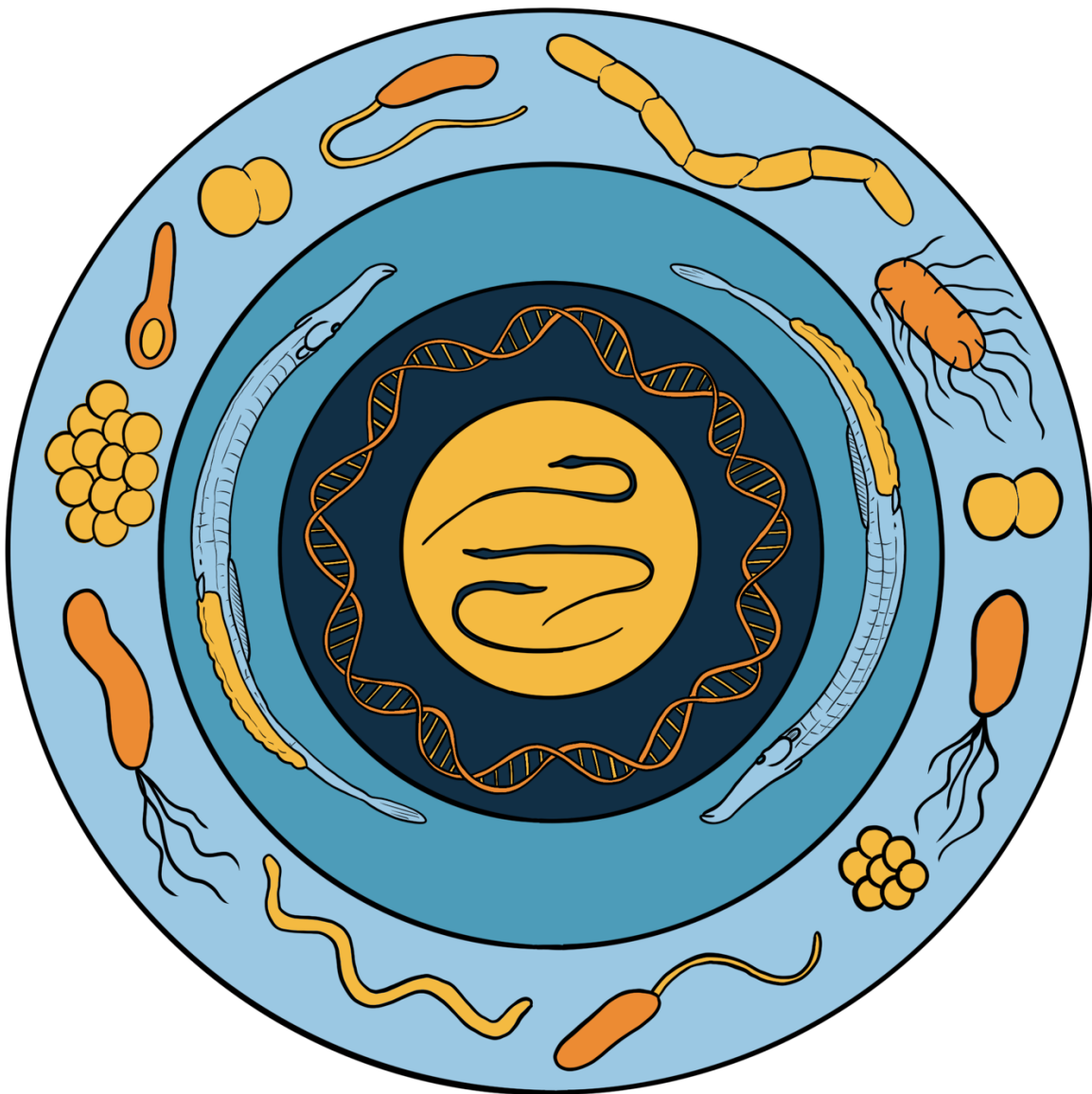


**DADS MATTER –
PATERNAL TRANSFER OF MICROBIOTA
THROUGH MALE PREGNANCY IN THE SEX ROLE-
REVERSED PIPEFISH *SYNGNATHUS TYPHLE***



Kim-Sara Wagner

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Vorgelegt von:

KIM-SARA WAGNER

Kiel, Januar 2025

Erste Gutachterin:

Prof. Dr. Olivia Roth

Zweite Gutachterin:

Prof. Dr. Ute Hentschel-Humeida

Tag der mündlichen Prüfung:

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Für Maëlie

“Messieurs, c'est les microbes qui auront le dernier mot”

Louis Pasteur (1822 – 1895)

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LIST OF ABBREVIATIONS

ANCOVA	Analysis of covariance
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ASV	Amplicon sequence variant
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
DADA2	<i>A software package that corrects Illumina-sequenced amplicon errors</i>
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpr	Days post-release
EPJ	Early pregnancy juveniles
EPP	Early pregnancy pouch
FG	Female gonads
GFP	Green fluorescent protein
GLS	Generalized least squares
IKMB	Institut für Klinische Molekularbiologie in Kiel
ISA	Indicator species analysis
LJP	Late pregnancy juveniles
LPP	Late pregnancy pouch
MANOVA	Multivariate analysis of variance
MB	Marine Broth
MCA	Multiple correspondence analysis
MD5	Message-Digest Algorithm 5
MiSeq	<i>A DNA-to-data NGS platform</i>
MULTIPATT	Multi-level pattern analysis
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NP	Non-pregnant males
NTC	No template control
OD _{600nm}	Optical density (<i>measured at a wavelength of 600 nm in 1 cm light path</i>)
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCA	Principal component analysis

List of Abbreviations

PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PSU	Practical salinity unit
PVP-I	Povidone-iodine
QIIME2	<i>A microbiome multi-omics bioinformatics and data science platform</i>
qPCR	Quantitative polymerase chain reaction
REML	Restricted maximum likelihood
rRNA	Ribosomal ribonucleic acid
S1 / S2	Biosafety level 1 / 2
SD	Standard deviation
SE	Standard error
SILVA	<i>A ribosomal RNA gene database project</i>
TCBS	Thiosulfate-citrate-bile salts-sucrose

SUMMARY

All living organisms host diverse microbial communities, ranging from pathogenic to commensal and mutualistic. Once primarily focused on pathogens, microbial research has increasingly revealed the pivotal role of beneficial microbes, or the microbiota, as a foundation for the evolution and persistence of life. These symbiotic microbial communities have profoundly influenced host development, immune regulation, behavior, and pathogen defense since the earliest stages of multicellular life. Through co-evolution, hosts and their microbiomes have formed intricate symbioses that enhance habitat colonization, nutrient acquisition, and behavioral interactions, resulting in long-lasting relationships. To understand how microbiomes form these highly specialized interactions with their hosts and their functional consequences, it is crucial to investigate host-microbe interactions starting at initial colonization. During embryonic development, primary colonizers adjust environmental conditions, paving the way for the establishment of the microbiota. Microbes are transmitted through vertical routes (e.g., eggs, pregnancy, birth, breastfeeding) and horizontal routes from the environment. Vertical transmission, closely linked to reproductive success, ensures the inheritance of beneficial microbes across generations. However, in traditional female pregnancy systems, these vertical transmission pathways are intermingled within the maternal body, making it a challenge to disentangle sex-specific contributions. This limitation underscores the need to move beyond mammalian systems for advancing microbial transmission research.

Pregnancy has evolved independently in over 150 lineages. Among these, the Syngnathidae, a family of marine teleost that includes pipefishes and seahorses, is the only group in which male pregnancy evolved alongside protective brood pouches and placenta-like tissues. Based on the hypothesis that this unique system allows disentangling the roles of sex (female egg production) and sex role (male pregnancy), this thesis presents the first experimental assessments of microbial transfer in the sex role-reversed broad-nosed pipefish (*Syngnathus typhle*). This work focuses on pathways and implications of vertical paternal microbial transfer, highlighting the role of male pregnancy in microbial transmission and paving the way for future sex-specific investigations that additionally consider maternal contributions.

Chapter I laid the groundwork for investigating paternal microbial transfer by characterizing the microbiota of females and males across different pregnancy stages and cultivating key bacterial strains. This baseline study provided essential insights into microbiota dynamics in *S. typhle* and enabled the development of methods for manipulation. Additionally, the efficacy of antibiotics in

Summary

reducing the natural microbiota was evaluated as a critical step towards establishing *S. typhle* as a model system for host-microbe interactions. The first manipulation experiment then focused on altering the paternal microbiota through depletion and recolonization with selected bacterial strains. Microbial composition in the brood pouch and juveniles was monitored for up to six weeks after birth, alongside frequent measurements of life-history parameters. Results revealed that both antibiotic treatment and recolonization influenced the microbial composition of the brood pouch and the offspring. Remarkably, evidence was found that recolonization reduced pregnancy duration and improved offspring survival, demonstrating a functional significance of paternal microbial transfer.

Chapter II expanded these findings by incorporating horizontal microbial transmission during late pregnancy as an additional variable. This experiment furthermore introduced fluorescently tagged (GFP and mCherry) bacterial strains with the goal of visualizing and tracking microbial transfer during pregnancy and juvenile early microbial colonization. While detecting the tagged strains proved challenging, this study explored the potential of tracking vertical and horizontal microbial transfer in *S. typhle* using molecular markers. Evidence was found for distinct temporal dynamics of microbial transfer, with vertical transmission dominating during pregnancy and embryonic development and horizontal transmission shaping microbial diversity in juvenile fish after birth. Although the proof-of-concept for fluorescent visualization remains currently under development, this work underscores the complementary roles of these transmission modes and highlights the potential for refining molecular approaches to study microbial transfer.

This dissertation advances our understanding of microbial transfer in a non-mammalian, sex role-reversed system, identifying male pregnancy as a pathway for vertical microbiota transfer. By linking paternal microbiota to offspring development and microbiome composition, and establishing tools to study microbial transfer dynamics, this work sheds new light on host-microbe co-evolution and the implications of microbial inheritance.

ZUSAMMENFASSUNG

Alle lebenden Organismen beherbergen vielfältige mikrobielle Gemeinschaften, deren Interaktionen ein Spektrum von pathogen über kommensalisch bis hin zu mutualistisch umfassen. Während sich die mikrobiologische Forschung früher hauptsächlich auf Pathogene konzentriert hat, rückt zunehmend die zentrale Rolle von nützlichen Mikroben, der sogenannten Mikrobiota, als Grundlage für die Evolution und das Fortbestehen des Lebens in den Mittelpunkt. Diese symbiotischen mikrobiellen Gemeinschaften haben die Entwicklung von Wirten, die Immunregulation, das Verhalten und die Abwehr gegen Pathogene seit den frühesten Stadien des vielzelligen Lebens maßgeblich beeinflusst. Durch Koevolution haben Wirte und ihre Mikrobiome komplexe Symbiosen gebildet, die die Besiedelung von Habitaten, die Nährstoffaufnahme und das Verhalten fördern, was wiederum zu langandauernden Beziehungen führt. Für das Verständnis, wie Mikrobiome diese hochspezialisierten Interaktionen mit ihren Wirten eingehen und welche funktionalen Konsequenzen sich daraus ergeben, ist es entscheidend, Wirt-Mikrobiota-Interaktionen bereits während der initialen Besiedelung zu untersuchen. Während der embryonalen Entwicklung passen primäre Besiedler die Umgebung an und schaffen so die Grundlage für die Etablierung der Mikrobiota. Hierfür werden die Mikroben über vertikale Übertragungswege (z. B. Eier, Schwangerschaft, Geburt, Stillen) sowie horizontal aus der Umwelt übertragen. Die vertikale Übertragung, welche eng mit dem Fortpflanzungserfolg verknüpft ist, stellt somit die Weitergabe nützlicher Mikroben über Generationen hinweg sicher. In klassischen Systemen mit weiblicher Schwangerschaft sind diese vertikalen Übertragungswege jedoch alle im mütterlichen Körper verflochten, weshalb es deutlich schwieriger ist, geschlechtsspezifische Einflüsse zu identifizieren. Diese Einschränkung verdeutlicht die Notwendigkeit, die Forschung zur mikrobiellen Übertragung weiter voranzutreiben und über die Säugetiersysteme hinauszugehen.

Schwangerschaft hat sich in über 150 Abstammungslinien unabhängig entwickelt. Unter diesen ist jedoch die Familie der Syngnathidae (Seenadeln und Seepferdchen) die einzige Gruppe, bei der sich die männliche Schwangerschaft, zusammen mit schützenden Bruttaschen und plazentaähnlichem Gewebe, entwickelt hat. Basierend auf der Hypothese, dass dieses einzigartige System die Entwirrung der Rollen von Geschlecht (weibliche Eiproduktion) und Geschlechterrolle (männliche Schwangerschaft) ermöglicht, präsentiert diese Dissertation die ersten experimentellen Untersuchungen zur mikrobiellen Übertragung bei der geschlechterrollenumgekehrten Grasnadel (*Syngnathus typhle*). Der Fokus liegt hierbei auf den Routen der vertikalen mikrobiellen Übertragung durch die Väter. Dadurch soll speziell die

Zusammenfassung

Bedeutung der männlichen Schwangerschaft in der mikrobiellen Übertragung hervorgehoben werden, um die Grundlage für zukünftige geschlechtsspezifische Untersuchungen zu legen, in denen auch mütterliche Beiträge berücksichtigt werden.

Kapitel I bildet die Grundlage für die Untersuchung des Mikrobiotatransfers durch die Väter, indem die Mikrobiota von Weibchen und Männchen in verschiedenen Schwangerschaftsstadien charakterisiert und anschließend ausgewählte Bakterien kultiviert wurden. Diese Studie hat wichtige Einblicke in die Dynamik der Mikrobiota bei *S. typhle* geliefert und ermöglichte zudem die Entwicklung von Manipulationsmethoden. Darüber hinaus wurde die Wirksamkeit von Antibiotika zur Reduktion der natürlichen Mikrobiota untersucht und stellt damit einen entscheidenden Schritt zur Etablierung von *S. typhle* als Modellsystem für Wirt-Mikrobiota-Interaktionen dar. Im ersten Manipulationsexperiment wurde die mikrobielle Zusammensetzung in der Bruttasche durch Verminderung und anschließender Wiederbesiedlung mit ausgewählten Bakterienstämmen verändert. Die Mikrobiota in der Bruttasche und bei den Jungtieren wurde über einen Zeitraum von sechs Wochen nach der Geburt analysiert. Zusätzlich wurden regelmäßig Messungen von Lebenszyklusparametern bei den Nachkommen vorgenommen. Die Ergebnisse haben erste Hinweise geliefert, die darauf hindeuten, dass sowohl die antibiotische Behandlung als auch die Wiederbesiedlung die Mikrobiota in der Bruttasche und bei den Nachkommen beeinflusst haben. Bemerkenswerterweise führte die Wiederbesiedlung zu einer Verkürzung der Schwangerschaftsdauer aber auch zu einer Verbesserung des Überlebens der Nachkommen, was auf eine funktionelle Signifikanz der mikrobiellen Übertragung durch die Väter hindeutet.

Kapitel II integriert die horizontale mikrobielle Übertragung aus der Umwelt während der späten Schwangerschaft als zusätzliche Variable. In diesem Experiment wurden fluoreszenzmarkierte (GFP und mCherry) Bakterienstämme genutzt, um den Mikrobiotatransfer während der Schwangerschaft und die mikrobiologische Besiedlung bei Jungtieren zu visualisieren und nachzuverfolgen. Es konnte gezeigt werden, dass die mikrobielle Übertragung zeitlich unterschiedlich verläuft: Während der Schwangerschaft und embryonalen Entwicklung dominiert die vertikale Übertragung, während die horizontale Übertragung die mikrobielle Diversität der Jungtiere nach ihrer Geburt verstärkt prägt. Der Nachweis der fluoreszenzmarkierten Bakterien stellte sich jedoch als kompliziert heraus und muss daher noch weiterentwickelt werden. Dennoch konnte dieses Experiment die Rollen verschiedener mikrobieller Übertragungsrouten hervorheben und wertvolle Erkenntnisse liefern, mit denen molekulare Methoden zur Untersuchung mikrobieller Übertragung angepasst und präzisiert werden können.

Zusammenfassung

Diese Dissertation vertieft unser Verständnis in Bezug auf die Übertragung der Mikrobiota in einem Nicht-Säugetier-System mit umgekehrter Geschlechterrolle, indem die männliche Schwangerschaft als ein Weg für den vertikalen Mikrobiotatransfer identifiziert wird. Diese Arbeit beleuchtet, wie die väterlichen Mikroben die Entwicklung und Zusammensetzung des Mikrobioms der Nachkommen beeinflussen und präsentiert neue Ansätze zur Untersuchung des Mikrobiotatransfers. Damit liefert sie wichtige Einblicke in die Koevolution von Wirt und Mikrobiota sowie in die Bedeutung mikrobieller Vererbung jenseits klassischer Säugetiermodelle.

1 INTRODUCTION

1.1 We are not alone: Beneficial microbes and metaorganisms

Interactions between microorganisms and their hosts have profoundly formed life on Earth, driving the evolution of complex organisms and ecosystems (Sieber et al. 2021). These intricate relationships, ranging from beneficial to harmful, have shaped ecological dynamics and evolutionary pathways for billions of years (Wiesmann et al. 2023; Bontemps et al. 2024). Remarkably, every living organism is home to an immense and diverse microbial community – including bacteria, viruses, fungi, archaea, and eukaryotes (Rosenberg and Zilber-Rosenberg 2016). These microbes engage in highly dynamic and multifaceted interactions, acting as pathogens, neutral passengers, or indispensable allies, highlighting the extraordinary complexity and significance of host-microbe relationships (Bosch and Hadfield 2020). For decades, research predominantly focused on pathogenic, or harmful, microorganisms, largely overlooking the non-pathogenic, mutualistic members of the microbial community (Lozupone et al. 2012; Soni et al. 2024). This focus has heavily influenced public perception, where microbes are often viewed as dangerous threats to health. Public awareness is often shaped by media coverage reporting outbreaks of flesh-eating *Vibrio* bacteria in lakes or oceans (Grimes 2020), microbial food-borne illnesses (Ünüvar 2018) and global pandemics (Ciotti et al. 2020), reinforcing a predominantly negative image. The COVID-19 pandemic, in particular, highlighted the general fear, uncertainty and risk perceptions associated with microbial agents, when entire societies mobilized to control the spread of a virus worldwide (Carvalho and Lima 2022). These perceptions, while grounded in legitimate health concerns, often overshadow the equally significant truth that many microorganisms are crucial for health.

Beneficial microbes, collectively known as the microbiota, are nearly equal in number to human cells (Sender et al. 2016) and shape their host's development, digestion, immune regulation, behavior, and pathogen protection (Esser et al. 2019; Zheng et al. 2020). The broader understanding of these positive relationships has emerged over the years, challenging the singularly negative view of microbes and revealing their fundamental role in the evolution of life (Karl 2016). Furthermore, recognizing the importance of such symbiotic relationships has restructured our understanding of individual organisms, suggesting that we are not alone: all multicellular life forms should be considered as metaorganisms as they describe entities comprised of the host and its associated microbial communities (Rohwer et al. 2002; Bosch and McFall-Ngai 2011; Rees et al. 2018). The term “metaorganisms” was originally coined by Bell

Introduction

(1998) to describe multicellular hosts co-evolving with their microbiota, but now broadly refers to any host with its distinct microbial community (Biagi et al. 2012).

Advances in our understanding of how microbial communities impact host health and disease have accelerated significantly in recent decades. This strong emergence in microbiome research has largely been facilitated by the development and increased accessibility of various analytical methodologies, including, but not limited to, next-generation sequencing (NGS), metagenomics and single-cell analyses encompassing genomics, transcriptomics, and spatial resolution techniques (Wei et al. 2021). Microbiomes are present in various tissues and organs. The gastrointestinal tract of vertebrates, however, is the most studied example of how metaorganisms function and represents the starting point for many studies in the emerging field of microbiota research. Within this dynamic microbial ecosystem, the gut microbiota harbors an extensive variety of complex host-microbial interactions that typically operate in homeostasis (Schaedler et al. 1965; Mazmanian and Lee 2014; Wang et al. 2018). Studies have demonstrated that these interactions significantly influence evolutionary processes, marking a shift from microbes serving solely as prey to becoming providers of essential digestible molecules within the host's digestive system. This fundamental change was driven by the evolution of one-way, tube-shaped digestive tracts (McFall-Ngai et al. 2013). Recent research has also established that the gut microbiota functions as a vital organ for host-microbe interactions, engaging in communication with neural, immune, and metabolic pathways (Afzaal et al. 2022).

Such perspectives – viewing the microbiota as a singularity, a collective, or even a supplementary organ – underscore the vast unknowns surrounding its functional roles and versatility. Exploring these aspects is critical to understanding the full extent of its influence on host health and development. These foundational discoveries have expanded the scope of research to include other tissues and species, emphasizing the intricate, lifelong connections between hosts and their microbiota. The exploration of these interactions continues to reveal the diverse ways in which microbial communities contribute to both the health and disease of their hosts, underscoring the dynamic nature of these associations.

1.2 Host-Microbe interactions: A lifelong partnership

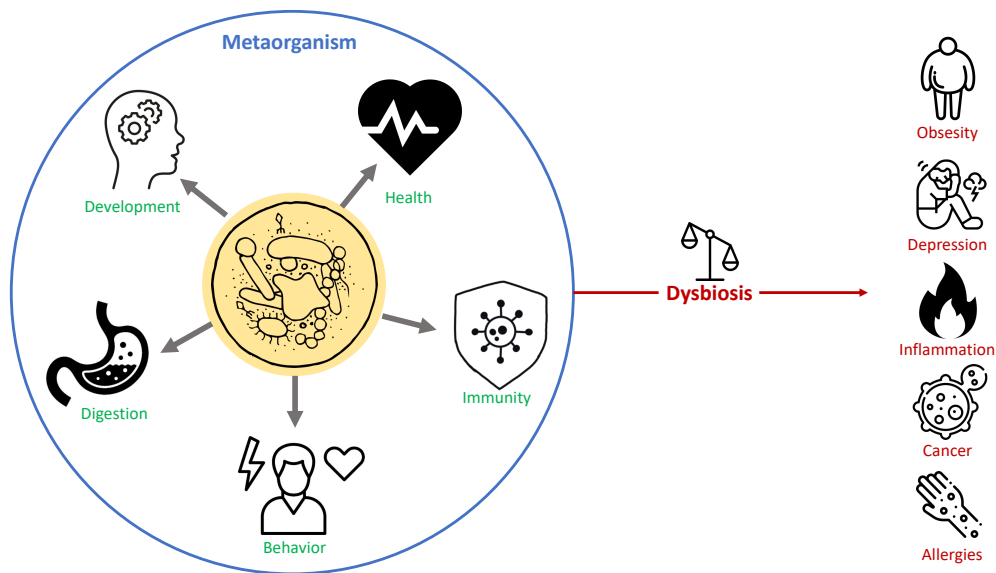


Figure 1: Functional interactions in metaorganisms and consequences of imbalances in homeostatic host-microbiome interactions. Adapted from Esser et al. 2019 (*J. Innate. Immun.*).

