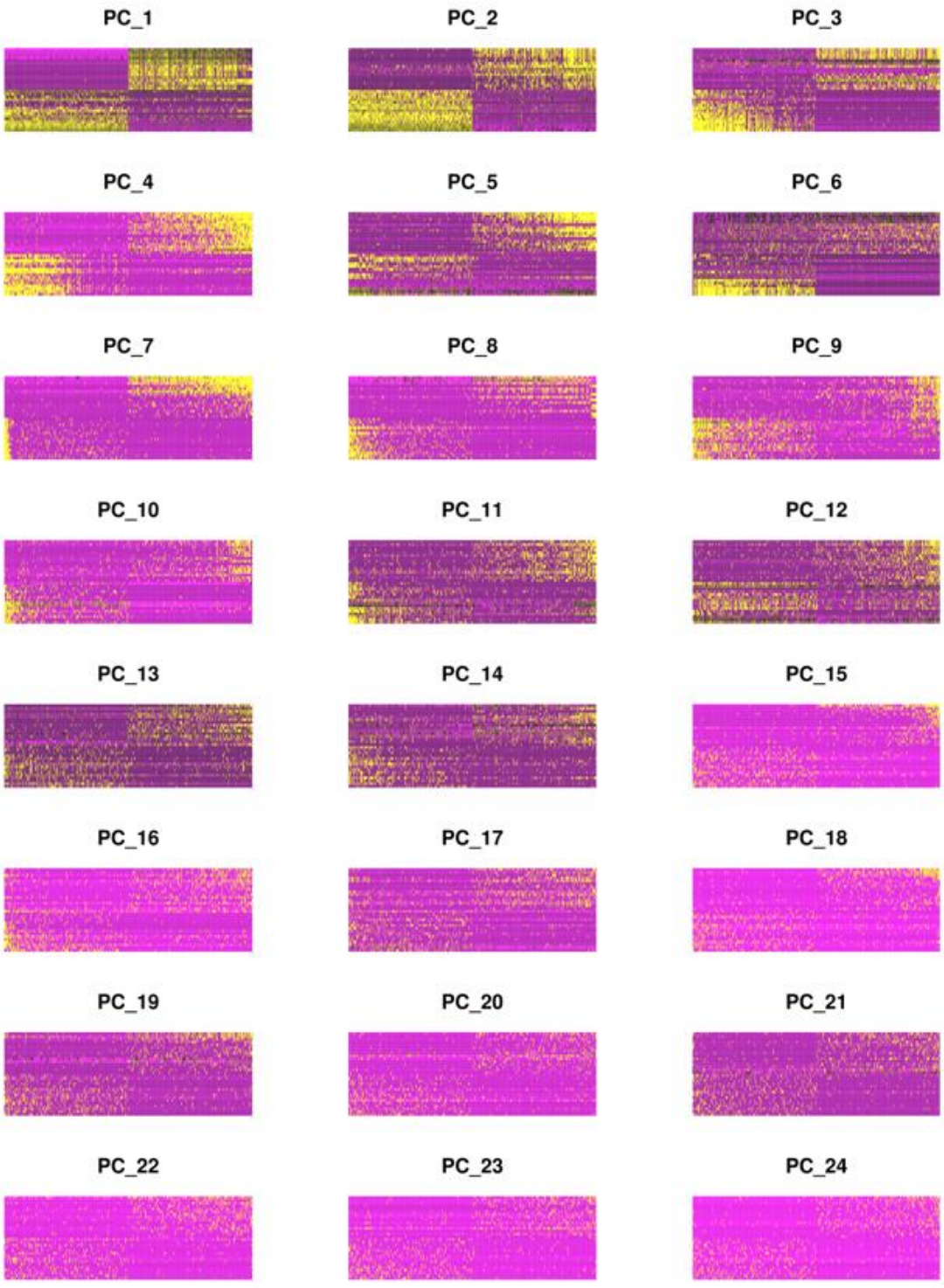


Figure 2. Differential gene expression heatmaps of the first 24 principle components, with genes representing rows and cells representing columns.