Building on the understanding of the importance of host-microbe interactions, it becomes obvious that these relationships are neither static nor singular. The interaction of the specific microbial community with their host is a complex and dynamic relationship that forms and evolves throughout life (Popkes and Valenzano 2020; Kalbermatter et al. 2021). Research on microbiota, particularly with an emphasis on the intestinal microbiota, has demonstrated that the coevolution of hosts and their associated microbial communities has given rise to intricate symbiotic relationships. These relationships have facilitated habitat colonization, influenced behavioral interactions, and improved nutrient acquisition (Lewin-Epstein and Hadany 2020; Ganesan et al. 2022) (Figure 1). Additionally, commensal microbes contribute significantly to the regulation of various metabolic pathways and have trophic effects on intestinal epithelial tissues (Montalto et al. 2009). The microbiome is fundamentally intertwined with processes traditionally associated with defining human identity. These include the adaptive immune system of vertebrates, which distinguishes between self and non-self with remarkable molecular accuracy, the neural mechanisms that shape personality and cognitive function, and the unique genetic blueprint of each individual, which directs distinct phenotypic characteristics (Rees et al. 2018). But this system is not invincible: studies have shown that disruptions within these microbial communities, or dysbiosis, can be associated with a range of health conditions, including inflammation, cancer, obesity, depression, allergies, and neurological disorders (Shu et al. 2019; Cryan et al. 2020; Hou

Introduction

et al. 2022) (Figure 1). Such imbalances may arise from failures in the host's control mechanisms over the microbial community (Ojetti et al. 2009; Esser et al. 2019).

It is evident that the continuous interaction between the microbiota and the host's immune system is of great importance. In vertebrates, from pregnancy to adulthood, the microbiota plays a crucial role in providing beneficial bioactive metabolites and micronutrients like lipids, polysaccharides, and vitamins (Patterson et al. 2014). During neonatal development, microbial metabolites are essential for the onset of the immune system and later for maintaining its maturation (Kalbermatter et al. 2021). The vertebrate's maturing immune system, in turn, not only regulates the presence, growth, and composition of host-associated microorganisms but also modulates their abundance and diversity, underscoring the complex interplay between microbial symbionts competing within the host milieu and the host's regulatory responses (Hooper and Macpherson 2012; Thaïss et al. 2016; Foster et al. 2017). This close relationship dynamically changes throughout life (Figure 2). External factors as well as lifestyle choices cause short- and long-term fluctuations in microbiota composition, impacting the host's immune function (Lloyd and Marsland 2017). Thus, these intricate, multifaceted interactions are thought to have driven the coevolution of host immune mechanisms and the microbiota, influenced by a multitude of environmental and lifestyle factors, with significant implications for health and disease (McFall-Ngai 2007; Lee and Mazmanian 2010).

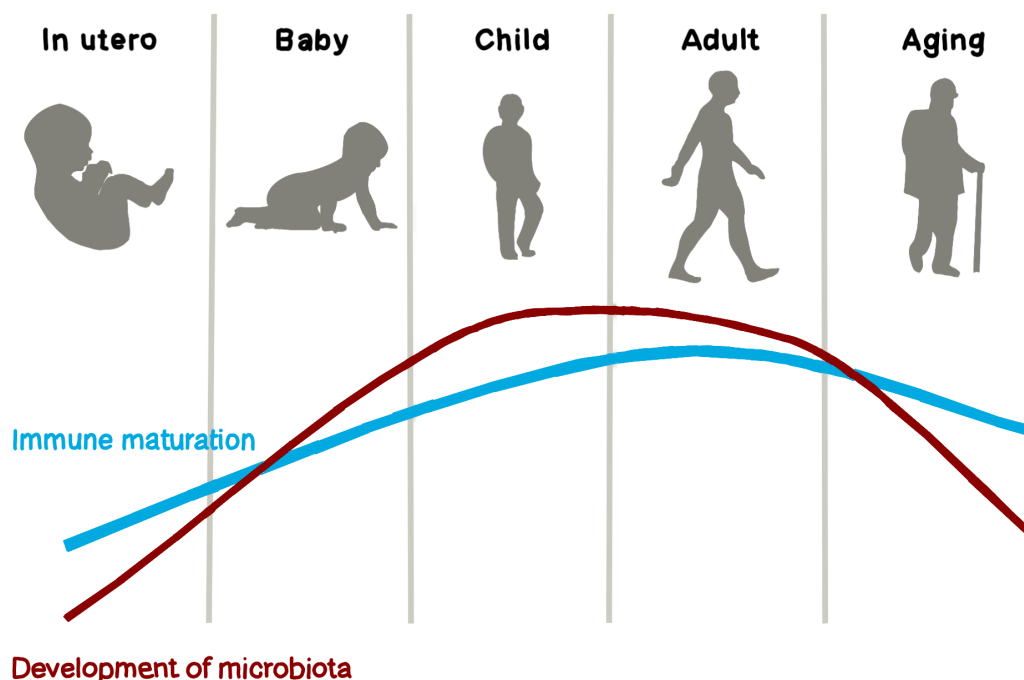


Figure 2: Interaction of immune system and microbiota over the course of life. Adapted from Llyod and Marsland 2017 (Immunity)

As most infections begin at mucosal interfaces, epithelial tissues play a key role in recognizing antigens and maintaining immune homeostasis by distinguishing between commensal and pathogenic microbes (McGhee et al. 1992; MacDonald 2003). This process relies on a dynamic mucosal immune system comprising innate and adaptive immune cells, which offer robust protection against pathogens while enabling selective responses to beneficial microorganisms. Simultaneously, commensal microbes have evolved mechanisms to suppress pathogenic traits, facilitating coexistence on mucosal surfaces (Gomez et al. 2013). Together, the host and its microbiota form a co-adaptive regulatory system, leveraging the microbiota's beneficial traits without the host needing to evolve them independently (Gill et al. 2006). While much is known about the significance of these interactions throughout life, understanding the initial establishment of microbial communities is equally critical. Through which mechanisms and pathways does early microbial colonization occur, setting the foundation for these lifelong interactions?

1.3 Transfer routes and early microbial colonization

To understand how microbiomes establish highly specialized interactions with their hosts and their functional consequences, we need to examine microbiome-host interactions beginning at initial colonization. Among vertebrates, some lizard and fish species exhibit a distinct *in ovo* microbiota, hypothesized to facilitate vertical transmission of symbionts (Nyholm 2020). Vertical transmission plays a pivotal role in establishing the microbiota across generations, ensuring the transfer of beneficial symbionts essential for offspring development and evolutionary stability (Douglas and Werren 2016). In mammals, for instance, the maternal microbiome contributes bioactive metabolites, lipids, and vitamins to the embryo during pregnancy, shaping the neonatal microbiota and preparing the immune system for lifelong function (Pradeu 2011; Patterson et al. 2014). Vertical transmission is thus directly tied to reproductive success (Ruiz-Guzmán et al. 2016). Horizontal transmission complements vertical transfer by introducing environmental microbes after birth, further enhancing microbial diversity of the skin, respiratory tract, and gut microbiomes in neonates (Leftwich et al. 2020). Together, these transmission routes contribute distinct microbial communities, which are essential for the early microbiome and its role in shaping host environments (Figure 3C).

Throughout early development, microbial colonizers play a critical role in shaping the host environment. They adjust conditions such as pH, compete for nutrients, and produce antimicrobial compounds, creating niches for beneficial populations (Senn et al. 2020). These early interactions also "educate" the immune system, teaching it to recognize and appropriately respond to various

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antigens, which is fundamental for long-term immune health (Zeissig and Blumberg 2014). Disruptions to early colonization, such as antibiotic exposure or reduced microbial diversity, can lead to immune dysregulation and increase the risk of chronic conditions, including allergies and autoimmune diseases (Keeney et al. 2014; Arrieta et al. 2015). The microbiota can be highly species- and tissue-specific (Larsen et al. 2013), forming long-lasting interactions with their hosts. Understanding the timing, mechanisms, and functional consequences of these interactions remains crucial for advancing our knowledge of how microbial influence shapes host biology. However, much of this understanding is derived from mammalian models, which present significant limitations for studying vertical transmission. In mammals, routes of contribution such as egg production, pregnancy, and parental care are intermingled within the maternal body, making it challenging to disentangle sex-specific parental roles. Historically, this limitation was often overlooked because microbial transfer was assumed to occur exclusively from mother to egg, with paternal contributions considered negligible (Sharma 2019). Yet, recent studies challenge this paradigm, suggesting the existence of a male gut-germline axis, which implies that paternal health could impact offspring health and pregnancy outcomes (Argaw-Denboba et al. 2024).

Given these constraints, it is clear that moving beyond well-studied classic female pregnancy systems is crucial to fully understand how mothers and fathers independently contribute to microbial transfer and early colonization. Systems where sex (egg production) and parental care (pregnancy) can be disentangled would provide the framework for such critical insights needed to address these gaps.

1.4 Dads matter: Sex role-reversed pipefish as model organisms

In the history of vertebrate evolution, viviparity – the development of the embryo inside the parent's body – has evolved independently in over 150 lineages, driven by the selection for increased parental investment (Blackburn 2015). However, members of the Syngnathidae family, a group of marine teleost fishes, are unique in exhibiting male pregnancy, which has driven the evolution of specialized brooding tissues and structures, analogous to those of mammalian female pregnancy systems (Whittington and Friesen 2020). This family displays diverse pregnancy modes with varying levels of parental investment. In the *Nerophinae* subfamily, eggs are attached externally to the male's trunk, leaving them fully exposed to the environment. At the opposite end of the spectrum, seahorses (*Hippocampinae*) have developed an impermeable, tightly sealed brood pouch with placenta-like structures (Figure 3A). Positioned between these extremes is my research organism, the broad-nosed pipefish *Syngnathus typhle* (Figure 4A). In this species, females deposit eggs into the male's inverted semi-sealed brood pouch, where fertilization occurs (Figure 3A, 4C), and the developing embryos are protected and nourished by the placenta-like tissue until birth (Stölting and Wilson 2007). As the embryos develop, the pouch becomes more permeable (Figure 3B), offering a unique opportunity to investigate environmental influences during late pregnancy. This reproductive strategy presents a promising perspective to disentangle the effects of sex (egg production) and parental investment (pregnancy) on offspring microbial composition, making *S. typhle* an excellent system for studying pregnancy and the evolution of sex roles (e.g. Keller and Roth 2020; Pappert et al. 2024).

I hypothesize that this system allows paternal transfer during pregnancy to be studied independently from maternal transovarial transfer, offering a unique opportunity to disentangle sex-specific contributions to microbial transfer. Unlike mammalian systems, where pregnancy is exclusively maternal and often confounded by shared physiology or lactation, the pipefish's male pregnancy simplifies these dynamics, allowing direct study of paternal microbial transfer during pregnancy. Furthermore, the semi-sealed brood pouch facilitates manipulations of microbial transfer, enabling controlled experimental designs not feasible in systems with internal gestation. The temporal separation of horizontal transmission adds another layer of experimental control, providing a rare opportunity to study how inherited (vertical) and environmental (horizontal) microbes are sequentially integrated into host-associated microbiomes. These unique features make the pipefish a powerful system for exploring microbial transmission dynamics and their role in host-microbe co-evolution across generations.

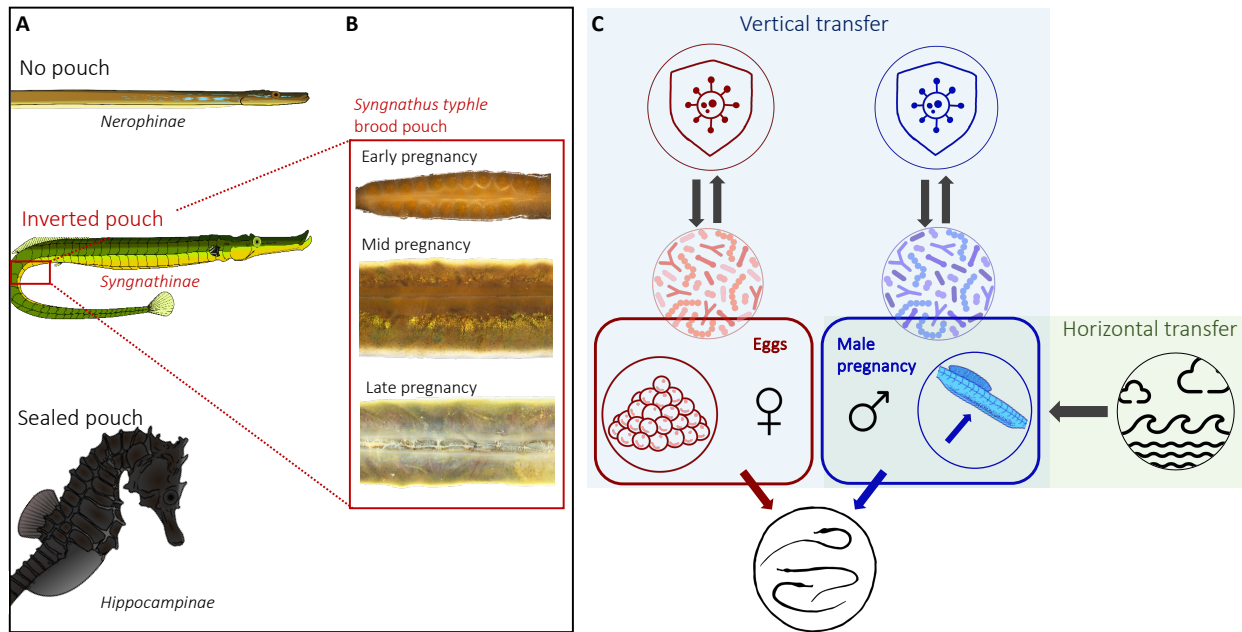


Figure 3: (A) The gradient of parental investment within the Syngnathidae family. (B) Anatomic changes of the brood pouch of *Syngnathus typhle* during pregnancy. (C) Pipefish male pregnancy as a method to study the influence of vertical sex-specific and horizontal contributions to early microbial colonization. Image credits: Jamie Parker, Ralf Schneider, Rebekka Leßke, Flaticon.com

1.5 From nature to lab: Establishing a new model organism for host-microbe interactions

The selection of suitable model organisms is fundamental to advancing our understanding of host-microbe interactions. While classical models on land and in the water such as mice (*Mus musculus*), roundworms (*Caenorhabditis elegans*), zebrafish (*Danio rerio*), and *Hydra* have significantly deepened our knowledge of microbiome functions (Bosch 2012; Zhang et al. 2017; Hugenholtz and de Vos 2018; Xia et al. 2022), novel systems are needed to explore less-understood biological phenomena. The broad-nosed pipefish (*Syngnathus typhle*) offers unique potential as a model organism due to its male pregnancy, which separates sex (egg production) and sex role (pregnancy). Furthermore, due to the semi-sealed anatomy of the brood pouch, this system allows a controlled manipulation of microbial transfer routes to assess the function of the microbiota.

Building on previous observational work by our group on pipefish microbiota (Beemelmans et al. 2019; Tanger et al. 2024), a focus of my thesis has been the development of novel methods to establish *S. typhle* as a model organism for experiment-based microbiota research. While our

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group has already successfully developed protocols for immunological studies in pipefish (Keller and Roth 2020; Parker et al. 2021), adapting the system for microbiota research required new approaches. These included constructing S1 and S2-compliant fish tanks to maintain biological safety standards (Figure 4B), designing experimental conditions to maintain fish health without water circulation, and exploring methods to deplete the natural microbiota. These efforts were challenging, as the full impact of environmental microbes from the marine environment on health and development remains unclear. By overcoming these challenges, our novel manipulation experiments provide valuable insights into sex-specific microbial transfer dynamics and early microbial colonization. *Syngnathus typhle* offers a unique opportunity to explore host-microbe interactions in a non-model organism with distinctive reproductive biology. Its establishment as a model for microbiota research is thus a significant step toward unraveling host-microbe interactions during pregnancy and early life. Ultimately, studying such a system does challenge established paradigms and inspires new research directions, offering innovative approaches to investigating microbiota in diverse reproductive and ecological contexts.

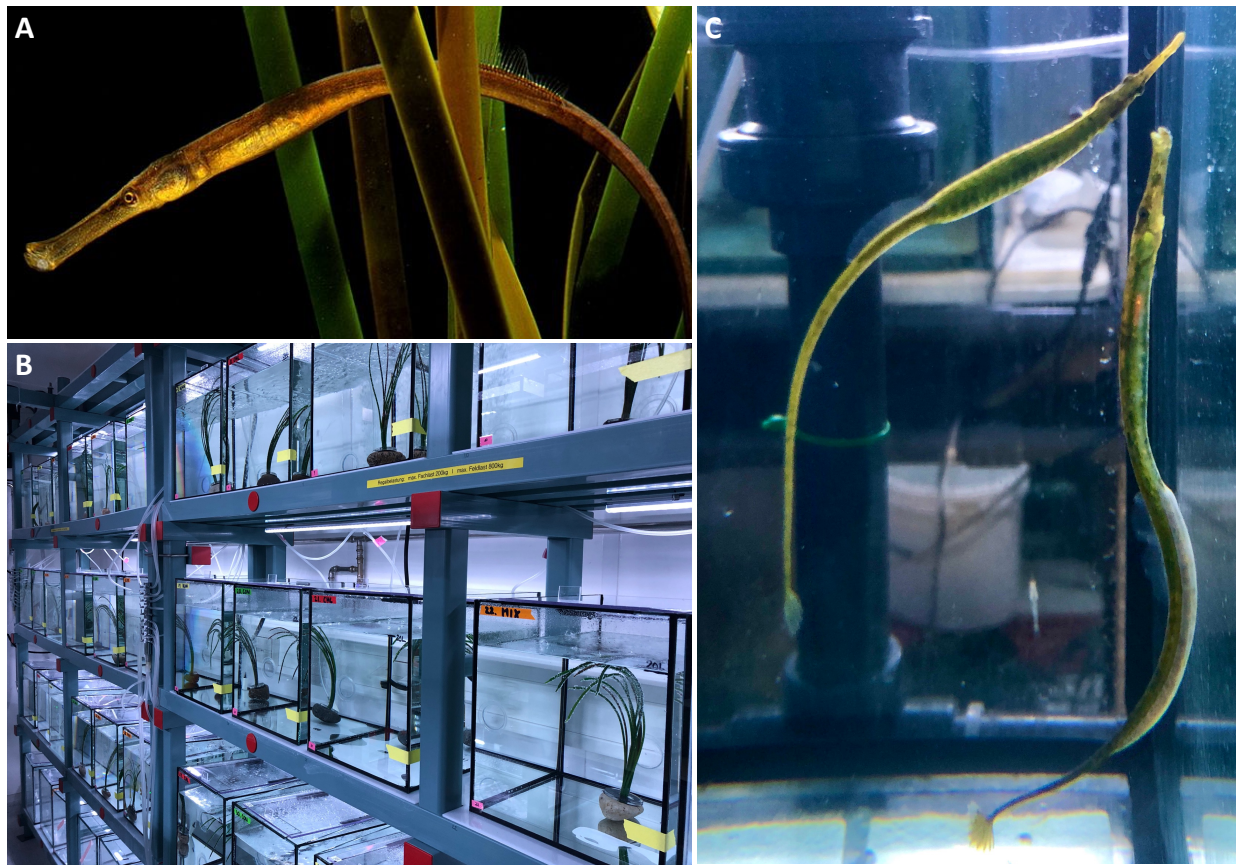


Figure 4: (A) The broad-nosed pipefish *Syngnathus typhle* hides in between seagrass (Photo: Uli Kunz), (B) S1-level research facilities for microbiota manipulation experiments at GEOMAR in Kiel, (C) Pipefish after mating: the male (right) remains in a distinct S-position for fertilizing the eggs.

2 THESIS OUTLINE

The study of host-microbe interactions has evolved into a significant interdisciplinary research area, emphasizing that the microbiota plays a critical role in the development and health of its host. To advance the current understanding of these interactions, particularly the sex-specific microbial transmission routes and the overall function of the microbiota during pregnancy and early microbial colonization, research needs to move beyond the classic mammalian female pregnancy model. The unique male pregnancy in the broad-nosed pipefish (*Syngnathus typhle*) offers a promising opportunity to investigate diverse aspects of microbial transmission and host-microbe interactions. In this thesis, the focus is placed on unraveling the mechanisms and significance of paternal microbial contributions during male pregnancy.

The central hypothesis is that paternal microbial transmission through male pregnancy represents a distinct pathway of vertical microbial transfer, which plays a crucial role in shaping offspring's early microbial colonization – independent from other transmission routes.

To test this hypothesis, the goals were to **(1)** establish *S. typhle* as a model for microbial transfer studies by developing experimental tools and validating their relevance for investigating host-microbe interactions; **(2)** to characterize the microbiota associated with the male brood pouch during pregnancy, female eggs, and the environment; **(3)** to experimentally disentangle the contributions of paternal (vertical) and environmental (horizontal) microbial transfer to offspring microbiota; and **(4)** to gain a basic understanding of the functional roles of paternal microbial transmission in shaping offspring development.

By examining the paternal component of microbial transfer, this thesis offers initial insights into vertical transmission and establishes a foundation for future studies that will incorporate maternal contributions and their potential combined effects.

2.1 Chapter I – Shaping tiny worlds: Paternal microbiota manipulation influences offspring microbial colonization and development in a sex role-reversed pipefish

Chapter I of this thesis combines three separate studies: two experiments that lead to the establishment of the pipefish *Syngnathus typhle* as a model organism for studying microbial transfer and a first manipulation approach to assess the influence of paternal microbiota manipulation on juvenile development and early microbial colonization. The sex-specific microbiota of the male brood pouch was characterized at different stages of pregnancy, potential candidate strains for vertical transmission were determined and different antibiotics were tested as means to deplete the natural microbiota. Using 16S rRNA amplicon sequencing data, this chapter shed light on the dynamics of paternal provisioning and the consequences of dysbiosis in the father's sex-specific tissues.

The first hypothesis of the manipulation experiment was that paternal microbiota manipulation will influence offspring microbiota and development. The second hypothesis was that microbe-host interactions are highly specific, therefore only paternal-specific bacterial strains would be able to colonize the paternal brood pouch tissue to be passed on to the next generation.

The results suggested that paternal manipulation does affect microbial composition of the brood pouch tissue and offspring microbiota development. Particularly the recolonization treatment reduced the length of pregnancy while boosting offspring survival. This study therefore provided first experiment-based evidence for vertical (paternal) microbial transfer through male pregnancy.

2.2 Chapter II – Visualizing temporal dynamics of vertical and horizontal microbial transfer during pipefish male pregnancy – a pilot study

Chapter II of this thesis presents a pilot study investigating the potential of fluorescently tagged bacterial strains as a method to track microbial transfer dynamics during male pregnancy. Two bacterial strains, naturally associated with the pipefish microbiota, were labeled with GFP or mCherry and introduced to the male brood pouch before mating (to study vertical paternal transfer) or to the water during late pregnancy (to study horizontal transfer). Microbiota sampling for 16S rRNA amplicon sequencing was conducted at key time points: during early and late pregnancy, and seven days post-release, to monitor early microbial colonization in the offspring. The hypothesis was that vertical and horizontal transmission routes distinctly influence the brood pouch and offspring microbiota, with tissue-specific impacts: vertical transfer predominantly shaping the juvenile whole-body microbiome, while horizontal transfer primarily affects the gut microbiome. Additionally, it was hypothesized that the fluorescently tagged bacteria would be detectable in both adult and juvenile tissues, serving as a proof of concept for tracking microbial transfer and colonization via fluorescent markers.

The results revealed that even subtle natural microbial shifts could influence the microbiota, highlighting the complexity of microbiota dynamics. While detecting the two experimentally introduced strains remained a challenge, the study identified critical methodological aspects requiring refinement, such as protocols for tagging and detecting the target strains. As a pilot study, it provided valuable insights into optimizing these techniques and lays the groundwork for further investigations into microbial transfer processes using molecular biology techniques.