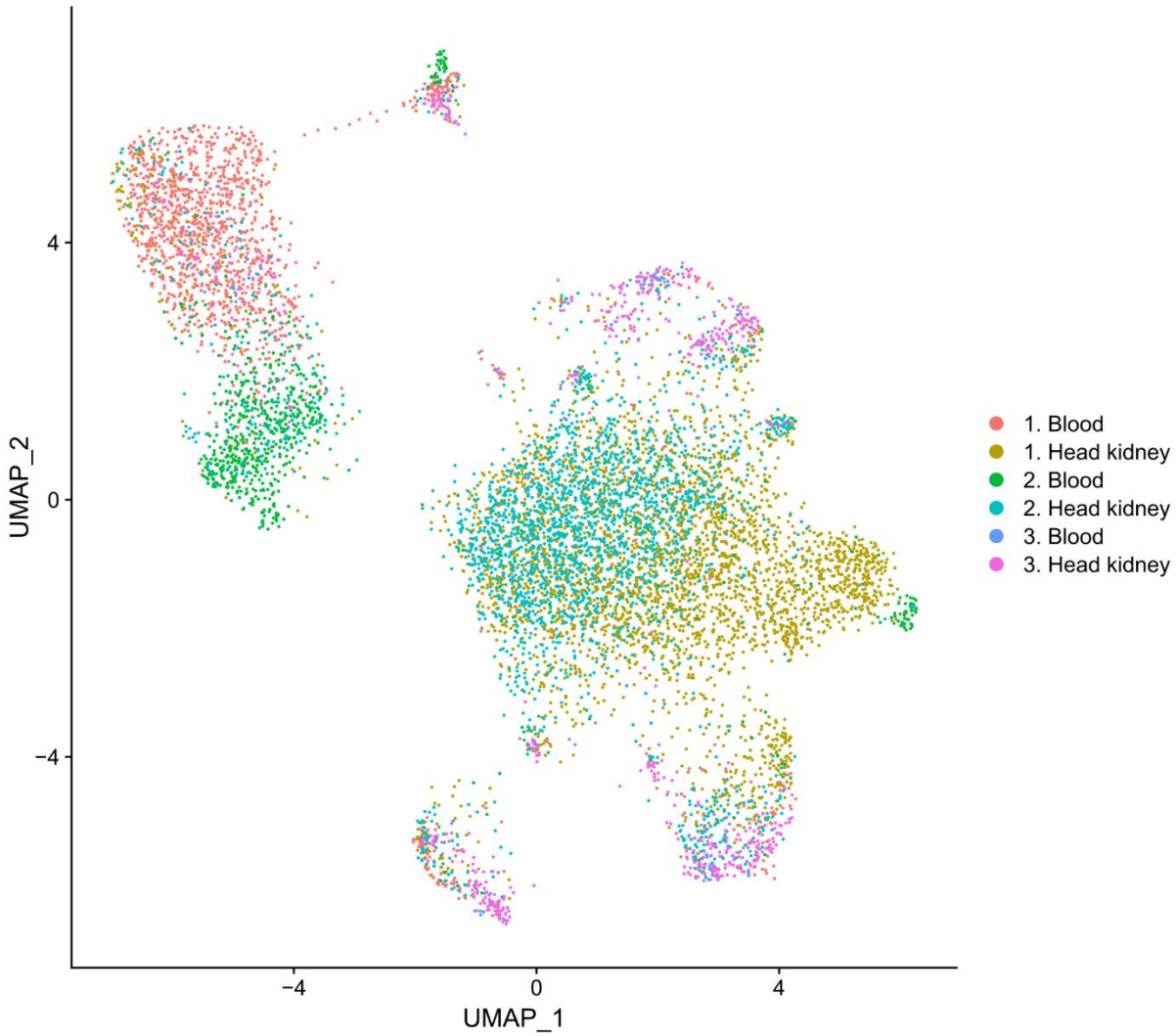


Figure 3. Uniform manifold approximation projection (UMAP) showing the extracted origin of cells, from three *Syngnathus typhle* individuals. Legend numbers (1, 2, 3) indicate the fish individual.