3 CHAPTER I

Shaping tiny worlds: Paternal microbiota manipulation influences offspring microbial colonization and development in a sex role-reversed pipefish

Kim-Sara Wagner¹, Frédéric Salasc^{1,2}, Silke-Mareike Marten¹ and Olivia Roth¹

1 – Marine Evolutionary Biology, Zoological Institute, Kiel University, 24118 Kiel, Germany

2 – present address: OSU Institut Pytheas, Aix-Marseille Université, 13009 Marseille, France

ABSTRACT

Microbes are acquired through vertical and environmental horizontal transmission. Vertical transmission is directly linked to reproductive success and entails early transmission, facilitating coexistence of host and microbes over generations. The multiple potentially interacting routes of vertical transmission are challenging to be disentangled in conventional sex-role species, as they are mostly intermingled on the maternal side, i.e., through egg production, pregnancy, birth and postnatal care. The evolution of male pregnancy in syngnathids (pipefishes and seahorses) offers an opportunity to separate vertical microbial provisioning through the egg (maternal) from provisioning through pregnancy (paternal). We experimentally evaluated the existence and role of paternal microbiota provisioning through male pregnancy on offspring development and microbial colonization. Male pipefish were exposed to antibiotics, and subsequently recolonized with bacteria of paternal, maternal, and environmental origin (spike treatment). After pregnancy, the microbiome (16S rRNA) of developing offspring was repeatedly ribotyped. Paternal antibiotic and spike treatments influenced the microbial composition of the brood pouch and offspring microbiome development. Paternal spike treatment gave offspring a kickstart in life by reducing pregnancy duration and inducing offspring survival. Expanding on how distinct vertical microbial transmission routes shape offspring microbiome will foster our understanding of the holobiont function in health and disease.

INTRODUCTION

The interplay of the host with its microbiome can be adaptive; facilitating long-lasting interactions that shape host development, digestion, immune regulation, behavior, and pathogen protection (He et al. 2020; Donald and Finlay 2023; Motta and Moran 2024). To understand the interactions of the host with its microbiome and their functional consequences on host development, we need to follow the microbes beginning at the initiation of their colonization. Vertical transmission fosters the initial microbial colonization as early as during egg development (Nyholm 2020), and can continue through the transfer in intimate parent-offspring interactions, such as pregnancy and postnatal parental care (Wosnick et al. 2022; Bogaert et al. 2023; Vitikainen et al. 2023). Vertical transmission routes are manifold and mostly bound to the maternal side impeding the disentangling of their roles in offspring microbial colonization and development. Therefore, the intertwining of egg production and pregnancy within the female body confounds the investigation of vertical transfer (Tanger et al. 2024), leaving us behind with open questions about the significance of the specific microbes transferred during egg production, pregnancy or postnatal parental care.

Throughout vertebrate evolution, viviparity – the development of the embryo inside the parent's body – has evolved independently in over 150 lineages (Blackburn 2015). While in almost all cases the female is the pregnant sex, the syngnathids, a group of teleost fishes, represent the unique evolution of male pregnancy (Whittington and Friesen 2020). In the broad-nosed pipefish, *Syngnathus typhle*, the female deposits her eggs into the semi-enclosed brood pouch of the male where they are fertilized, protected, and nourished until the male gives birth (Stölting and Wilson 2007). This characteristic reproductive strategy provides the opportunity to disentangle the typically intermingled vertical microbial transmission through eggs and pregnancy: Both, transovarial microbial transfer by mothers and transfer during pregnancy by fathers may be equally expected (Supplementary Figure 1)(Tanger et al. 2024). In contrast, horizontal transfer was suggested to occur only when the brood pouch becomes strained and permeable during late pregnancy (Beemelmans et al. 2019; Tanger et al. 2024) allowing to independently study the timing and role of vertical microbial transfer in depth.

The microbiome of *Syngnathus typhle* differs in composition between the female gonads and the male brood pouch (Beemelmans et al. 2019) While the maternal microbiota seems to shape the offspring gut microbiome, paternal microbes rather influence the establishment of the offspring whole-body microbiome (Tanger et al. 2024). We aimed to provide the first experimental

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assessment of paternal microbial transmission through male pregnancy, by manipulating the paternal brood-pouch microbiome and disseminating its impacts on offspring microbiome and health. We depleted the paternal natural brood-pouch microbiome with antibiotics, and subsequently recolonized the brood pouch with selected natural bacterial isolates before mating males with untreated females. We hypothesized that this paternal microbial manipulation would influence offspring microbiome establishment and offspring development. Given the intricate interplay of father and offspring during pregnancy, we expected paternal-specific microbes to successfully colonize the male tissue and be transferred to offspring. This study enhances the functional understanding of host-microbiota interactions in pregnancy and provides insights into the transmission and establishment of microbiota across generations.

MATERIALS & METHODS

To ultimately assess the transfer of microbes through paternal pregnancy, we conducted three experiments: **(1)** Cultivation and characterization of sex-specific microbiota, **(2)** effectiveness of antibiotics for depleting the natural pipefish brood-pouch microbiome, and **(3)** manipulating paternal sex-specific microbes to unravel their impact on the offspring. In all three experiments, we sequenced the 16S rRNA gene (Bouchet et al. 2008) with Sanger sequencing (experiment 1) and with Illumina MiSeq Amplicon (V3 / V4 region) (experiment 2 & 3). Data processing, visualization and statistical testing were performed in R Studio (R Core Team 2024).

Fish catching and rearing

Broad-nosed pipefish (*Syngnathus typhle*) were caught in the bay of Orth (Fehmarn, Germany) (54°26' N, 11°02'E). At the Helmholtz Centre for Ocean Research Kiel (GEOMAR), they were separated by sex, treated with formalin (1:8000 solution) on three consecutive days to remove potential ectoparasites, and kept in Baltic Sea water flow-through systems until allocation into the experimental facilities (16: 8 day-night; 15 PSU).

(1) Cultivation and characterization of sex-specific microbiota

We aimed to cultivate the sex-specific microbiota of *Syngnathus typhle* from female eggs, male brood pouches throughout pregnancy and environmental samples to compare findings to a cultivation-independent NGS study (Beemelmans et al. 2019). In total, 65 fish were used: 20

females, 17 non-pregnant, 16 early pregnant and 12 late pregnant males. Animals were euthanized with tricaine methane sulfonate (MS-222, 500 mg l⁻¹; Sigma-Aldrich, St. Louis, USA) followed by weight and length measurements. Microbiota samples were isolated from six groups: female gonads (FG), non-pregnant males: placenta-like tissue of the developing brood pouch (NP), early pregnant males: placenta-like tissue (EPP) and whole juveniles (EPJ) as well as late pregnant males: placenta-like tissue (LPP) and whole juveniles (LPJ). Tissue samples were homogenized in autoclaved phosphate buffered saline (PBS) and plated onto Marine Broth (MB, Carl Roth GmbH, Karlsruhe, Germany) or *Vibrio*-selective TCBS agar plates (Carl Roth GmbH). After incubation, bacterial colonies were prepared for amplification of the 16S rRNA (primers 27F and 1492R). The PCR product was purified and Sanger sequenced (Wendling et al. 2017). Raw sequences were aligned and edited with CodonCode Aligner (V8.0.2). Consensus sequences were exported and submitted to NCBI's BLAST for taxonomic identification within the 16S ribosomal RNA sequences (Megablast algorithm) (Altschul et al. 1997). The identified bacteria were compared to the sex-specific indicator species from the cultivation-independent study (Beemelmanns et al. 2019). Differences between stages and tissues were analyzed using PERMANOVA and ANOSIM on a ranked dissimilarity matrix and calculated with the Jaccard similarity coefficient (999 permutations). A multiple correspondence analysis (MCA), followed by pairwise Adonis analyzed microbial clusters of sexes, respectively pregnancy stages.

(2) Effectiveness of antibiotics for natural pipefish microbiota depletion

Microbiota sample collection

We assessed the effectivity of antibiotics in depleting the natural sex-specific microbiomes. We used 64 *Syngnathus typhle* (32 males, 32 females) and exposed them to antibiotics for 312 hours (13 days), consisting of 48 hours treatment, followed by 274 hours maintenance (reduction of antibiotic concentration by 75%). Pipefish were treated with chloramphenicol (treatment: 40 mg/L, maintenance: 10 mg/L), kanamycin (treatment: 10 mg/L, maintenance: 2.5 mg/L), a mixture of both antibiotics with identical concentrations (Munro et al. 1995; Verner-Jeffreys et al. 2003) or they were left untreated (control). Gut and sex-specific microbiota were sampled at 0, 6, 12, 24, 48, 192 and 312 hours. Gut microbiomes were sampled by gastric swabs using a sterile paper point (ANTÆOS® Absorbent Paper Points sterile, size 15/20/25, VDW GmbH). For assessing the reproductive sex-specific microbiomes, the inside of the brood pouch (male) and the surface of the ovipositor (female) were swabbed with sterile cotton swabs (Copan Italia, Thermo Fisher Scientific). Tank water antibiotic concentrations were assessed with agar well diffusion tests on

Epibacterium mobile lawns (prevalent in pipefish, susceptible to both antibiotics applied) (Balouiri et al. 2016). After 48 and 312 four pipefish per treatment were euthanized (MS-222) and sampled for final gastric as well as reproductive sex-specific microbiota swabs. DNA was extracted using DNeasy 96 Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) with enzymatic lysis buffer pre-treatment. The 16S rRNA V3/V4 was sequenced over Illumina Miseq amplicon sequencing at the Institute of Clinical Molecular Biology (IKMB, Kiel, Germany).

Data analysis and statistics

From demultiplexed paired-end fastq files, sequences were merged, quality filtered, and analyzed using QIIME2 version 2019.10 (Caporaso et al. 2010). Chimeras were removed using the “consensus” method within DADA2 (Callahan et al. 2016). Sequences were truncated 50 bases from forward and 70 bases from reverse reads in both runs. For taxa comparison, relative abundances based on all obtained reads were used. The Naïve Bayes classifier was trained on the SILVA138 99% OTUs full-length sequence database (Quast et al. 2013) and sequences from our dataset were assigned to ASVs from SILVA database with similarity on the 99% level. The phyloseq package v.1.46.0 (McMurdie and Holmes 2013) was used for further analyses and data visualization. The data set was subdivided for the reproductive sex-specific (brood pouch and ovipositor) and gut microbiomes. Count data was normalized, log-transformed and prevalence filtered to only preserve ASVs that are present in >20% of the samples. Alpha diversity indices were estimated for Shannon. Indicator species analysis (ISA) using MULTIPATT with 999 permutations was performed using the R package indicpecies (Cáceres and Legendre 2009). PCAs and PCA biplots were calculated and visualized with FactoMineR (Lê et al. 2008). A mixed ANOVA (factors: treatment x time) tested for differences in Shannon diversity between treatment groups over time points. A repeated measured PERMANOVA evaluated the impact of treatments on sex-specific microbiota composition (beta diversity).

(3) Manipulating paternal sex-specific microbiota to unravel impact on the offspring

In the last experiment, the methods established in the two previous experiments were applied: male pipefish were fully reciprocally treated with antibiotics (chloramphenicol) and recolonized with candidate strains for vertical and horizontal transfer (spike treatment) (Figure 1). We aimed to assess the potentially interacting impact of paternal antibiotic and spike treatment, on microbial colonization and offspring development.

Antibiotic treatment of male fish

72 *S. typhle* (36 males, 36 females) were used in this experiment. Half of the male fish were exposed to chloramphenicol in a high dose (40 mg/L) for 48 hours, followed by a low dose (10 mg/L) for 144 hours (6 days) (details: experiment 2). Females and untreated males were kept in separate circulation systems. Water temperatures were slowly increased from 11°C to 17°C. Fish were fed with live *mysid spp.*, mysids for the antibiotic-treated males were treated with chloramphenicol (40 mg/L) for 1 hour prior to feeding.

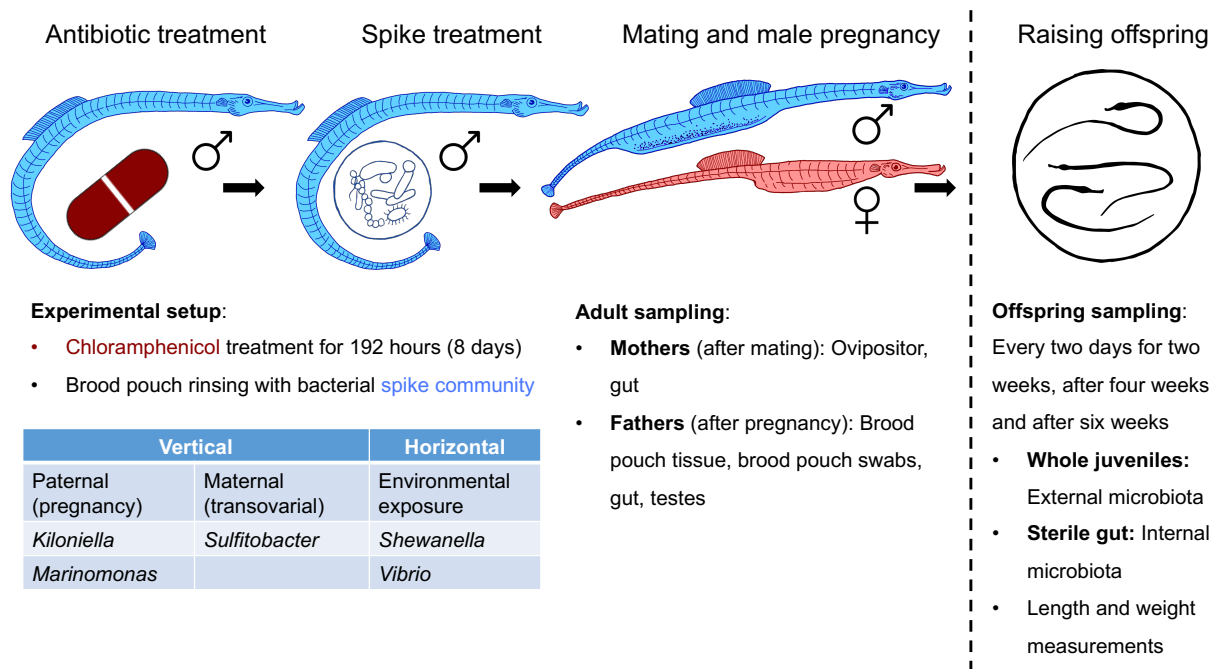


Figure 1: Setup of experiment 3: Manipulating paternal sex-specific microbiota to assess the impact on offspring development and early microbial colonization. Fathers received a treatment (antibiotics and/or spike) and were mated with untreated females. Offspring were kept in separate tanks and were repeatedly sampled for 45 days.

Spike treatment of male pipefish, pregnancy, and microbiota sampling of adults

Prior to mating, half of the male pipefish received a spike-bacteria treatment resulting in four different treatment groups: Control, Antibiotics (antibiotics applied, no spike bacteria added), Spike (no antibiotics applied, spike bacteria added) and AntiSpike (antibiotics and spike treatment). The spike community consisted of five selected bacterial strains cultivated during experiment 1: *Sulfitobacter* (vertical maternal transfer candidates), *Kiloniella* and *Marinomonas* (vertical paternal transfer candidates), *Shewanella* (horizontal transfer candidate) and *Vibrio* (prevalent in *S. typhle*). Overnight cultures were separately diluted to OD_{600nm} 0.5, washed with

phosphate-buffered saline (PBS), mixed equally and 30x concentrated (Supplementary Figure 7). Brood pouches of half of the males were flushed with 50µl spike culture. The other half was flushed with 50µl PBS (sham control). After treatment, pipefish pairs were put into 40 L glass mating tanks. After mating, females were removed and samples taken for gut and ovipositor microbiome ribotyping. After two weeks of pregnancy, the male fish were moved into individual 20 L tanks. Immediately after birth, placenta-like tissue, brood pouch surface, hind gut, and testes were sampled from the male pipefish. Tissues were flash frozen and stored at -80°C.

Juvenile time series

10 juveniles from each batch were moved to 400 ml beakers for daily monitoring of length and weight for 45 days (Figure 1). From the remaining offspring (kept in their birth tanks), microbiota was sampled directly after birth, and then every two days for two weeks, after four weeks (28 days) and six weeks (45 days). Tissues were stored at -80°C. The fish were fed daily with live *Artemia*. At each sampling, two offspring were sacrificed (one for whole-body and one for gut ribotyping), as well as food and seawater of each tank were collected. DNA was extracted and sequenced from all tissue and water samples for 16S rRNA ribotyping as in experiment 2. We only discuss relevant time points (complete dataset: supplementary spreadsheets 6 and 7).

Data analysis

Paternal treatment effects on gestation length and number of offspring were assessed with a one-way ANOVA. Influence of paternal treatment on juvenile survival, weight and size development were tested in an ANCOVA with treatment as fixed factor and time as covariable, followed by pairwise comparisons and contrasts analyses. Processing of raw 16S rRNA amplicon sequences using the QIIME2 workflow followed the procedures described above. Truncation lengths were: run 63: forward 250, reverse 195; run 64: forward 260, reverse 190; run 65: forward 275, reverse 220; run 79: forward 250, reverse 195. Whole microbiome composition was assessed with a PCA biplot and MANOVA, followed by ANOVA. To identify sequences stemming from the recolonization spike culture, QIIME2 sequences were aligned to the sequences of the spike community. Relative abundance of spike community strains within the microbiome were analyzed with a generalized least square model (GLS) using normalized, log-transformed counts. Differences between treatment groups were assessed with a Kruskal-Wallis test followed by a Dunn's post-hoc test.

RESULTS

The sex-specific microbiota: Cultivation vs. Sequencing

We aimed to compare the isolated, cultivated and characterized sex-specific microbiota 16S rRNA Sanger sequences of *Syngnathus typhle* to the NGS dataset from Beemelmans et al. (2019) facilitating the selection of strains suitable for manipulating the paternal sex-specific microbiota (experiment 3). 767 full length 16S rRNA sequences were trimmed (clip ends) and assembled, resulting in 376 consensus sequences (presence-absence matrix, species and genus level). From 79 tissue samples (13 female gonads (FG), 16 non-pregnant (NP), 11 early pregnancy pouch (EPP), 10 early pregnancy juveniles (EPJ), 12 late pregnancy pouch (LPP), 12 late pregnancy juveniles (LPJ)), 92 strains from 38 genera were identified. This corresponds to approximately 2.97% of the total number of 3090 OTUs (97% cut-off) identified in the NGS approach by Beemelmans et al. (2019). Another six bacterial isolates from four genera were isolated from the seawater. Microbiome composition differed between stages (ANOSIM ($p = 0.011$); PERMANOVA ($p = 0.001$)): FG – LPJ: $p = 0.020$; NP – LPJ: $p = 0.015$; EPP – LPJ: $p = 0.015$; EPJ – LPJ: $p = 0.023$ (pairwise Adonis, Supplementary Table 2).

The MCA visualized associations between sexes or developmental stages with the 20 mainly contributing bacterial isolates (Supplementary Figure 3). Dimension 1 drove the microbiota of the female gonads (FG) away from the remaining five developmental stages, whereas dimension 2 segregated the microbiota associated to the late pregnancy stage (LPP, LPJ) from the other stages. Microbiota associated with non-pregnant (NP) males and the early pregnancy stage (EPP, EPJ) showed the strongest overlap. This pattern resembles the pattern identified by (Beemelmans et al. 2019). *Vibrio* predominantly distinguished female eggs from other tissues, while *Pseudoalteromonas* was overrepresented in male brood pouches, particularly towards late pregnancy (Supplementary Figure 3B). In the NGS study, female eggs were represented by *Brevinema* and *Halomonas*, whereas the male brood pouch was characterized by *Marinomonas*, *Kiloniella*, *Ruegeria* and *Pseudoalteromonas* (Beemelmans et al. 2019) (Complete dataset: supplementary spreadsheet 1).

For manipulating the sex-specific microbiota of *S. typhle* through recolonization, we pooled five bacterial strains with a potential role in transgenerational transfer and early microbial colonization (hereafter called “spike community”). As a similar pattern was identified in both studies but, not entirely driven by the same strains, we selected indicator strains from the NGS study

(Beemelmans et al. 2019) that were represented within the 2.97% cultivable part of the sex-specific pipefish microbiota. For paternal vertical transfer, we chose *Kiloniella*, *Marinomonas* and *Pseudoalteromonas*, indicators for the male brood pouch microbiome from non-pregnant to late pregnancy. For maternal vertical transfer, we selected *Sulfitobacter*, a strain present in female gonads and the brood pouch during late pregnancy but absent in non- or early pregnant males. We also included *Shewanella*, ubiquitous in aquatic ecosystems (Lemaire et al. 2020) isolated from water samples and in pipefish highly prevalent *Vibrio sinaloensis*.

Impact of antibiotics on microbial diversity and on target strains for parental transfer

This experiment aimed to investigate how antibiotic treatments influenced sex-specific microbial diversity of *Syngnathus typhle* (males: brood pouch, females: ovipositor surface, pooled samples). 754 samples were submitted to 16S rRNA MiSeq amplicon sequencing. A total of 19,837,424 reads were returned (mean run 47: 28,517 reads/sample, mean run 48: 21,022 reads/sample), along with 10,688 representative sequences (Supplementary Table 1). The duration of the antibiotic treatment (time) as well as time x antibiotic interaction influenced sex-specific microbiota alpha diversity (Shannon) (ANOVA Type III: treatment: $p = 0.629$; time: $p < .001$; time x treatment: $p < .001$). No significant difference in alpha diversity were identified for timepoints T0 ($p = 0.828$), T6 ($p = 0.165$), T12 ($p = 0.82$) and T48 ($p = 0.156$) whereas from T24 ($p = 0.002$) onwards, diversity indices decreased (T192: $p < .001$; T312: $p < .001$). Within the first 48 hours (treatment), alpha diversity fluctuated (Figure 2A). After 8 days (maintenance), diversity was lowest in the treatments Chloramphenicol and Mix. After 13 days Chloramphenicol and Mix microbial diversities increased slightly but remained lower than diversities of Control and Kanamycin treatments, which remained high throughout the experiment (Figure 2A). This outcome corresponds to the well diffusion test results. Plates with water containing chloramphenicol (Chloramphenicol and Mix) showed clear zones of inhibition, whereas they were non-existent for in the other treatment groups (Kanamycin and Control) (Supplementary Figure 3). The alpha diversity of the gut microbiota was accordingly (Mixed ANOVA Type III on Shannon diversity: treatment: $p = 0.276$; time: $p < .001$; time x treatment: $p < .001$) (Supplementary Figure 4), which underlines the effectiveness of our chosen treatment (Complete dataset: supplementary spreadsheet 2).

To select spike community strains for manipulating the paternal sex-specific microbiota (experiment 3), we validated their successfully depletion by the antibiotic treatments. Five bacterial strains (Paternal: *Kiloniella*, *Marinomonas*; Maternal: *Sulfitobacter*; Environmental/ubiquitous: *Shewanella*, *Vibrio*) were depletable by chloramphenicol and could not

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be detected after 192 hours (Figure 2B-F). *Pseudoalteromonas* could not be depleted by chloramphenicol treatment and was therefore excluded from the spike community (Supplementary Figure 5).

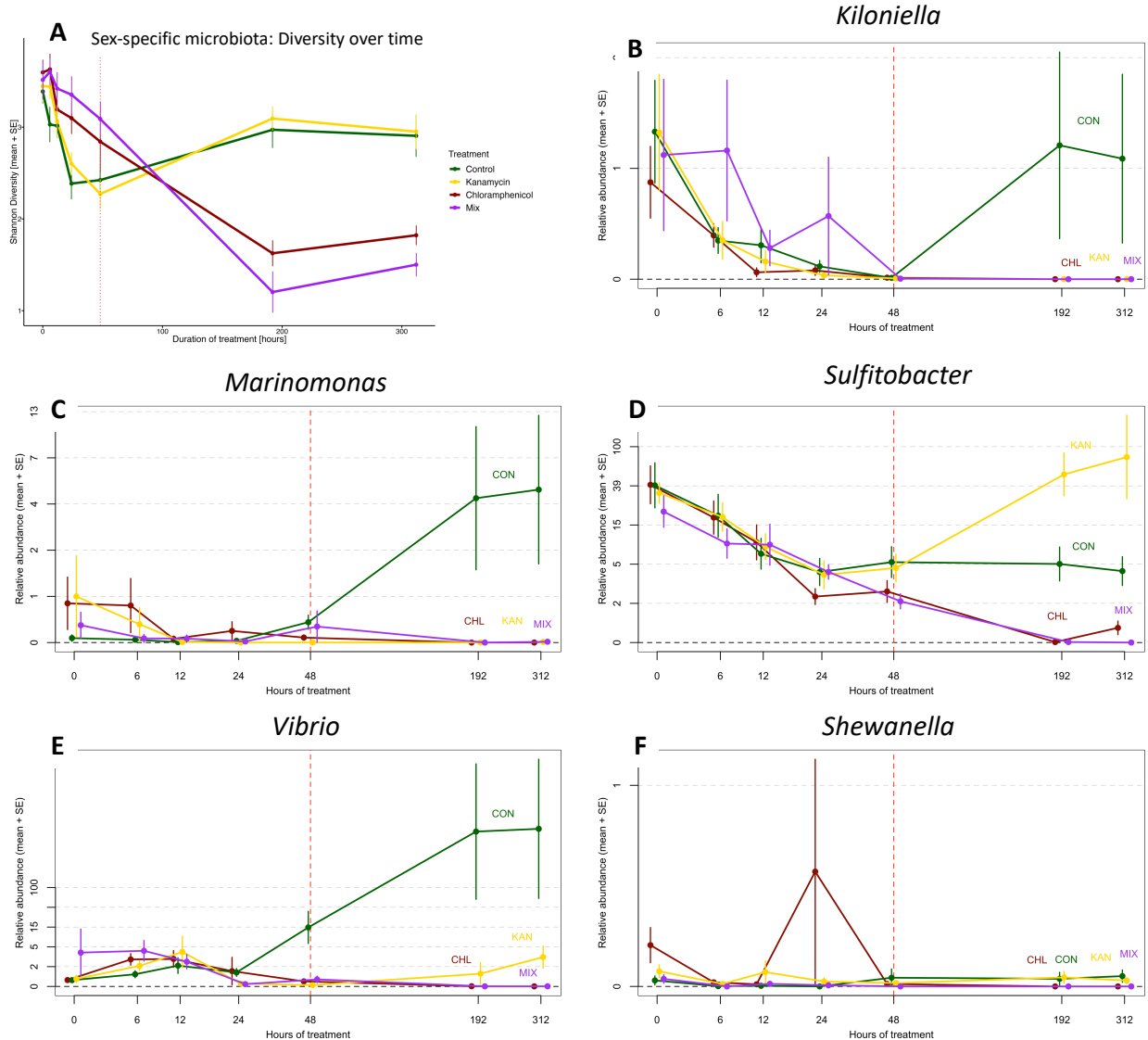


Figure 2: Impact of antibiotics (mean + SE) on Shannon diversity of sex-specific microbiota (A) and on relative abundance of spike community strains (B-F) over a period of 312 hours. Colours indicate treatment groups: Control (green), Kanamycin (yellow), Chloramphenicol (red) and Mix (purple). Red vertical line represents time of treatment dose (48 hours after treatment start).

Impact of antibiotics on microbial community structure (beta diversity)

Similar to alpha-diversity, a repeated measures PERMANOVA suggested significant differences in treatment, time and the treatment x time interaction (all $p < 0.001$) of the sex-specific microbiota composition (beta-diversity, Supplementary Table 3).

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Principal component analyses (PCA) of four time points (treatment: 6, 48 hours; maintenance: 192, 312 hours) suggested a similar microbiota composition in all treatments at the onset of the antibiotic application (MANOVA: T6: $p = 0.457$, Figure 3A). After 48 hours microbial communities separated into Chloramphenicol and Mix treated individuals vs. Kanamycin treated and control individuals (T48: $p = 4.577\text{e-}09$, Figure 3B). This separation intensified over time and was strongest after 192 hours (T192 and T312: $p < 2\text{e-}16$, Figure 3C-D).

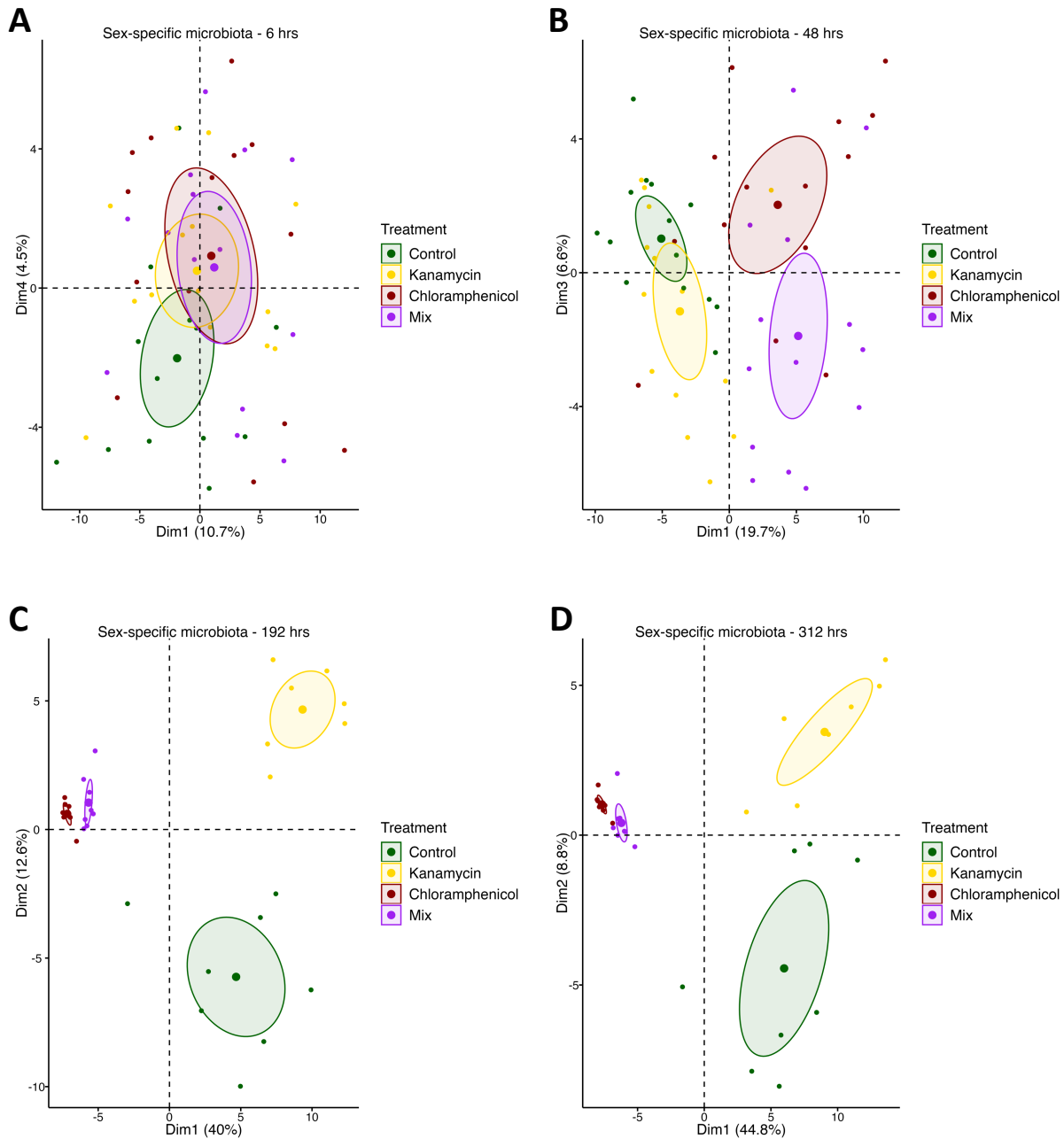


Figure 3: PCAs of antibiotic treatment (A: 6 hours, B: 48 hours, C: 192 hours, D: 312 hours) represent effectiveness of chloramphenicol. Normalized, log-transformed data include ASVs present in >20% of samples. Colours represent treatment group and ellipses 95% confidence intervals.

An indicator species analysis (ISA) for T192 displayed associations between species patterns and combinations of groups, the 30 strains with the highest contribution to the observed patterns are displayed (Supplementary Figure 6). At T192, the Chloramphenicol-treated bacterial composition was driven by *Paraglaciicola* and *Maribacter*, whereas Kanamycin and Control composition were driven by *Sulfitobacter* and *Pseudoalteromonas* (Complete dataset: supplementary spreadsheet 3).

The antibiotic application modified the bacterial composition and reduced its diversity. Chloramphenicol was suitable to deplete a large number of strains within the characterized microbiota and depleted the five isolates chosen as spike community for experiment 3. 192 hours antibiotic treatment appeared most suitable for experiment 3, minimizing stress and disease risk as opposed to longer treatments.

Impact of antibiotic and spike treatments on pregnancy and parental tissues

Experiment 3 aimed to assess the impact of paternal antibiotic and spike treatment on pregnancy, early microbial colonization and offspring health from mating until 45 days post release (dpr). Fish were pregnant for 29.18 ± 1.62 days (mean + SD), spike treatment reduced the length of gestation (28.47 ± 0.8 days) (SP: $p = 0.01$). Proportion of brood pouch filled with eggs and number of offspring born was not influenced by treatments (Figure 4, Supplementary Table 4) (Complete dataset: supplementary spreadsheet 4).

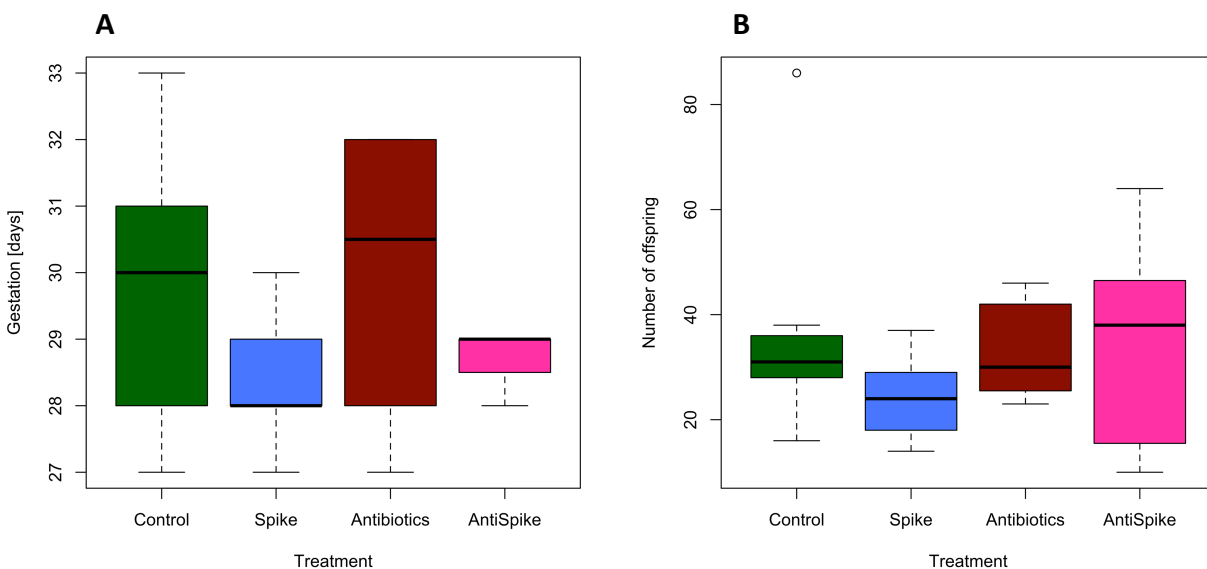


Figure 4: Gestation length (days) (A) is significantly shorter for males who received a spike treatment. In contrast, number of offspring (B) does not differ between males from different treatment groups

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For microbiota ribotyping, a total of 1080 samples, including adult tissue, juvenile tissue and water samples, were 16S rRNA MiSeq amplicon sequenced. 34,021,625 reads were returned (mean run 63: 29,912 reads/sample, mean run 64: 27,566.5 reads/sample, mean run 65: 23,589 reads/sample, mean run 79: 135,136.5 reads/sample), along with 23,841 representative sequences (Supplementary Table 1). Male pipefish were sampled after giving birth (approximately day 30 post-treatment). We analyzed alpha diversity (Shannon) and beta diversity of the placenta-like tissue microbiome and its surface. Alpha diversity of the males was not influenced by treatment (Supplementary Figure 8, Supplementary Table 5). Antibiotic treated fish (Antibiotics and AntiSpike group) showed a significantly different microbial composition (beta diversity, $p = 0.002$) on the surface (brood pouch swabs) and within the placenta-like tissue (Figure 5A and 5C) compared to non-antibiotic treated fish (Control and Spike) (Figure 5A and 5C). Male gut microbiome was not influenced by the antibiotic treatment (Figure 5B). In contrast, the Spike group overlapped with the AntiSpike group and differed from the other groups (SP: $p = 0.027$). In the testes, all groups overlapped in their microbiome and no statistical differences were identified (Figure 5D). While males were sampled after giving birth, females (all untreated) were sampled directly after mating. Ribotyping the female gut and ovipositor microbiota allowed a) to illuminate sexual dimorphism in gut microbiome when comparing to gut microbiome of untreated males, and b) provided a positive control for the presence of our spike community, as the ovipositor of a mated female had been in direct contact with the spike-treated male brood pouch when mated with males from AntiSpike and Spike treatment groups (treatment assignment thus refers to the treatment of the male mating partner).

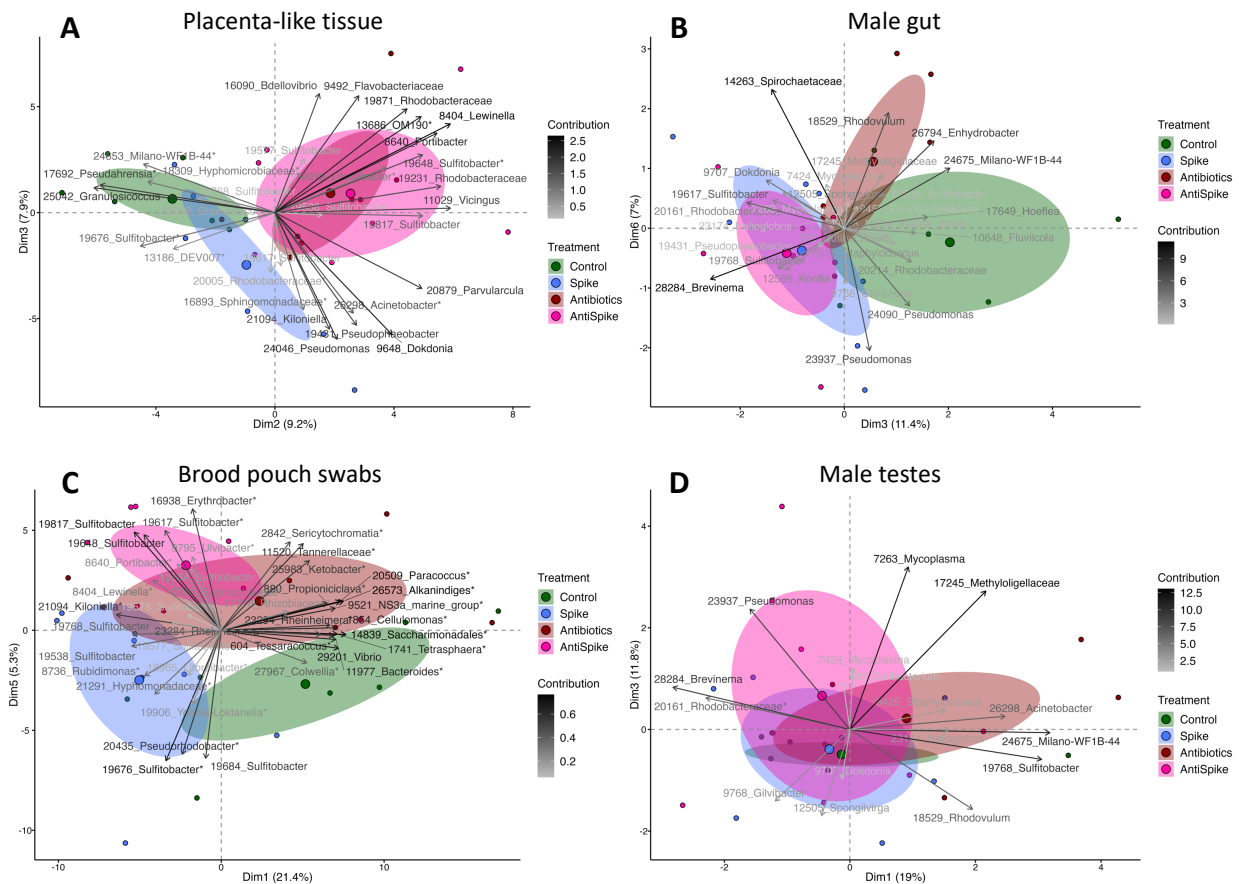


Figure 5: PCA biplots displaying treatment effects (antibiotics and spike) on paternal tissue. In the placenta-like tissue (A) and on the surface of the brood pouch (B), antibiotics affected the microbial composition. In the male gut (C) the spike treatment had a significant impact whereas in the testes (D), none of the treatments showed an effect. Colours represent the paternal treatment group: green = Control, blue = Spike, red = Antibiotics, pink = AntiSpike, Ellipses show a confidence interval around the group's mean. Factor map shows ASV ID and genus name respectively family name.

Previous treatment of the male pipefish did not influence female pipefish microbiomes (Supplementary Figure 10).

Sex was an important factor explaining variation in alpha ($p = 0.0002$, Supplementary Table 6) and beta diversity of gut microbial composition ($p = 0.026$, Supplementary Figure 11, Supplementary Table 7), suggesting a sexual microbiome dimorphism in untreated pipefish. In the gut tissue, this dimorphism was driven by the indicator species *Aliivibrio* in the female gut as well as *Rhodobacteriaceae*, *Maribacter*, *Enhydrobacter* and *Milano-WF1B-44* in the male gut (Supplementary Figure 12) (Complete dataset: supplementary spreadsheet 5).

Impact of antibiotic and spike treatments on life-history and offspring microbiome

Offspring gained weight over time (time: $p < 2e-16$) (Figure 6B, Supplementary Table 8), in contrast, offspring size was influenced by time and its interaction with treatment (time: $p < 2e-16$, time*treatment: $p = 0.045$) (Figure 6C, Supplementary Table 9). Treatment, time and their interaction significantly affected offspring survival (treatment: $p = 1.85e-14$; time: $p < 2e-16$; treatment*time: $p = 8.03e-10$) (Supplementary Table 10). Offspring of spike-treated fathers survived better than all other groups (Contrasts: Spike vs Antibiotics, AntiSpike and Control (all $p < 0.0001$, Figure 6A, Supplementary Table 11) (Complete dataset: supplementary spreadsheet 4).

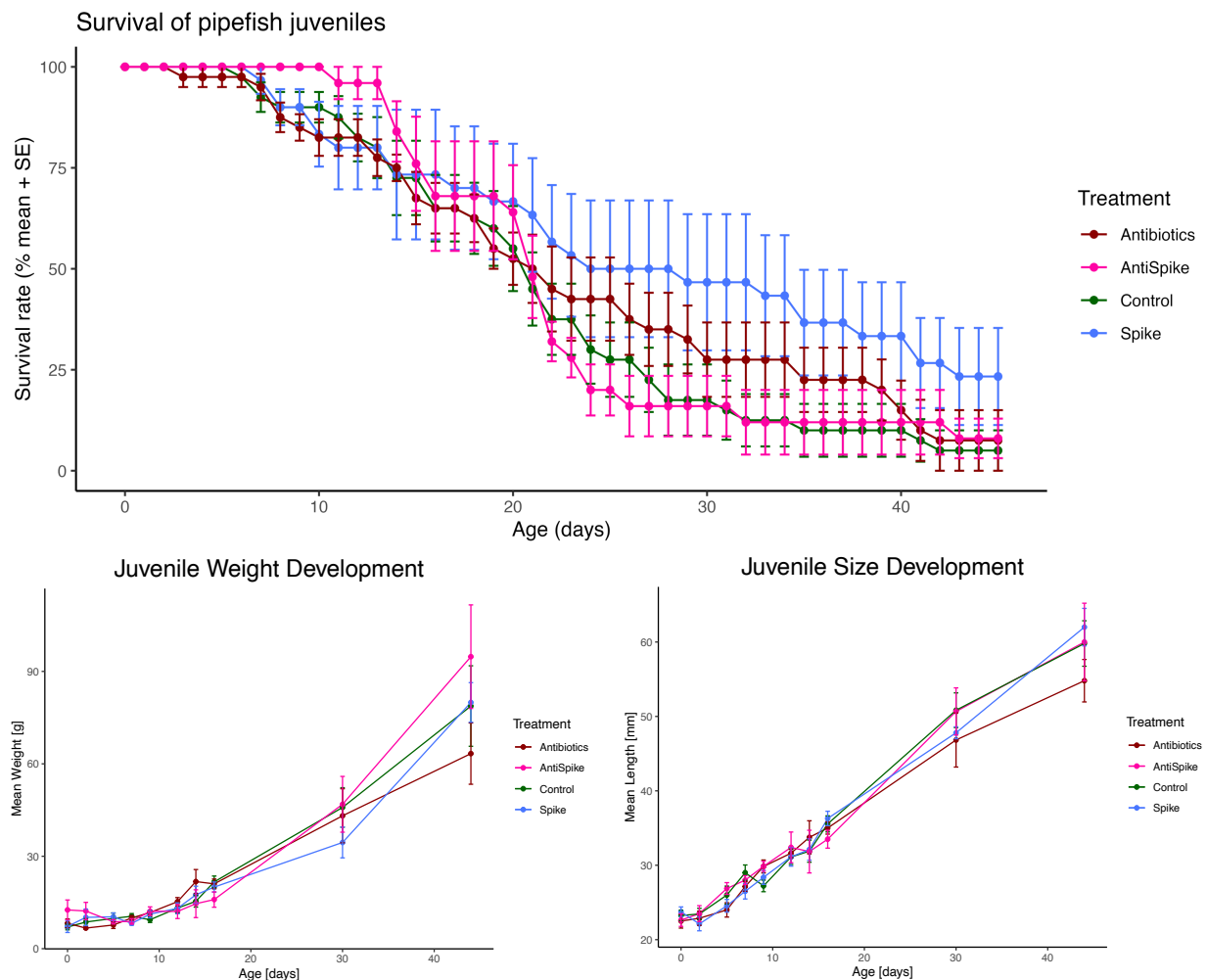


Figure 6: Life-history data of pipefish juveniles from birth until 45 days after birth. Survival rate (A), Weight development (B) and Size development (C). All plots show mean \pm standard error (SE). Colour represents paternal treatment.

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Upon offspring birth, juvenile whole-body and gut microbiomes were sequenced every second day for two weeks, after 30 and 45 days. Offspring from Control fathers had lower whole-body microbiota diversity across all time points ($p = 0.039$) (Alpha diversity, factor treatment), while the time point (age) itself had no significant impact (Figure 12A, Supplementary Table 12). Conversely, offspring gut microbiome changed (Alpha diversity) over time ($p < 0.001$), unaffected by the paternal treatment (Figure 12B, Supplementary Table 13). At birth, offspring whole-body microbiome was unaffected by paternal treatment (dpr 0). However, two days after birth, the spike treatment significantly shaped offspring microbiome ($p = 0.013$), while paternal antibiotics treatment indicated a trend ($p = 0.052$). Paternal treatment effects were strongest between six and ten days after birth (antibiotics: dpr 6: $p = 0.039$; dpr 10: $p = 0.025$) and (spike: dpr 6: $p = 0.006$; dpr 10: $p = 0.017$), but remained detectable up to 30 days after birth (antibiotics: $p = 0.025$; spike: $p = 0.009$) (Supplementary Table 14, Figure 7). The absence of a treatment effect at dpr 8 and dpr 12 indicates fluctuations in paternal treatment effects. Paternal spike treatment did not shape offspring gut microbiome, with the exception of 12 days post-release ($p = 0.044$; Supplementary Table 15, Supplementary Figure 13) (Complete dataset: supplementary spreadsheet 6 and 7).

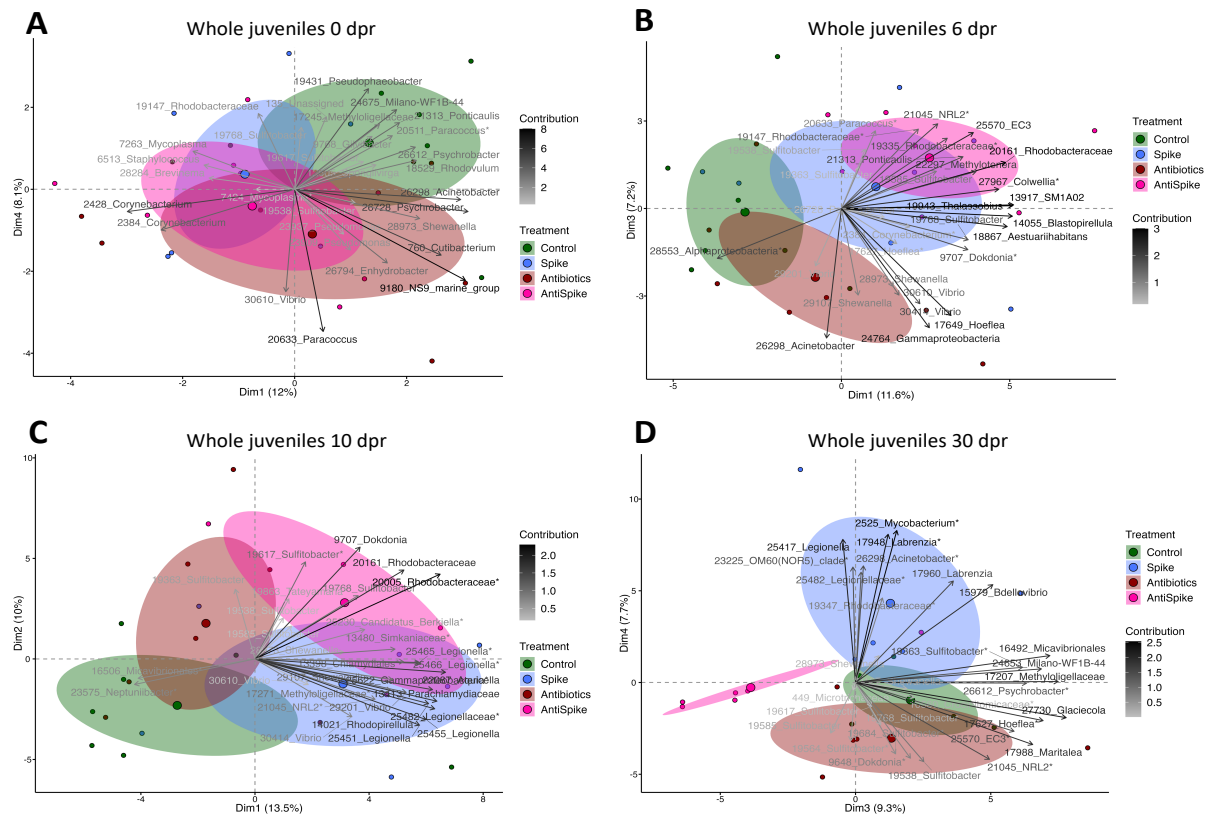


Figure 7: Shifts in offspring whole body microbiome composition from birth (A), 6 days (B), 10 days (C) up to 30 days (D) after birth. Colours represent the paternal treatment group. Ellipses show confidence intervals. Factor map shows ASV ID and genus name respectively family name.

Searching for the spike bacteria in the adults and offspring

When examining the 16S rRNA sequences of our spike community in the relevant tissues after approximately two (females) respectively 30 days of pregnancy (males), we found identical sequence hits across all tissues (both adults and juveniles) and treatment groups, underlying that the microbes used in our spike community are members of the natural pipefish microbiome. In tissue of spike-treated fathers (Spike and AntiSpike), relative abundances of spike community members were undistinguishable from untreated fathers (Control and Antibiotics) (Figure 8A, Supplementary Figure 14). Normalized counts of Spike members were higher on the surface of male brood pouches (brood pouch swabs) from spike treated fathers ($p = 0.044$), a pattern driven by high *Shewanella* prevalence ($p = 0.018$). However, across all adult tissues and treatments *Shewanella* counts were high, while *Marinomonas* was mostly absent indicating a lack of colonization (Supplementary Table 16).

Paternal spike treatment (spike and AntiSpike) induced spike community prevalence in offspring whole-body microbiome at 10 days after birth ($p = 0.013$), driven by high counts of *Shewanella* ($p = 0.018$), particularly in the AntiSpike treatment group (Figure 8D). No similar tendency was identified for offspring gut microbiome. *Kiloniella* was absent from juvenile gut samples, suggesting a lack of transfer respectively colonization. Regardless of paternal treatment, *Marinomonas* was absent in the majority of offspring samples (both whole-body-microbiome and gut-microbiome), but prevalent in the microbiome of the food (artemia, Figure 8D). Since this strain did not colonize adult tissue (Supplementary Table 16), the rare counts for this strain in our offspring dataset probably stem from food. *Vibrio* counts in the offspring should be treated with caution, as a ubiquitous strain in the natural environment of the pipefish, *Vibrio* was particularly abundant in artemia (Complete dataset: supplementary spreadsheet 8).

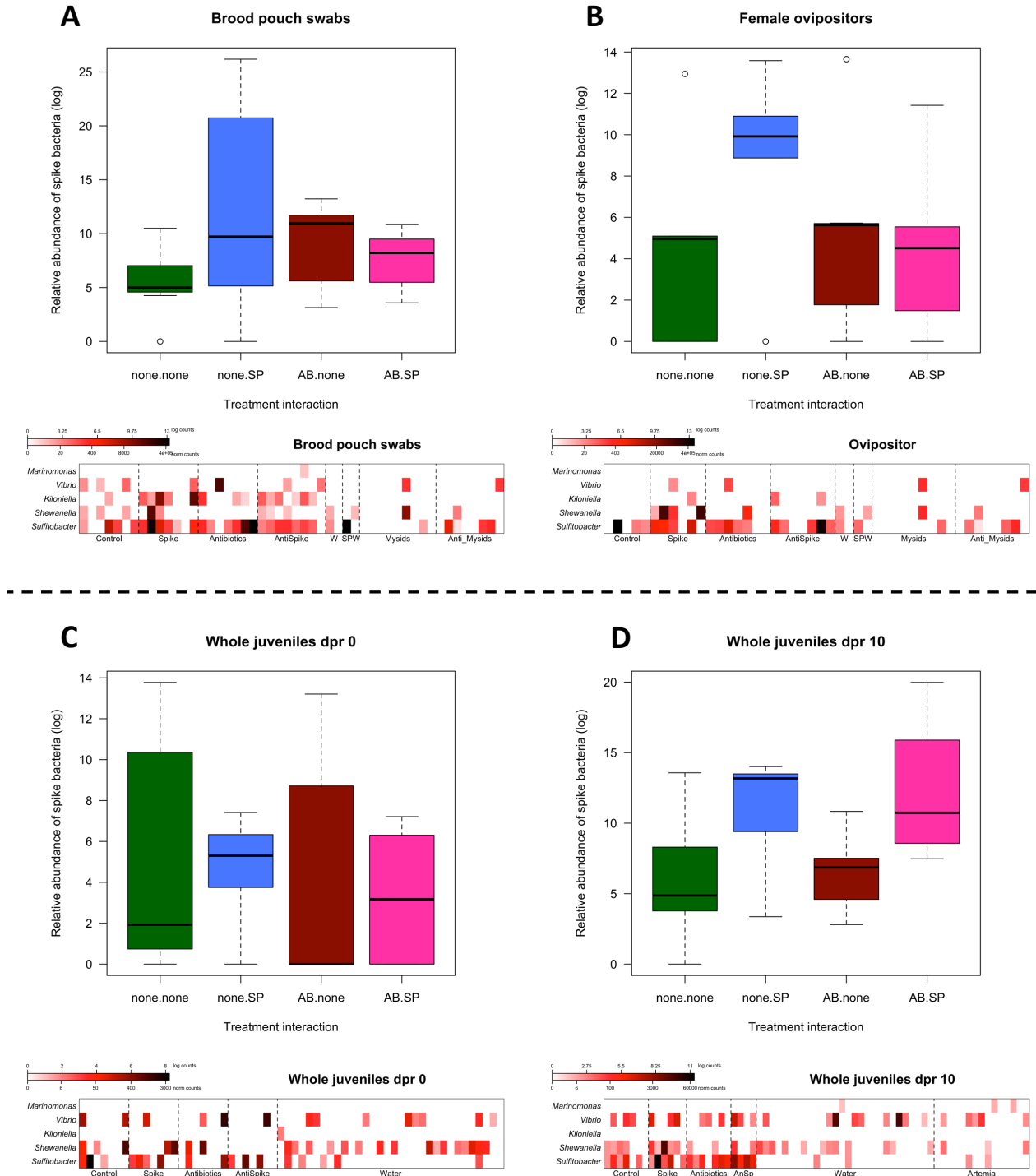


Figure 8: Prevalence of spike bacteria. Upper boxplots show relative abundance of spike bacteria within the whole microbiome of the respective tissue. Colours represent paternal treatment group. Lower heatmaps show individual counts (normalized) per sample. Darker colour indicates higher counts. A: Swabs of brood pouch surface, B: Female ovipositor after contact with treated male brood pouch, C: Whole body of juveniles after birth, D: whole body of juveniles 10 days after birth.

DISCUSSION

We aimed to experimentally assess the existence and role of paternal microbiota provisioning on offspring life-history and early microbial colonization through male pregnancy in the sex-role reversed pipefish *Syngnathus typhle*. To achieve this goal, we cultivated and characterized the sex-specific pipefish microbiota (experiment 1) and studied the effectiveness of antibiotics in depleting its microbiota (experiment 2). This facilitated to select microbes for paternal microbial manipulation (experiment 3) to unravel how paternal microbial provisioning influenced offspring development and microbiome establishment.

Both cultivation-dependent and cultivation-independent approaches (Beemelmanns et al. 2019) supported the presence of distinct microbial communities in female eggs, the brood pouch of non-pregnant males, and throughout male pregnancy. Early offspring microbial colonization was tissue-specific, shaped rather independently by mother and father (Tanger et al. 2024).

The here observed sexual dimorphism in microbial communities, coupled with a decreased beta diversity in females (Supplementary Figure 11), strikingly contrasts with findings from conventional sex role species (de la Cuesta-Zuluaga et al. 2019; Valeri and Endres 2021). Sex roles and life history, rather than sex alone, seem to be the drivers of immunological activity and microbial diversity (Roth et al. 2010; Pappert et al. 2024). The differences in investment into parental care and secondary sexual ornaments appear to extend into the complex interactions between the microbiome and the immune system. Investigating parental microbial provisioning will help to understand how sex-role dynamics influence health and development across generations.

By treating pipefish with chloramphenicol, we successfully decreased pipefish brood pouch microbial diversity. Chloramphenicol was particularly effective against the microbes later assigned as experimental spike community, consisting of microbes from parental origin and isolates ubiquitous in pipefish and their natural environment. Achieving a completely germ-free status in our fish remained unattainable with antibiotic treatment alone. Working with *S. typhle*, a non-model marine species, presents challenges for gnotobiotic rearing due to its obligatory male pregnancy and the largely unknown impact of environmental microbes on its development and health. We thus acknowledge the inherent bias of persistent microbes, that we were unable to exclude throughout the conducted experiments.

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Treating paternal pipefish with antibiotics influenced the composition and diversity of their microbiomes. The antibiotic treatment effect remained detectable 30 days post-treatment (after male pregnancy) when assessing the microbiome of the placenta-like tissue and its surface, but was absent when assessing the microbiome of the male gut or testes. This suggests that the antibiotic treatment affected the parts of the body, which were in direct contact with the water, and penetrated the underlying tissues, while it did not have a long-lasting effect on internal organs. After the antibiotic treatment, the gut microbiome might have reverted towards a stable core microbiome during the four weeks of pregnancy. In contrast, a long-lasting influence of the spike treatment on the microbiome composition was observed in the male gut despite that bacteria were added directly into the brood pouch. To shed light on time dynamics of potentially interacting antibiotic and spike treatments on parental tissues, ribotyping the microbial composition of paternal tissue throughout male pregnancy would be necessary. We have decided against this approach in the concurrent experiment to avoid disturbing or sacrificing the developing embryos due to limited housing opportunity in our experiment (36 breeding pairs in separate tanks, 9 per treatment).

Paternal spike treatment (Spike and AntiSpike) shortened paternal pregnancies compared to those of unexposed fathers (Control and Antibiotics). Also, in mammalian pregnancy various types of bacteria have been linked to preterm labor (Daskalakis et al. 2023). However, contrary to health challenges observed upon mammalian preterm birth, offspring from spike-treated fathers survived better than those from all other groups, suggesting spike treatment to be adaptive for offspring health.

After offspring left the paternal brood pouch and the associated protection from environmental microbial colonization, paternal spike as well as antibiotic treatment started to influence the whole-body microbiome up to 30 days after birth (Figure 7B-D, Supplementary Table 14). This suggests that the shift in the paternal brood pouch microbiome upon paternal treatment must have influenced the offspring microbial composition and the susceptibility of the offspring towards microbial colonization. The latter could explain why paternal treatment effects only emerged after birth, when offspring were suddenly exposed to a cocktail of surrounding environmental bacteria. In contrast, the offspring gut microbiome was not influenced by paternal treatment. While an impact on offspring gut microbiome by paternal treatment was potentially masked by a strong influence of the bacteria consumed over artemia feeding, this finding supports the notion that pipefish fathers primarily shape the whole-body microbiome, while mothers shape the gut microbiome (Tanger et al. 2024).

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Despite initiating the spike community treatment with a mixture of pure bacterial cultures (Supplementary Figure 7), sequences matching our spike bacteria were found across all tissues (adults and juveniles) and treatment groups. This either suggests that the respective bacteria also colonized through environmental sources, making experimentally applied and natural strains difficult to detect. Or, non-mutually exclusive, that the sequences obtained from 16S V3/V4 ribotyping were too conserved to reliably detect the specific strains provided in the spike community. In particular *Shewanella* and *Vibrio* often exhibit high 16S rRNA copy numbers, leading to taxonomic classification differences for the same strain (Větrovský and Baldrian 2013). Metagenomics could have better distinguished bacterial strains, however the high sample size ribotyped throughout the experiment ($n = 1080$) rendered this impossible.

Although this experiment focused solely on paternal microbial transmission during pregnancy, the selected bacteria originated from paternal, maternal, and environmental sources, permitted to assess the microbial specificity of transgenerational effects. *Sulfitobacter*, identified as maternally-specific strain in experiment 1, exhibited high prevalence upon inoculation (Spike and Antispike) both in paternal tissue post-pregnancy and offspring tissues. Also, in offspring tissues of Control and Antibiotics treated fathers, it was present, albeit in lower counts. This may suggest that the extent of its colonization and transfer depended on the overall prevalence of the strain, especially given its high concentrations in the spike treatment, or that *Sulfitobacter* was simply ubiquitous in the environment and therefore horizontally colonized the tissues after antibiotic effects ceased. Conversely, *Marinomonas*, previously identified as paternal-specific in all studies on *S. typhle* microbiota, was found only in low abundances on the brood pouch surface within the AntiSpike group and was absent in all offspring tissues. Similarly, *Kiloniella*, which was present in high abundances in the paternal placenta-like tissue upon spike exposure, could not be detected in offspring. It is tempting to speculate that while these isolates are naturally sex-specific, they were here outcompeted by other isolates of maternal or environmental origin upon paternal inoculation.

In line with earlier studies, paternal spike treatment only shaped the whole-body microbiome (Tanger et al. 2024). This underlines the importance in disentangling routes of vertical bacteria transfer, as maternal transfer was suggested to rather shape offspring gut microbiomes (Tanger et al. 2024), giving both parents specific roles in microbial inheritance. In natural systems such as *S. typhle*, differentiating between environmental and parental effects on microbial colonization and development is essential. The interplay between these factors is complex and requires comprehensive analysis to fully understand the patterns involved. Due to the discussed limitations

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of our approach, future research should apply genetic-fluorescent- and antibiotic resistance-tagged spike strains. This would facilitate a detailed exploration of microbial transfer pathways from parents to offspring, providing precise insights into how specific bacteria are transmitted and established across generations, as well as pinpointing their functions in both paternal as well as offspring microbiomes.

By assessing the parental as well as offspring immune responses upon both antibiotic and spike exposures, we could gain deeper insights into the complex interactions between microbial communities, environmental factors, and host immune systems across generations, linking to previously suggested bi-parental trans-generational immune priming (Roth et al. 2012; Beemelmans and Roth 2016a, 2017). Paternal spike bacteria did not only shape offspring microbiomes but also increased offspring survival at a larger size. Insights into adaptive paternal effects on offspring microbiomes can add to our fundamental understanding of the ecological and evolutionary contexts of microbial interactions enlightening the adaptive significance of microbiota in host biology.

CONCLUSION

Our study underscores the significant role of both paternal and environmental factors in shaping the microbiota of offspring. It also highlights the necessity for advanced methodologies and comprehensive studies to unravel the complexities of microbial transfer and its implications for host health. Continued research in this area will not only enhance our understanding of microbial ecology but may also contribute to managing microbial communities for promoting health and preventing disease.

This work highlights the role of paternal provisioning during male pregnancy, demonstrating that early colonization patterns in pipefish juveniles are primarily tissue- rather than sex- or bacterial strain-specific. This indicates that a dysbiosis in the father's sex-specific tissues may notably influence offspring microbial colonization, as demonstrated here for the maternal indicator *Sulfitobacter*. While these findings might slip our attention in a balanced ecosystem, they could gain significance in the context of global change, potentially leading to the transfer of an altered microbial community with unknown impacts on offspring health and development. Our study examines only a small part of a complex and dynamic system, and the full impact of various microbial sources, including environmental bacteria, remains to be predicted in future studies. This underscores the need for advanced methodologies, such as fluorescent tagging, to better understand microbial transfer pathways in more detail. Future research should focus on exploring these transfer mechanisms and their long-term effects on offspring health to provide a more comprehensive understanding of the ecological and evolutionary implications of microbial inheritance.

ETHICS STATEMENT

The work was carried out in accordance with the German animal welfare law and with the ethical approval given by the *Ministerium für Landwirtschaft, ländliche Räume, Europa und Verbraucherschutz des Landes Schleswig-Holstein* (MLLEV-SH) (permit no. 63-7/18). No wild endangered species were used in this investigation.

4 CHAPTER II

Visualizing temporal dynamics of vertical and horizontal microbial transfer during pipefish male pregnancy – a pilot study

Kim-Sara Wagner¹, Rebekka Leßke^{1,2}, Silke-Mareike Marten¹, Nils F. Hülter³ & Olivia Roth¹

1 – Marine Evolutionary Biology, Zoological Institute, Kiel University, 24118 Kiel, Germany

2 – present address: Natural History Museum Denmark, 1350 Copenhagen K, Denmark

3 – Institute of Microbiology, Kiel University, 24118 Kiel, Germany

ABSTRACT

Microbial colonization during early development is shaped by vertical and horizontal transmission, processes that are essential for host health and development. Yet, the influence and timing of different microbial transmission routes remain poorly understood, particularly in classic female pregnancy systems where provisioning through eggs and pregnancy is intermingled. The evolution of male pregnancy in syngnathids (pipefishes and seahorses) provides a unique opportunity to disentangle these routes, enabling the study of vertical microbial transfer through paternal provisioning during pregnancy, distinct from maternal provisioning during egg production. Horizontal microbial transmission from the environment, suggested to occur during late pregnancy, represents an additional pathway influencing colonization. In this study, we investigated temporal microbial transfer dynamics in the sex-role-reversed pipefish *Syngnathus typhle*, focusing on vertical (paternal) and horizontal (environmental) transmission pathways.

Using targeted additions of fluorescently tagged bacterial strains (spike treatment: *Marinomonas* GFP – vertical; *Ruegeria* mCherry - horizontal) before and during late pregnancy, we found that both transmission modes contribute distinctly to juvenile microbiome composition. While vertical transfer exerted a strong initial impact on the microbiome of the developing embryos during male pregnancy, this influence diminished by birth when horizontal transfer became the predominant factor shaping microbiome diversity. Our results indicate that even minor shifts in natural microbes can significantly alter community structure. However, the limited number of introduced strains should be considered when interpreting the results. While this study aimed to evaluate a “proof-

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of-concept” method for visualizing the vertical and horizontal transfer of fluorescently tagged microbes in *S. typhle*, it also provides insights into the distinct temporal dynamics of microbial transfer during early host colonization. Although detecting the fluorescently labeled bacteria posed certain methodological challenges, this pilot study offered valuable experience and highlighted key areas for method optimization. This work highlights the importance of exploring microbial transmission in complex reproductive systems to understand its evolutionary implications.

INTRODUCTION

The microbiome shapes host development, digestion, immune regulation, behavior, and pathogen protection (He et al. 2020; Donald and Finlay 2023; Motta and Moran 2024). As such, the coevolution of hosts with their microbiome has led to complex symbioses, facilitating habitat colonization, behavioral interactions or advancements in nutrient acquisition (Lewin-Epstein and Hadany 2020). The microbiota can be highly species-, sex-, tissue-, and development stage-specific, forming intimate, long-lasting interactions with their hosts (Larsen et al. 2013; Zhang et al. 2021). However, dysbioses, i.e., imbalances in these communities, can induce inflammation, cancer, obesity, depression, and allergies (Hou et al. 2022).

To fully understand how these specialized microbial communities form and function, it is essential to examine the initiation of microbial colonization. Among vertebrates, some lizard and fish species show *in ovo* transfer of microbes that is suggested to serve for vertical transmission of symbionts (Nyholm 2020). From mammals, in particular, we know that already during pregnancy the microbiome plays a crucial role in providing bioactive metabolites and micronutrients like lipids, polysaccharides, and vitamins to the embryo (Wosnick et al. 2022; Vitikainen et al. 2023). During embryonic development, primary colonizers adjust environmental conditions to facilitate the establishment of the microbiome. They can be acquired through multiple vertical transmission routes, e.g., eggs, pregnancy, birth and postnatal care (Bogaert et al. 2023). Ultimately, these microbes are essential for the onset of the immune system and play a critical role in offspring development (Kalbermatter et al. 2021).

Vertical transmission is closely tied to reproductive success (Ruiz-Guzmán et al. 2016) and reflects a long history of host-microbe coevolution across generations. However, horizontal transmission – the acquisition of microbes from the environment – also plays an equally crucial role in shaping early microbial communities (Leftwich et al. 2020). Horizontal transmission typically occurs shortly after birth or hatching, when offspring come into contact with their external environment. This can include interactions with water, soil, food or conspecifics (Rosenberg and Zilber-Rosenberg 2021). Unlike vertical transmission, which remains relatively stable across generations, horizontal transmission can lead to significant fluctuations due to dynamic environmental changes (Coyte et al. 2022). These horizontally acquired microbes play key roles in enhancing phenotypic plasticity for acclimatization to changing conditions, offering flexibility in response to environmental challenges.

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The interplay between vertical and horizontal transmission, so-called mixed mode transmission (Ebert 2013), creates a specific microbial community for the offspring, incorporating both inherited, coevolved microbes and environmentally sourced microbes that increase the host's ability for acclimatization (Breusing et al. 2022). Understanding the interacting effects of these transmission routes is essential for a comprehensive view of microbial inheritance and its impact on early development.

In most viviparous animals, vertical transmission routes are uniquely bound to the maternal side, making it difficult to differentiate between sex and sex role and therefore understanding the influence of maternal and paternal provisioning on the establishment and development of the offspring microbiome (Arora et al. 2017; Mahyuddin et al. 2020). In vertebrate evolution, viviparity – the development of embryos inside the parent's body – has independently evolved in over 150 lineages (Blackburn 2015). However, the Syngnathidae family, which includes seahorses and pipefish, displays the unique evolution of male pregnancy, alongside the development of specialized brooding tissues on the paternal side (Whittington and Friesen 2020). In these fishes, pregnancy has evolved on a gradient of parental investment, ranging from an external attachment of eggs in *Nerophinae* to an internal pregnancy with a placenta-like structure in *Syngnathinae* and *Hippocampinae* (Keller and Roth 2020; Roth et al. 2020). In our research organism, the broad-nosed pipefish *Syngnathus typhle*, the female deposits eggs into the male's inverted brood pouch, where they are fertilized, protected and nurtured until birth (Stölting and Wilson 2007). Juveniles hatch inside the brood pouch after 14–17 days, but actual birth occurs later after approximately 30 days (Schneider et al. 2023). During early stages of pregnancy, the semi-sealed brood pouch remains relatively impermeable, presumably allowing vertical transmission of microbes solely from the father to the developing embryos. As pregnancy progresses, the brood pouch becomes physically strained, and its permeability increases, allowing water and environmental bacteria to penetrate the pouch. This stage of increased permeability marks the onset of microbial priming through horizontal transmission (Beemelmans et al. 2019; Schneider et al. 2023). Shortly before birth, the sealing of the brood pouch-forming skin flaps detaches, allowing even closer contact with environmental microbes (Figure 1).

In this system, the temporal separation of transmission modes is particularly valuable. Vertical transmission from mothers and fathers is assumed to occur during early pregnancy, while horizontal transmission is likely to be initiated later (Beemelmans et al. 2019; Tanger et al. 2024). Additionally, as juveniles open their mouths inside the pouch after approximately three weeks of pregnancy, their gut becomes exposed to these environmental microbes, facilitating colonization

of the gut and the establishment of the gut microbiome (Schneider et al. 2023). This temporal distinction provides a unique opportunity to study the diverse microbial transmission routes independently along a defined timeline, shedding light on their respective contributions to early microbial colonization.

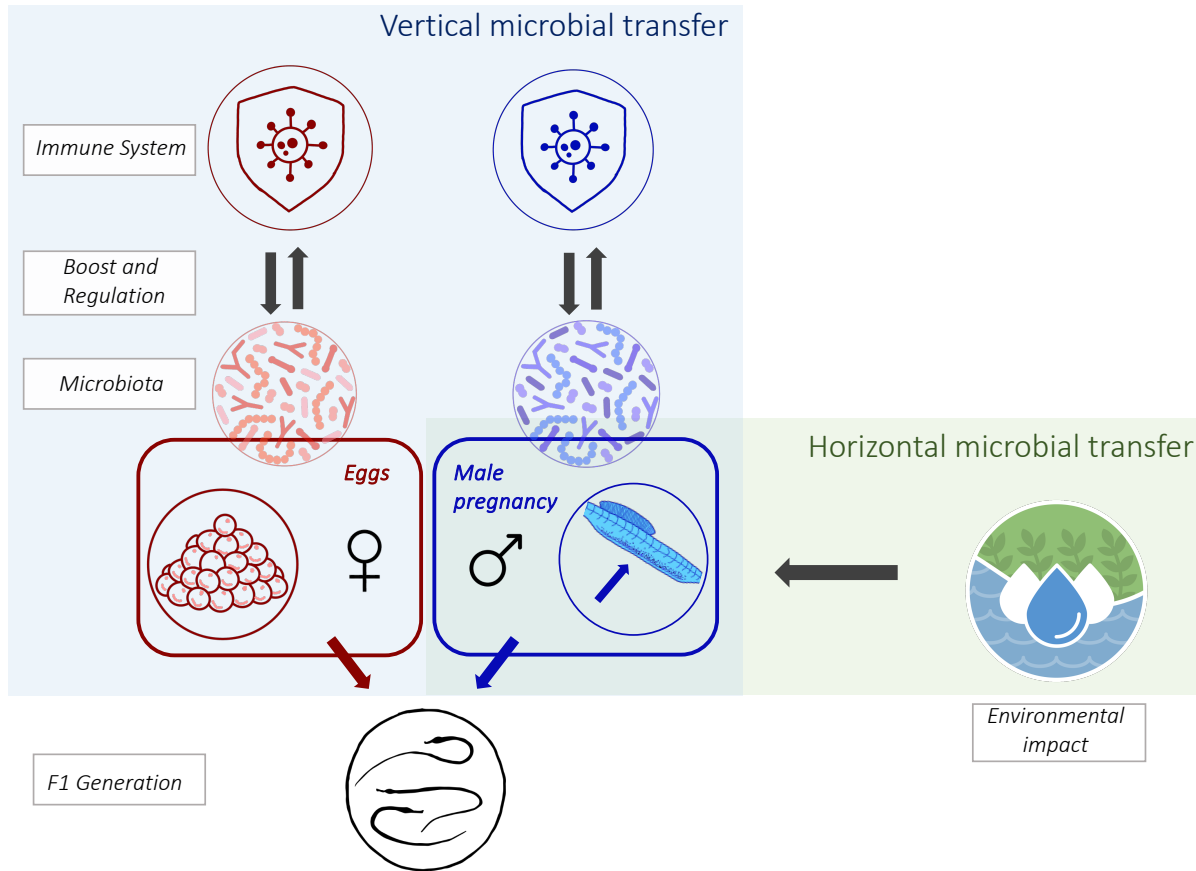


Figure 1: Microbial transfer during male pregnancy in *Syngnathus typhle*. Interaction of immune system with microbiota and inheritance (vertical transfer) from parents to offspring (blue shaded box). During male pregnancy, environmental microbes can colonize the developing offspring, particularly towards the end of pregnancy (horizontal transfer, green shaded box).

In a previous experimental study (Wagner et al. 2024), we assessed the effect of paternal microbiota manipulation on offspring development and microbial colonization. By employing a combination of antibiotic treatment and recolonization techniques, we demonstrated that paternal treatments influenced the microbiota of adult tissues and the whole-body microbiota of juveniles. Remarkably, it was the recolonization treatment that led to shorter pregnancies and higher offspring survival. In the present study, we wanted to shed light on the temporal dynamics of vertical and horizontal transmission on early microbial colonization in pipefish juveniles by assessing different timepoints throughout pregnancy and early development from mating until 7 days post-release (7 dpr). In addition, we aimed to establish and test an approach to visualize

microbial transfer using fluorescently-tagged bacteria, similar to methods used in other studies investigating host-microbe interactions in fish (Wiles et al. 2018; Basheer et al. 2020). We hypothesized that both vertical and horizontal transmission influence the microbiota composition of the brood pouch and the developing offspring, with each transmission route leading to distinct effects on microbial colonization. Additionally, we hypothesized that the influence of transmission route is tissue-specific, with vertical transmission primarily impacting the juvenile whole-body microbiome and horizontal transmission more significantly affecting the gut microbiome. We also anticipated that our experimentally introduced fluorescent bacteria would be detectable in both adult and juvenile tissues, providing evidence of successful microbial transfer and colonization through these distinct transmission routes.

This study deepens our understanding of host-microbiota interactions during pregnancy and offers valuable insights into the distinct but potentially interacting roles of vertical and horizontal transmission in shaping microbiota establishment across generations. By utilizing fluorescently tagged bacteria, we explored the possibility of tracking microbial transfer from parents to offspring, aiming to shed light on these complex transmission pathways.

MATERIALS & METHODS

Using a reciprocal experimental approach, we investigated vertical and horizontal microbial transfer in *Syngnathus typhle* by introducing fluorescently tagged bacterial strains (*Marinomonas*-GFP and *Ruegeria*-mCherry) to track microbial transmission routes during male pregnancy and assess their colonization in the paternal brood pouch, developing embryos, and juvenile fish up to 7 days post-release (7 dpr). This study should therefore act as a proof-of-concept test to assess the visualization of vertical transfer using fluorescently tagged microbes. Antibiotic treatment was not applied, as our previous study (Wagner et al. 2024) demonstrated recolonization effects regardless of prior antibiotic exposure. Moreover, the fluorescence of the tagged strains was expected to allow for clear differentiation from the natural microbiome. Considering the stress induced by antibiotic treatment (Bojarski et al. 2020), we chose to omit it, relying on the distinct labeling of our experimentally-added bacterial strains.

Fluorescent strain construction

To separately track vertical and horizontal microbial transfer, two fluorescently tagged bacterial strains (“spike strains”) were established: GFP-expressing *Marinomonas* for vertical transfer and mCherry-expressing *Ruegeria* for horizontal transfer. Both strains were equipped with derivatives of the mobilizable broad host range plasmid RSF1010 carrying either GFP (pRL153-GFP; Tolonen et al. 2006), or mCherry (pTW1-mCherry; Wein et al. 2018), respectively. These plasmids carry the kanamycin resistance gene *nptII* with its original Tn5 promoter and are otherwise isogenic, except for the respective fluorophore-encoding gene expressed from the P_{trc} promoter. The plasmids (pRL153-GFP and pTW1-mCherry) were transferred into the respective target strain (*Marinomonas* or *Ruegeria*) by biparental mating with *E. coli* MFD π (Ferrières et al. 2010) as plasmid donor. To account for the marine origin of *Marinomonas* and *Ruegeria* strains, which require lower temperatures for optimal growth, we adapted the original mating temperature of 37°C, typically used for *E. coli*, to 25–30°C, depending on the strain. Both fluorescently tagged strains were routinely cultivated over night at 20°C in Marine Broth (MB) medium (Carl Roth GmbH, Karlsruhe, Germany) with gentle shaking or on MB agar plates, both under constant aeration. When required, kanamycin (5 $\mu\text{g ml}^{-1}$) was added to select for plasmid carriage. Colonies displaying a uniform fluorescence were selected using fluorescent microscopy and additionally confirmed through an oxidase test (ROTITEST Oxidase strips, Carl Roth GmbH, Karlsruhe, Germany).

Fish catching and rearing

This study was conducted on adult broad-nosed pipefish (*Syngnathus typhle*) from the family Syngnathidae, which inhabit local shallow seagrass meadows. A total of 96 fish (48 males and 48 females) was used. Pipefish were collected shortly before the breeding season in spring from the bay of Orth, located on the island of Fehmarn, southwestern Baltic Sea (54°26' N, 11°02'E). At the Helmholtz Centre for Ocean Research Kiel (GEOMAR), the fish were sex-separated and treated with a 1:8000 formalin solution for three consecutive days to remove potential ectoparasites. They were kept in large flow-through Baltic Sea water systems with salinity around 18 PSU, and the water temperature, initially at an ambient temperature of 13°C, was gradually increased by 1°C per day until reaching 18°C. Under these conditions, male brood pouches matured, preparing the fish for mating. A 16:8 light-dark cycle was applied to mimic natural summer conditions in Northern Germany. Tanks were cleaned daily and the fish were fed twice a day with small frozen crustaceans (family Mysidae).

Paternal transfer treatment and mid-pregnancy sampling (Day 0-10):

Prior to the paternal treatment, the GFP-expressing *Marinomonas* strain was cultured in an overnight culture and transferred to fresh liquid medium until reaching the log growth phase. The replicated cultures were diluted to an OD_{600nm} of 0.5, washed thoroughly with sterile phosphate-buffered saline (PBS), and concentrated 30-fold. Half of the male fish received 50 µl of the concentrated *Marinomonas*-GFP culture via flushing of their brood pouch to track vertical microbial transfer through paternal provisioning. The other half of the males were flushed with 50 µl of sterile PBS, serving as a sham control. Following treatment, each male was transferred to a 20 L glass tank and paired with an egg-bearing female for mating. Three days after mating, females were removed from the tanks. Out of the 48 pairs, 44 successfully mated, with 23 spike-treated males and 21 PBS-treated males.

Ten days into pregnancy, at the transition from early to mid-pregnancy, the fish were sampled, at which point there were only two experimental groups: paternal transfer (*Marinomonas*-GFP spike treatment) and no transfer (PBS treatment). Seven males from each group were randomly selected. The animals were euthanized with an overdose of tricaine methane sulfonate (MS-222, 500 mg l⁻¹; Sigma-Aldrich, St. Louis, USA) and decapitated. Brood pouch microbiota samples were collected using sterile cotton swabs (Copan Italia, Thermo Fisher Scientific), which were first streaked on kanamycin-selective MB agar plates (5 µg/ml) to selectively cultivate fluorescent colonies and then frozen at -20°C for further analysis. Agar plates were incubated at room temperature for 48 hours. Developing embryos were extracted from the anterior brood pouch. Half of the embryos were surface-sterilized with povidone iodine (PVP-I), following protocols by Tanger et al. (2024) (hereafter called sterile embryos), while the other half were left untreated (hereafter called embryos). The tissues were homogenized with a pestle, one part streaked on kanamycin agar plates and the other part stored at -20°C for later 16S rRNA amplicon-sequencing. Additionally, 200 ml of water from each tank was filtrated using sterile 0.2 µm nylon membranes to assess the environmental microbial composition.

Horizontal transfer treatment, late-pregnancy sampling, raising of juveniles (Day 21-30):

At three weeks of male pregnancy (day 21), 50 µl of a highly concentrated mCherry-expressing *Ruegeria* culture (cultured and concentrated as described above) was added to the water of half of the tanks in each treatment group, creating four treatment groups: no transfer (None), vertical transfer (Paternal), horizontal transfer (Horizontal), and combined transfer

(Paternal-Horizontal). The second treatment was scheduled on day 21 because the brood pouch of the males is significantly stretched at this stage, becoming more permeable to environmental microbes. Additionally, the developing embryos are suggested to open their mouths around this time during late pregnancy, allowing for early microbial colonization of the gut (Schneider et al. 2023). Two days after this treatment (day 23), pregnancy was terminated to avoid additional contamination of the newborn juveniles with environmental microbes. The remaining males and water were sampled following the same procedures as for the mid-pregnancy sampling. Two juvenile fish from each father were dissected into gut and body tissues and stored separately. The remaining juveniles were transferred to small plastic tanks and raised for an additional seven days (7 dpr). At the end of this period (day 30), another two juveniles per tank were dissected into gut and body tissues, while the remaining juveniles were plated on kanamycin-selective MB agar or preserved in RNAlater for future analysis.

Sample processing

DNA was extracted from brood pouch swabs, juvenile tissues, water filters and pure spike bacteria cultures with the DNeasy 96 Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany), following the pre-treatment for gram-positive bacteria using enzymatic lysis buffer. The 16S rRNA V3/V4 hypervariable regions were sequenced over Illumina Miseq amplicon sequencing at the Institute of Clinical Molecular Biology (IKMB, Kiel, Germany) using the primer pairs 341F and 806R as described by Tanger et al. (2024). In addition, the extracted DNA was analyzed twice using quantitative PCR (qPCR) aiming to detect the fluorescent GFP and mCherry genes of the experimentally introduced bacterial strains. Both approaches utilized the same primers (Supplementary Table 1) but differ in the composition of the template DNA. In the first approach, qPCR was performed directly on the extracted DNA, containing all DNA present in the sample. In the second approach, the DNA was pre-treated with Plasmid-Safe™ ATP-Dependent DNase (LGC Ltd, Teddington, England) to selectively degrade linear DNA from the pipefish host and other microbes while preserving circular plasmid DNA. This enzymatic digestion step was applied to reduce the background of fish genomic DNA, thereby enhancing the detection of the target genes located on the plasmid. Standard curves were generated using serial dilutions of plasmid DNA containing the GFP and mCherry genes. No-template controls (NTCs) were included to monitor potential contamination during qPCR.

Data analysis and statistics

Demultiplexed paired-end fastq files were used as input files. Sequences were merged, quality filtered, and analyzed using QIIME2 version 2019.10 (Caporaso et al. 2010). The DADA2 software package within QIIME2 was used for modeling and correcting the sequenced fastq files including the removal of chimeras using the “consensus” method (Callahan et al. 2016). Due to a drop of quality scores, sequences were truncated 40 bases from forward and 105 bases from reverse reads (forward 260; reverse 195). For taxa comparison, relative abundances based on all obtained reads were used. The QIIME2 q2-feature-classifier plugin was used. The Naïve Bayes classifier that was trained on the SILVA138 99% OTUs full-length sequence database (Quast et al. 2013) and subsequent sequences from our dataset were assigned to ASVs from SILVA database with similarity on the 99% level. The phyloseq package v.1.46.0 (McMurdie and Holmes 2013) was used for further analyses and data visualization of the QIIME2 output. Microbial count data was normalized, log-transformed and filtered to only preserve ASVs that are present in >20% of the samples. The core microbiota was identified to determine microbial taxa consistently present across non-treated tissues, as these are thought to play essential roles in maintaining function, resilience, and health (Salonen et al. 2012; Neu et al. 2021). To identify the core microbiota, an abundance threshold of 0.0001 was used to detect low-abundance strains, and a prevalence threshold of 0.5 ensured strains were present in over 50% of samples as suggested in previous studies (Astudillo-García et al. 2020; Hoffbeck et al. 2023). The analysis was conducted at ASV level, with results collapsed at genus level for interpretation, following a workflow described by Shetty et al. (2017). Alpha diversity indices were estimated for Shannon. Influence of treatment on juvenile alpha diversity was tested in an ANCOVA with treatment as fixed factor and time as covariable and their interaction. When day 10 was included, a fully reciprocal analysis was performed with only the paternal and control treatments. For analyses including all treatment groups, day 10 was excluded due to the horizontal treatment being applied only later in pregnancy. Indicator species analysis using MULTIPATT with 999 permutations was performed using the R package indicpecies (Cáceres and Legendre 2009). This analysis utilizes the relative abundance and frequency of species occurrence within defined (treatment) groups to identify key species that are most characteristic of those groups. PCAs and PCA biplots were calculated and visualized with FactoMineR (Lê et al. 2008). Differences between treatment groups were statistically assessed with a MANOVA for PCA dimensions 1 to 6, followed by an ANOVA to obtain p-values. In addition, treatment differences were statistically assessed with a PERMANOVA on normalized, log-transformed counts.

To assess the presence and abundance of the experimentally introduced *Marinomonas*-GFP and *Ruegeria*-mCherry spike cultures, a total of 10 ASVs were identified through sequence analysis of the pure cultures (Supplementary Table 2, Supplementary Figure 1 and 2, Supplementary Spreadsheet 1). These ASV IDs were then used to screen the microbiota samples collected during the experiment. ASV3404 (*Ralstonia* sp.) was excluded from this screening, despite its high abundance in the pure cultures, due to its well-documented role as a common contaminant in DNA extraction kits and PCR reagents, which can result in its erroneous detection in microbiota datasets (Salter et al. 2014). Relative abundance of spike community strains within the microbiome were analyzed with generalized least squares (GLS) fit by restricted maximum likelihood (REML) using normalized counts and visualized as box plots and heatmaps on a log-transformed scale due to extremely high variances between individual samples of a treatment group.

RESULTS

This experiment aimed to investigate how different pathways (vertical vs. horizontal and their combination) of transgenerational microbial transfer influenced early microbial colonization of developing embryos during pregnancy and juveniles of *Syngnathus typhle* until 7 days post release (7 dpr). In addition, an approach to visualize microbial transfer using fluorescently tagged bacterial strains was tested. 200 samples were submitted to 16S rRNA MiSeq amplicon sequencing. A total of 5,190,229 reads were returned (24598.24 ± 888.67 mean reads/sample \pm SE), along with 4508 representative sequences (Supplementary Table 3).

Untreated control group as a baseline

Previous studies of the sex-specific microbiome in pipefish have demonstrated sex-, tissue-, and pregnancy stage-specific variations in microbial composition (Beemelmans et al. 2019; Tanger et al. 2024). Focusing on the untreated microbial control samples as well as the water samples of this experiment, permitted to support previous findings and to establish a baseline for the present study.

Tissue and time, respectively pregnancy stage (d10: mid; d23: late) both strongly influenced microbial beta diversity (ANOVA: tissue: $p < 0.001$; time / pregnancy stage: $p < 0.001$; PERMANOVA: tissue: $p = 0.001$; time / pregnancy stage: $p = 0.009$), however, the interaction of

tissue x time had no effect. Microbial communities of adults and embryos overlap at day 10, which suggests that at mid pregnancy embryos harbor a similar microbiota composition as the paternal brood pouch. In contrast, surface-sterilized embryos (sterile embryos) at this stage cluster further from the paternal pouch and the non-surface-sterilized embryos (embryos), and resembled the paternal brood pouch microbiota during late pregnancy (d23). This indicates that the microbiome inside the embryo (sterile embryos) differs from the whole-body microbiome (embryos), and that the microbial community composition changes throughout male pregnancy, a result consistent with earlier observations (Beemelmans et al. 2019; Tanger et al. 2024). These results were supported by distinct clusters of juvenile fish (whole-body and gut) microbiomes at birth (d23) that were distinct from both, adults and embryos, which suggests that a unique, tissue-specific offspring microbiome already developed within the brood pouch. Also, water samples taken during mid and late pregnancy formed distinct clusters, which emphasizes the difference between environmental microbes and pipefish-specific microbes but also indicates a temporal shift in the water microbiome (Figure 2).

The core microbiota revealed insights into consistent taxa characteristic of *S. typhle* in general, as well as those specific to each tissue type and time point. Overall, the *S. typhle* core microbiota (adults + juveniles) is characterized by strains from the genera *Flavobacterium*, *Vibrio*, and *Pseudoalteromonas*. While overlap among core taxa exists, each tissue exhibited distinct microbial communities with temporal and tissue-specific patterns, aligning with the observed differences in beta diversity. In the male brood pouch, the core microbiota shifts from a dominance of *Vibrio*, *Pseudoalteromonas*, and *Pseudomonas* as the most prevalent genera during mid pregnancy (d10) to *Sulfitobacter*, *Litoreibacter*, and *Defluviimonas* in late pregnancy (d23); *Spongiivirga* was prevalent at both stages. Developing embryos (d10) had a core microbiota resembling the surrounding brood pouch, dominated by *Vibrio*, *Pseudoalteromonas*, *Pseudomonas*, and *Spongiivirga*, while surface-sterilized embryos showed a distinct internal microbiota composition, characterized by *Acinetobacter*, *Bacteroides*, and *Alkalibacterium*. At birth (d23), the juvenile core microbiota, both whole-body and gut, included *Acinetobacter* and *Flavobacterium*. By 7 days post-release (7 dpr, d30), distinct differences emerged between whole-body and gut microbiomes: the former was shaped by high prevalences of *Pseudoarcobacter*, *Labrenzia*, and *Alteromonas*, while the latter showed a strong presence of *Vibrio*, along with *Bacteroides*, *Pseudomonas*, and *Labrenzia*. The water microbiome showed the most striking shift over time suggesting a dynamic restructuring, with a reduction in diversity and a shift toward specific dominant taxa. At day 10, the core microbiota comprised a large and diverse group of 77 ASVs, including *NS3a_marine_group*, *Flavobacterium*, *RS62_marine_group*, *SAR92_clade*,

Vibrio, and *Pseudohongiella* as the most prevalent genera. By day 23, the core group had narrowed to 34 ASVs, dominated by *Glaciecola*, *Pseudorhodobacter*, *Celeribacter*, and *Oleispira*. An overview of the top five core microbiota strains for each tissue by relative abundance across all samples is provided in Supplementary Table 4. Heatmap visualizations depicting the core microbiota composition across different tissues are presented in Supplementary Figure 3. The complete list of ASVs contributing to the core microbiota is available in Supplementary Spreadsheet 2.

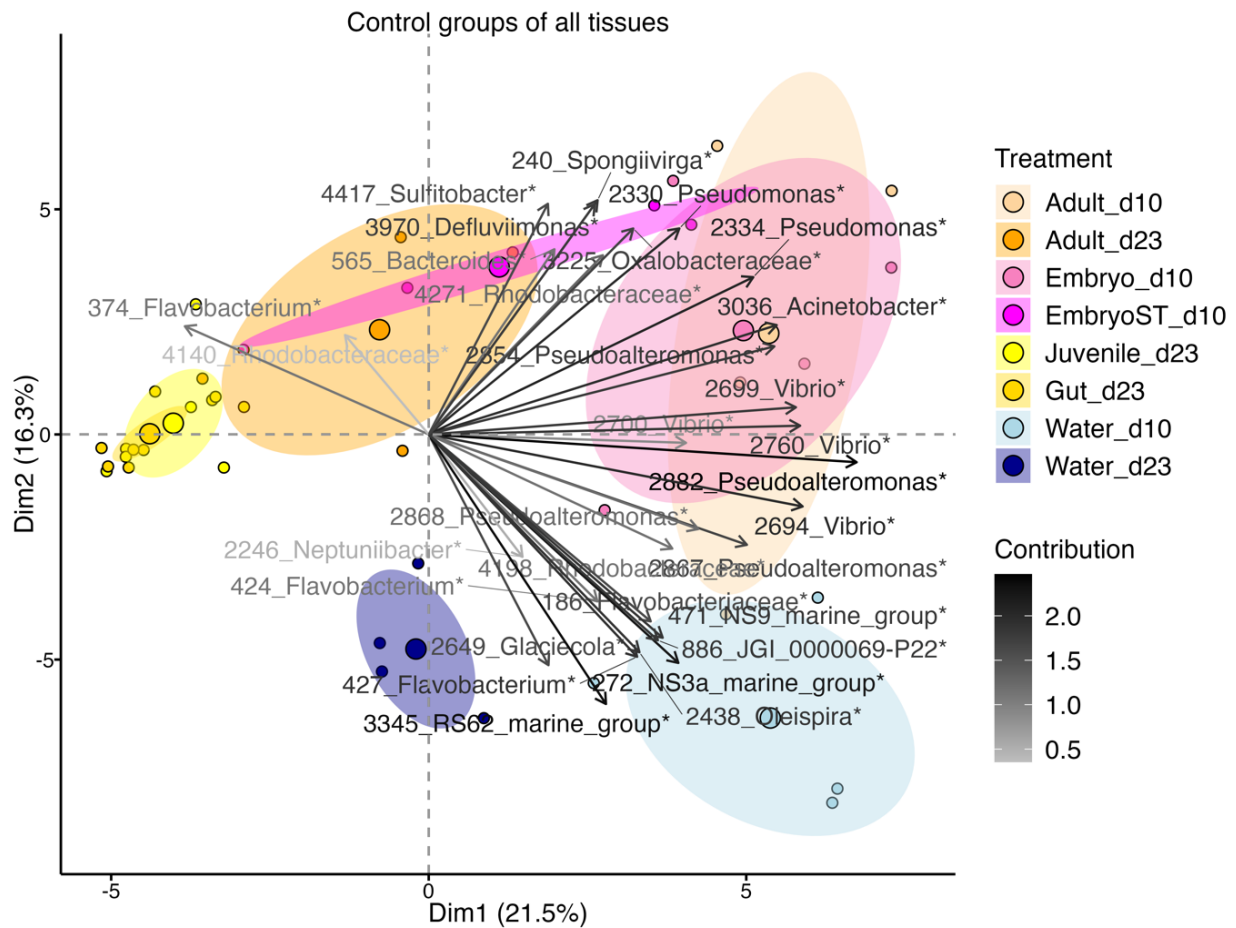


Figure 2: PCA biplot showing the microbial composition of untreated control samples from different tissue types from mid (d10) to late pregnancy (d23). Color tones represent tissue groups (red = adults, orange = developing embryos, green = newborn juveniles, blue = water). Ellipses show a confidence interval around the group's mean. Factor map shows ASV ID and genus name respectively family name

Having established the baseline microbiota composition and its temporal shifts in the untreated control groups, we then focused on the effects of the experimental treatments. First, we examined the impact of paternal microbial transfer throughout pregnancy. Second, we explored the effects of horizontal transfer that was introduced during late pregnancy only.

Paternal microbial transfer (d0 – d10)

Here, we intended to unravel the impact of vertical transfer by treating the paternal brood pouch with GFP-expressing *Marinomonas* prior to mating. We ribotyped (16s rRNA amplicon sequencing) and compared the microbiota of the paternal brood pouch and developing embryos during mid-pregnancy (d10). At this stage, a clear separation was observed in the microbial beta diversity between the *Marinomonas*-GFP spike treatment and the control across all studied tissues (adults, embryos, and sterile embryos). Particularly in the visual representation (Figure 3A) the paternal spike treatment appeared to reduce group variance, however, there was no statistical difference (Levene's test: $p = 0.34$; Fligner-Killeen test: $p = 0.46$).

Both treatment (paternal vs. control) and tissue type (adult vs. embryo vs. sterile embryo) significantly influenced microbial composition (ANOVA: tissue: $p = 0.003$; treatment: $p = 0.0004$; PERMANOVA: tissue: $p = 0.001$; treatment: $p = 0.004$). The interaction of tissue* treatment was not significant. In the paternal transfer group, where fathers received a spike treatment (Adult_pat, Embryo_pat, and EmbryoST_pat; Figure 3A), the microbiota is now dominated by *Sulfitobacter* and *Marinomonas*, contrasting with the *Pseudomonas*, *Acinetobacter* and *Pseudoalteromonas* dominance observed in the untreated group. Notably, ASVs classified as *Marinomonas* appeared frequently in the paternal transfer group, however, while they match at the genus level, most ASV sequences differed from those of our experimentally added strains (Supplementary Table 2), therefore the results have to be treated with caution. Focusing only on the microbiota composition of the developing embryos, there was a clear clustering that distinguished both embryo types (surface-sterilized vs. untreated) as well as treatment groups (paternal transfer vs. control) (Figure 3B). This separation is statistically supported, with both embryo type and treatment showing significant effects on microbial composition (ANOVA: tissue: $p = 0.026$; treatment, $p = 0.01$; PERMANOVA: tissue: $p = 0.006$; treatment: $p = 0.028$). The interaction of tissue* treatment was not significant.

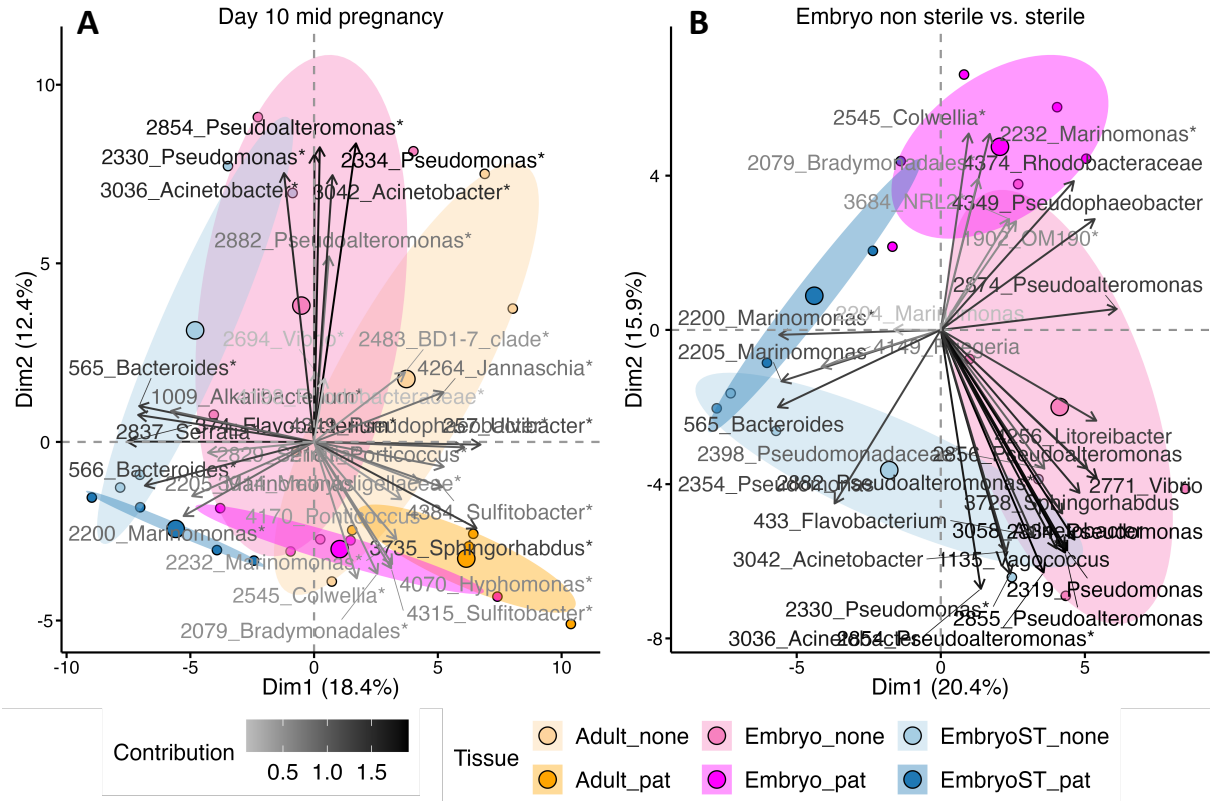


Figure 3: Microbiota of paternal brood pouch and embryos during mid pregnancy (A) and detailed visualization of embryonal microbiota (B). Color tones represent tissue groups (yellow = adults, pink = developing embryos, blue = surface-sterilized embryos). Ellipses show a confidence interval around the group's mean. Factor map shows ASV ID and genus name respectively family name

When comparing the external and internal microbiota of developing embryos (embryo vs. embryo sterile) to juvenile pipefish (juvenile vs. gut) at birth (d23), we observed a clear temporal shift in microbial composition during late pregnancy. In the PCA biplot (Figure 4), this shift is evident along dimension 1, which strongly separates the tissues of embryos at day 10 from the tissues of juveniles at day 23 (ANOVA: tissue: $p < 0.001$, PERMANOVA: tissue: 0.001). In contrast, treatment had a much smaller (ANOVA: treatment: $p = 0.02$), or, depending on the analysis chosen, even neglectable impact (PERMANOVA: treatment: $p = 0.177$), the treatment effect was potentially masked by the strong tissue, respectively time effect. The interaction of tissue*treatment did not reach significance in both analyses. In embryos from treated fathers (Embryo_pat) *Sulfitobacter* were particularly numerous, consistent with the treatment-specific strains described earlier.

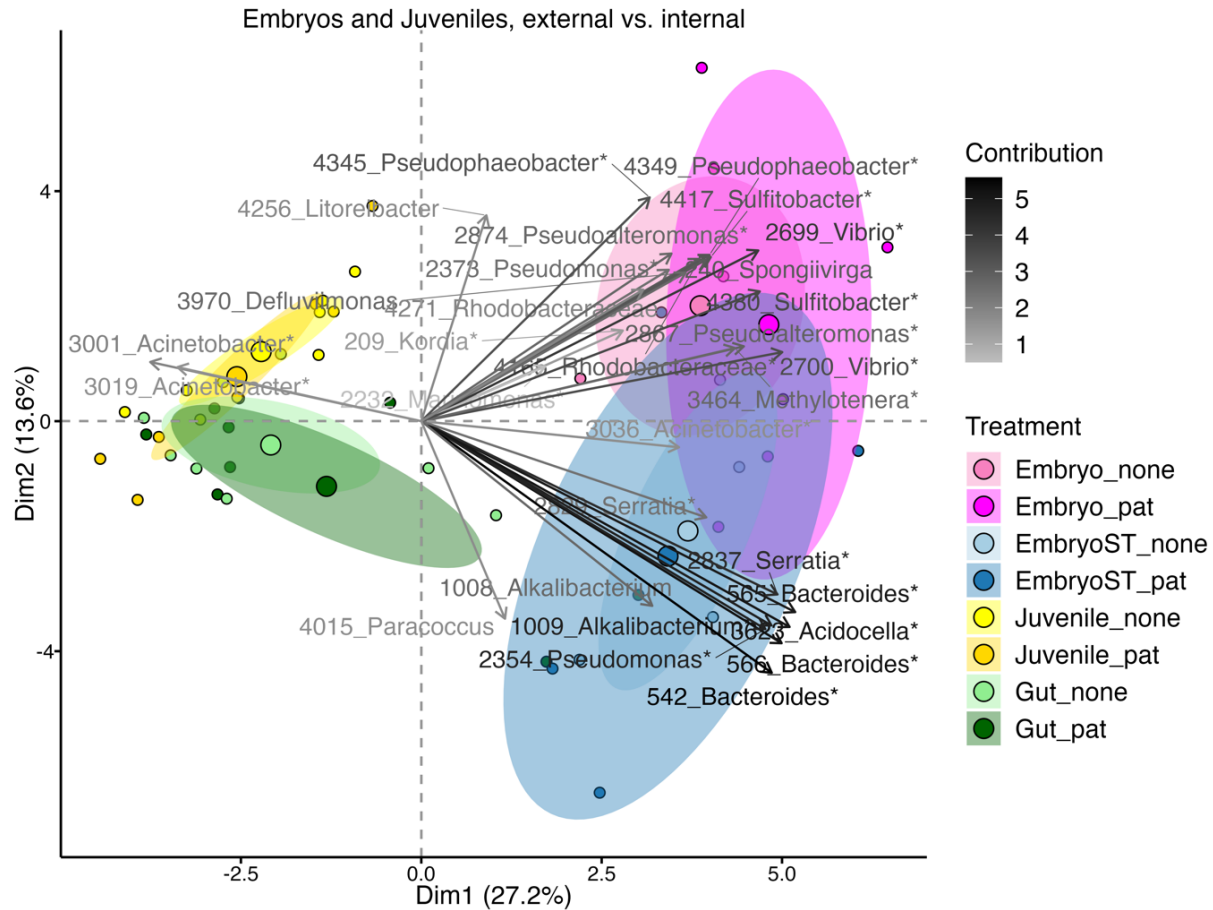


Figure 4: PCA biplot of developing embryos and juveniles at birth comparing external and internal microbiota composition. Color tones represent tissue groups (pink = developing embryos, blue = surface-sterilized embryos, yellow = whole-body juveniles, green = juvenile gut). Ellipses show a confidence interval around the group's mean. Factor map shows ASV ID and genus name respectively family name.

Paternal and horizontal microbial transfer (d21 – d30):

We aimed to shed light on horizontal microbial transfer by adding mCherry-expressing *Ruegeria* to the water during late pregnancy (d21) when the brood pouch becomes permeable for colonization by environmental microbes and to study its interaction with the earlier vertical transfer initiated by a paternal brood pouch treatment. A highly significant temporal shift in microbiota composition was observed between mid- and late pregnancy (ANOVA: $p < 0.001$; PERMANOVA: $p = 0.001$). Despite some visual overlap among treatment groups during late pregnancy, both horizontal (ANOVA: $p = 0.0002$; PERMANOVA: $p = 0.047$) and vertical (ANOVA: $p = 0.02$; PERMANOVA: $p = 0.422$) treatments influenced brood pouch microbiota, though the vertical effect was only significant in the ANOVA. This suggests that both treatments occurred at a critical time for microbial colonization in the brood pouch tissue (Figure 5). However, the interaction of paternal*horizontal transfer remained non-significant (ANOVA: $p = 0.6$; PERMANOVA: $p = 0.7$).

Tissues that were exposed to the horizontal-transferred *Ruegeria* strain (Horizontal, Paternal-Horizontal) were dominated by *Flavobacterium*, *Methylobacteriaceae* and *Parvibaculales*.

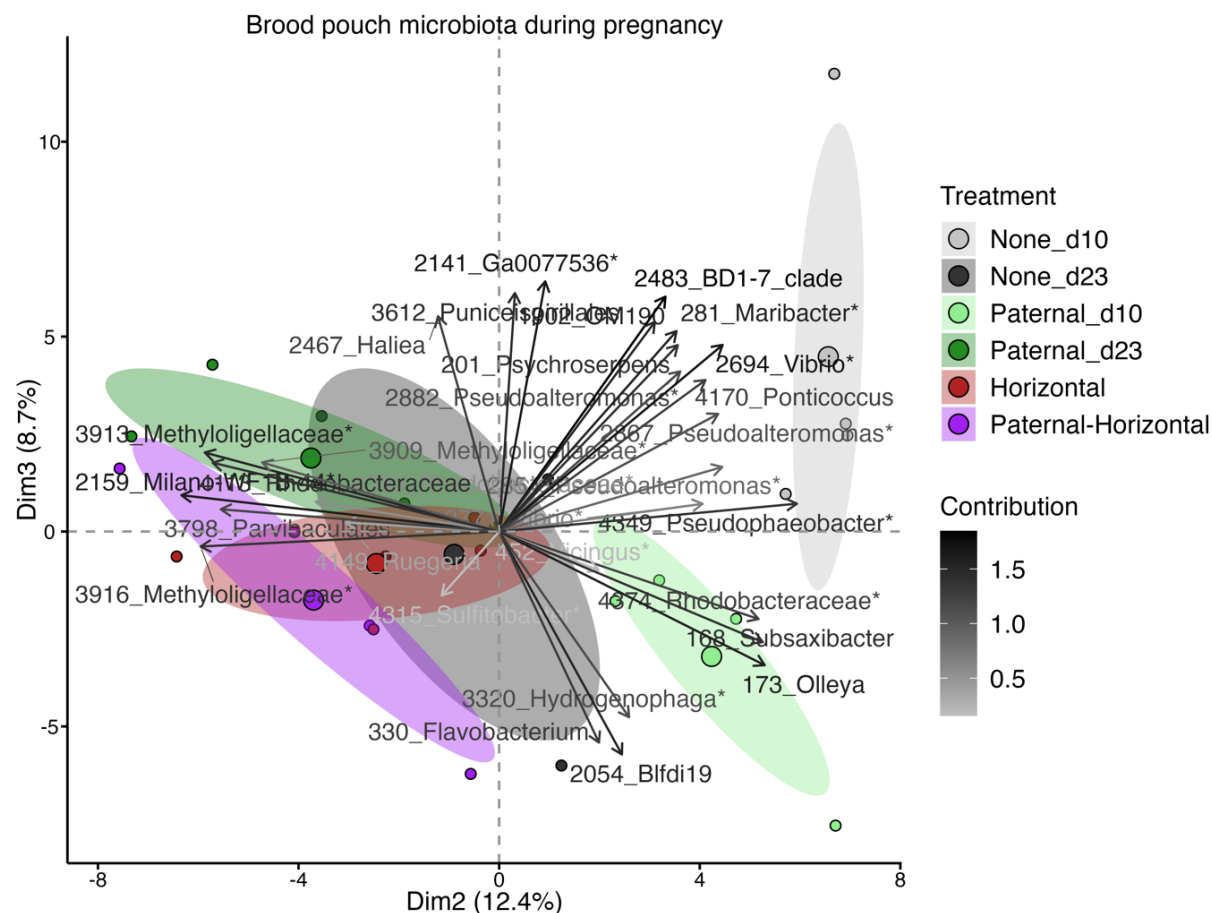


Figure 5: Shifts in microbiota composition (beta diversity) during pregnancy. Colors represent treatment groups and pregnancy stages. Ellipses show a confidence interval around the group's mean. Factor map shows ASV ID and genus name respectively family name.

Development of juvenile microbiota post release

At birth (day 23), both the juvenile whole-body and gut microbiota were ribotyped, with sampling repeated at 7 days post-release (7 dpr, day 30 of experiment) to evaluate microbial colonization during the first days of life. Alpha diversity (Shannon) of the whole-body microbiota was significantly affected by the horizontal treatment, resulting in increased diversity over time compared to the control and paternal treatment groups (ANCOVA paternal: $p = 0.41$; horizontal: $p = 0.002$). In the visual representation (Figure 6A) this effect was particularly pronounced in the combined Paternal-Horizontal group, which showed the highest alpha diversity at 7 dpr, indicating an interaction with the previously applied paternal treatment, however, the statistically-tested

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interaction of paternal*horizontal treatment was not significant (ANCOVA: $p = 0.23$). While time showed a suggestive trend towards influencing microbial alpha diversity, the effect was not statistically significant, suggesting that colonization may have already occurred before birth (ANCOVA time: $p = 0.054$). In contrast, alpha diversity of the juvenile gut (Figure 6B) showed a strong time effect, steadily increasing its microbiome diversity in all four groups, unaffected by treatment (ANCOVA paternal: $p = 0.83$; horizontal: $p = 0.44$; time: $p < 0.001$; paternal*horizontal: $p = 0.36$).

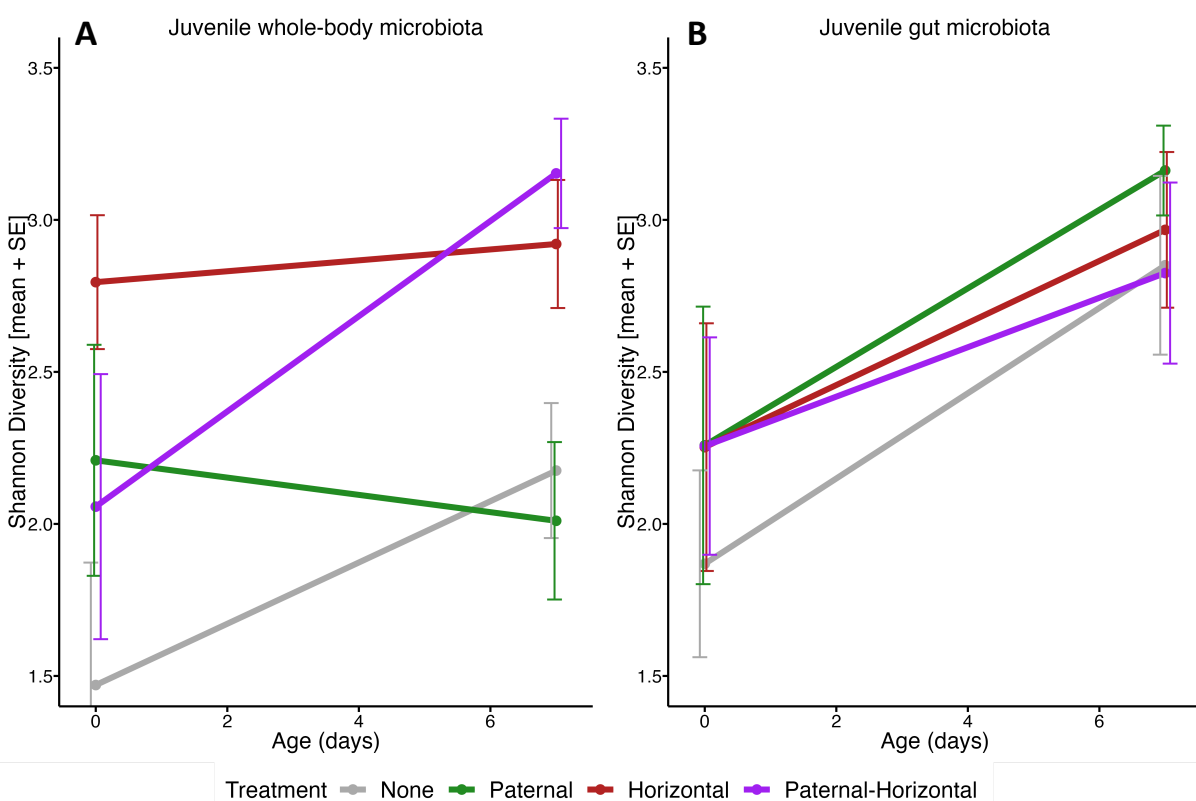


Figure 6: Alpha diversity (Shannon index) of juvenile whole body (A) and gut (B) microbiota from birth until 7 days post release. Colors indicate treatment group (during pregnancy). Data points represent mean diversity \pm standard error (SE).

Embryonal microbial diversity, both external (embryos) and internal (embryos sterile), appeared much higher during pregnancy than at birth in the visualizations that included all treatment groups (Supplementary Figure 4). For fully reciprocal statistical testing, only the paternal and control groups were analyzed for time points during pregnancy (day 10), revealing no significant effects of treatment or time on alpha diversity in either the external or internal microbiota. The full test statistics for alpha diversity can be found in Supplementary Spreadsheet 5.

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At birth, the interaction of paternal and horizontal treatments showed a trend towards influencing the beta diversity of the whole-body microbiota (paternal*horizontal; ANOVA: $p = 0.091$), PERMANOVA: $p = 0.093$) (Figure 7A), however, the main effects, i.e., paternal and horizontal, were not significant. By 7 days post-release, horizontal transfer significantly shaped offspring whole-body microbiota (ANOVA: $p = 0.04$; PERMANOVA: $p = 0.05$), while the paternal treatment showed a non-significant trend toward shaping the microbiota in ANOVA ($p = 0.09$) but not in PERMANOVA ($p = 0.121$) (Figure 7C). The strongest visual separation in the biplot displaying the whole-body microbiomes of the juveniles was along dimension 1, separating paternal from horizontal transfer. Surprisingly, the horizontal transfer group (red ellipse) was dominated by *Marinomonas*, the strain originally applied to examine paternal transfer. The juvenile gut microbiota, in contrast, was primarily shaped by the horizontal treatment. This effect became already evident at birth (ANOVA: horizontal: $p = 0.01$, PERMANOVA: horizontal: $p = 0.26$) (Figure 7B) and persisted until 7 days post-release (ANOVA: p horizontal = 0.03; PERMANOVA: p horizontal = 0.74) (Figure 7D). The paternal treatment, however, did not influence the gut microbiota in this context. At 7 days post-release (7 dpr), gut tissue diversity was markedly higher, indicated by a broader array of indicator species distributed across treatments in the biplot (Figure 7D). Notably, the Paternal-Horizontal transfer group (indicated by the purple ellipse) was initially dominated by *Bacteroides* at birth, but a shift to *Vibrio* was observed later. *Vibrio* is commonly found in both pipefish and its surrounding environment, but its high prevalence was notably restricted to this specific group and time point. Additionally, in this tissue, strain 2847_ *Pseudoalteromonas* was identified as the only indicator species within the entire dataset, specifically associated with the horizontal transfer group both at birth (0 dpr) and at 7 days post-release (7 dpr).

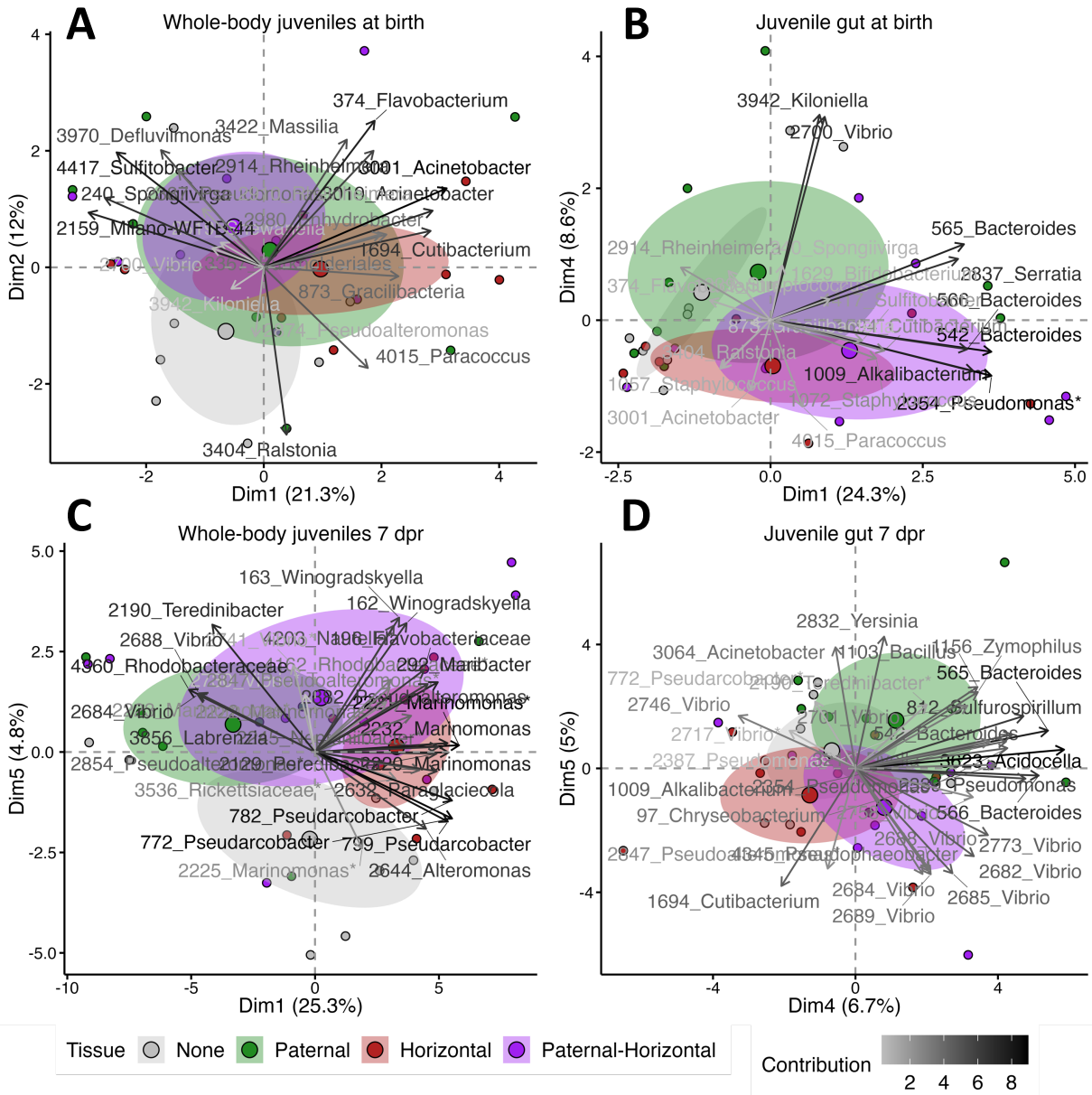


Figure 7: PCA biplots showing microbial beta diversity of whole-body juveniles at birth (A), after 7 days (C) and juvenile gut at birth (B) and after 7 days (D). Colors indicate treatment during pregnancy, ellipses show a confident interval around the group's mean. Factor map shows ASV ID and genus name respectively family name.

Indicator species analysis

To identify bacterial strains characteristic of each treatment group, an indicator species analysis was performed for each tissue and time point. Strains identified as significant indicator species are marked with an asterisk (*) in the PCA biplots. The remaining species displayed were sorted based on their contribution to the observed patterns, with species of highest contribution added sequentially until a total of 30 species were included in each biplot. A full list of indicator species

and their associations with treatment groups can be found in Supplementary Spreadsheet 3 (sampling during pregnancy) and Supplementary Spreadsheet 4 (post-release sampling). Despite introducing high concentrations of spike bacteria into the brood pouch (*Marinomonas*-GFP) and water (*Ruegeria*-mCherry), neither strain appeared as a significant indicator species. Only one of the spike strains (ASV2200_*Marinomonas*) was detected in both untreated embryos and embryos from spike-treated fathers. While its presence was anticipated in both groups, given that this strain had previously been identified as a member of the natural brood pouch microbiota, we would have expected a higher number of hits for the introduced *Marinomonas*-GFP strain at ASV level following its direct introduction to the pouch.

Searching for the spike bacteria in the adults and offspring

This study aimed to establish a proof-of-concept for tracking bacterial transfer throughout pregnancy and early microbial colonization using fluorescently tagged strains. These strains were originally isolated from Baltic Sea pipefish and identified in a previous study on the sex-specific microbiome of *Syngnathus typhle* (Wagner et al. 2024) as potential candidates for paternal transfer. *Marinomonas* and *Ruegeria*, two marine bacteria commonly found in the male brood pouch, are considered key early microbial colonizers (Beemelmans et al. 2019). While *Marinomonas* was selected to represent paternal transfer due to its high prevalence in the brood pouch and therefore its potential role in paternal provisioning (Beemelmans et al. 2019), *Ruegeria* was chosen to study horizontal transfer because of its versatility in the marine environment and frequent association with the gastrointestinal tracts of marine fishes, such as cod larvae (Porsby et al. 2008; Romero et al. 2014). To track the introduced spike strains – *Marinomonas* (paternal transfer via the brood pouch, introduced pre-mating) and *Ruegeria* (horizontal transfer via water, introduced at day 21) – samples were screened using three methods: direct plating on kanamycin-selective agar plates, qPCR with GFP- and mCherry-specific primers, and amplicon sequencing data analysis based on ASV IDs. Since the fluorescently tagged strains carried kanamycin resistance genes, we expected only these strains to grow on the agar plates. However, after incubation, all plates were overgrown with a bacterial lawn, making it impossible to identify the fluorescently tagged bacteria or proceed with further analysis. The first qPCR approach, performed on untreated extracted DNA, yielded unclear patterns, likely due to non-specific binding of the primers to the fish's genomic DNA. In contrast, the second approach, which involved enzymatic digestion to selectively remove linear genomic host DNA, appeared more promising, as only plasmid DNA remained after digestion. Despite this improvement, the results were still at the lower detection limit or showed ambiguous patterns.

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Additionally, a high number of potential false positives were observed, suggesting that either the primers lacked sufficient specificity or contamination occurred during sampling or sample processing.

Consequently, we proceeded with screening the amplicon sequencing data. Neither *Marinomonas* nor *Ruegeria* could be detected in the paternal brood pouch tissue or water during mid-pregnancy (d10), nor in the juvenile gut samples at 7 days post-release (7 dpr, d30). However, both strains were identified in nearly all sterile embryo samples (d10), regardless of the treatment group, while they were absent in non-sterile embryos, except for a single control sample. This suggests the possibility of maternal transfer ovarian transfer through the inside of the developing embryos, which may have only become detectable after surface sterilization. During late pregnancy (d23), *Ruegeria* strains were identified in the water samples from the expected horizontal treatment groups. This confirms that the strain could be identified in the environment (GLS horizontal: $p = 0.04$). While no treatment effects were observed in any of the tissues (Supplementary Spreadsheet 6), ASVs assigned to the experimentally added *Ruegeria* strain were detected in approximately half of the samples across the Horizontal, Paternal-Horizontal, and Control groups. In the whole-body microbiota of juveniles at birth, ASVs assigned to the *Marinomonas* strain were prevalent in the paternal treatment group, while ASVs assigned to *Ruegeria* were detected in one sample from the treatment groups None, Paternal, and Paternal-Horizontal. However, it is important to note that *Marinomonas* ASVs were not detected in the brood pouch during pregnancy. This discrepancy indicates that prevalence filtering, while necessary to avoid unspecific patterns masking treatment effects, may act as a confounding factor in interpreting these results. However, by 7 days post-release (7 dpr), only *Marinomonas* remained detectable across the all groups except Paternal-Horizontal. In contrast, in the juvenile gut at birth, ASVs assigned to *Ruegeria* – the horizontally transferred strain – were detected, with the highest counts in the paternal treatment group, despite the horizontal treatment showing a significant ANOVA result ($p = 0.03$) which indicates a treatment effect. This finding indicates that the paternal treatment may have facilitated increased colonization by *Ruegeria*, a natural member of the pipefish microbiome. Matching *Ruegeria* ASVs were already present in developing embryos (both sterile and non-sterile) but were not detected in the water of non-horizontally treated fish at any of the time points. Although we did not observe a significant interaction between paternal and horizontal treatments, this potential connection highlights the need for further investigation to unravel the underlying mechanisms of microbial colonization. While we observed treatment effects on the overall microbiota composition, no consistent patterns emerged when screening for the spike strains across treatment groups.

DISCUSSION

This study aimed to assess the temporal dynamics of vertical and horizontal microbial transmission routes, and to explore their influence on early microbial colonization in the sex role-reversed pipefish, *Syngnathus typhle*. For visualization, fluorescently tagged strains were used. Prior to mating, male pipefish were treated with concentrated cultures of a GFP-expressing *Marinomonas* strain to track vertical transmission throughout pregnancy. Shortly before the males gave birth, the water was supplemented with a mCherry-expressing *Ruegeria* strain to additionally assess the effects of horizontal transfer and the interaction between these two transmission routes. After birth, the offspring were raised for one week to further monitor potential early-life microbial shifts.

Core microbiota

Natural microbial communities are highly complex, comprising hundreds to thousands of species across various taxa, leading to significant analytical challenges due to dynamic interactions (Astudillo-García et al. 2017). The “core” microbiota concept helps focus on persistent community members to identify key species. We could identify a specific *S. typhle* core microbiota for all our samples, comprising the genera *Flavobacterium*, *Vibrio*, and *Pseudoalteromonas*. However, highest prevalences (80-100%) were detected at very low relative abundance thresholds ($\leq 0.01\%$) (Supplementary Figure 3), suggesting that either a microbial community does not need to present in high abundances in order to be persistent across all samples or that a stable microbiome is possibly not required for the host’s lifestyle. Highly prevalent taxa found in the untreated male brood pouch were *Vibrio*, *Pseudoalteromonas*, *Sulfitobacter* and *Spongiivirga* (Supplementary Table 4). All of these strains are common marine bacteria that are associated with marine animals and are typical indicator species for late pregnancy in *S. typhle* (Beemelmanns et al. 2019). *Vibrio* is a consistently present member of the pipefish microbiome, representing a group of opportunistic bacteria with some strains fluctuating between beneficial or pathogenic effects on their host, depending on environmental conditions (Goehlich et al. 2021; Chibani et al. 2023). *Pseudoalteromonas* is known for its antibacterial and antifungal properties, serving as a natural antagonist to harmful microbes (Bowman 2007). *Sulfitobacter* and *Spongiivirga* are known for their roles in nutrient and sulfur cycling, vital for balancing the marine ecosystem (Sorokin et al. 2015; Kiran et al. 2018). The developing embryos during mid-pregnancy (d10) had a core microbiota similar to the male brood pouch which is reasonable given the close contact of the paternal tissues. The surface-sterilized embryos, however, had a different core

microbiota that included *Acinetobacter* and *Bacteroides*. *Acinetobacter* is a common member of the male brood pouch microbial community although in literature it is more recognized for its environmental role in biodegradation and its infectious potential in clinical settings (Doughari et al. 2011). *Bacteroides* can represent opportunistic microbes and are commonly found in the gut microbiota, assisting digestion and immune modulation (Wexler 2007). The juvenile core microbiota was largely different from the one of the paternal brood pouches. Here, we frequently found *Flavobacterium*, *Labrenzia*, *Pseudomonas* and *Alteromonas*. *Alteromonas* is known as a commensal fish gastrointestinal strain, whereas *Pseudomonas* was described to be highly prevalent in late pipefish male pregnancy, particularly following an immune activation of the adults (Beemelmanns et al. 2019). *Flavobacterium* is known for organic matter degradation and nutrient cycling but includes some fish pathogenic members (Bernardet and Bowman 2006). In contrast, *Labrenzia* is characteristic for its symbiotic and protective role in marine ecosystems, potentially enhancing resilience in marine hosts (Zhong et al. 2021). In conclusion, the core microbiota of *S. typhle* comprises diverse bacterial genera, each fulfilling unique roles that support host health and ecological balance ranging from nutrient cycling and pathogenic dynamics to providing antimicrobial benefits.

Treatment-specific bacterial strains and niche colonization

Our data provided evidence for shifts in microbial communities following paternal (*Marinomonas*) or environmental (*Ruegeria*) exposures. Although the experimentally added strains were not frequently detected, this pattern suggests underlying mechanisms that may drive specific microbial interactions in response to treatment conditions. It is possible that colonization was mainly transient, and the sampling intervals (day 10, birth at day 23, and 7 days post-release) have missed the critical time points for detection in the respective tissues.

Furthermore, the introduced strains may have colonized niches temporarily but were subsequently outcompeted by other microbes. This aligns with the principles of colonization resistance and competitive exclusion, where pre-existing strains in stable microbiomes often outcompete new strains (Caballero-Flores et al. 2023). In our previous study (Wagner et al. 2024), while antibiotics were applied to the fathers, the most pronounced effects on spike strain colonization were observed in groups that were not exposed to these antibiotics. This stark contrast may reflect the introduction of a microbial community in the prior study, compared to single strains in the present study, which possibly limited their competitive advantage. Additionally, the metabolic burden of maintaining fluorescent proteins might have reduced the fitness of spike strains compared to wild-type bacteria (Rang et al. 2003). Despite these

challenges, paternal treatment with *Marinomonas* led to an increased presence of *Sulfitobacter* and *Marinomonas* in male and embryo tissues. However, most ASVs identified as *Marinomonas* did not match the specific IDs of the introduced strains, resulting in negative outcomes in the ASV-level spike screening. This discrepancy could be due to sequence deviations, as ASV IDs are unique MD5 hashes based on representative sequences and generated during the QIIME2 workflow, where even a single base difference produces a new ASV ID (Schillebeeck 2018). Horizontal treatment with *Ruegeria* resulted in an increased prevalence of *Flavobacterium*, *Methylobacteriaceae*, and *Parvibaculales*. These shifts may reflect niche dynamics triggered by introduced bacteria or competition with resident strains. Throughout colonization, it is likely that the introduced bacteria modified certain environmental parameters – such as nutrient availability, pH, or oxygen levels – or depleted specific resources, creating conditions that promoted colonization by other strains that are more successful in exploiting these altered niches (Ratzke and Gore 2018; Baquero et al. 2021) by which the respective spike strains were outcompeted. Remarkably, *Marinomonas* was unexpectedly prevalent in whole-body juveniles from horizontal treatment groups, while *Vibrio* dominated juvenile gut samples in the paternal-horizontal group 7 days post-release (7 dpr, d30). Both strains are members of the natural pipefish microbiota – *Marinomonas* as an initial colonizer and *Vibrio* as a ubiquitous strain – whose competitive advantage may have been enhanced by treatment conditions, allowing these bacteria to grow and dominate within the juvenile microbiome. *Marinomonas* may facilitate early niche establishment (Beemelmanns et al. 2019), while *Vibrio*'s metabolic flexibility and adaptability (Liang et al. 2022) could enable it to exploit altered environmental conditions and outcompete other strains in the gut. The increased permeability of the male brood pouch during late pregnancy may have further facilitated these environmental shifts. Future studies could address these dynamics by testing competition between tagged strains and their wild-types through competition assays, e.g., using flow cytometry as described by (Wendling et al. 2022), introducing higher concentrations of spike bacteria, or manipulating niches with antibiotics to temporarily reduce competition. Additionally, understanding the temporal dynamics of colonization remains a critical challenge. The current sampling intervals may have missed key time points when colonization effects or interactions were most pronounced. Incorporating a more frequent sampling scheme would increase accuracy and provide valuable insights into when microbial colonization occurs, which could clarify the dynamics driving these patterns.

Shifts in water microbial composition and potential implications on the fish

Our data indicates a strong shift and a decrease in microbial diversity in the water between day 10 and day 23, observed throughout significant differences in alpha diversity, beta diversity, and core microbiota analyses. Throughout male pregnancy, fish were kept in 20 L tanks with filtered, aerated Baltic Sea water. Typically, in larger aquaria systems, tanks are connected and water is circulated via a biofilter to regulate ammonia levels, which are generated as a byproduct of protein catabolism in fish (Bartelme et al. 2017). In our experimental setup, we used single unconnected tanks for each breeding pair, and decided against a biofilter to avoid potential impact of additional bacteria and to ensure consistency across tanks, especially regarding the horizontal transfer treatment. This environment, a closed system without water circulation, presumably led to several factors contributing to the microbial diversity shift. First, nutrient depletion over time may have favored competitive species adapted to low-nutrient conditions, which reduced the microbial diversity as less competitive strains were outcompeted (Ma et al. 2023). Although daily cleaning with a vacuum siphon was performed, the lack of water changes had possibly caused a slow accumulation of organic material from the fish and their food. This accumulation could enrich bacteria that have metabolized these compounds, contributing to shifts in community composition (Quero et al. 2020; Wu et al. 2022). Lastly, the limited influx of new microbes in the tank environment reduced diversity further as dominant strains occupied niches while others disappeared (Zhang et al. 2024). Together, these factors potentially contributed to a stable, low-diversity bacterial community adapted to the specific conditions of the tanks, which explains the observed microbial shifts throughout the experiment. We recognize that the enclosed tank conditions may have introduced a confounding factor in our analyses, as water beta diversity at day 23 increasingly resembled the microbial composition found in the fish. When comparing the top five most prevalent water core microbiota members from day 10 to day 23, distinct shifts are evident, as no genera were shared between the two time points. The only overlap was observed at day 10 with *Pseudoalteromonas* detected in both, fish and water microbiota. This suggests that the microbial composition in the water evolved over time, possibly influenced by fish activity or their metabolic byproducts. Additionally, by day 23, there was a notable divergence between the fish and the water microbiomes, pointing to specific community changes likely prompted by tank conditions and the interactions within (Supplementary Table 4). In natural larger water bodies, such as oceans or even lakes, continuous microbial input and water flow result in distinct ecological dynamics (Betiku et al. 2021). Our setup, however, lacked these characteristics and thus did not maintain a stable baseline. This may have led to potential biases in our observations and stresses the importance of acknowledging these constraints when interpreting the results. To

better address these limitations, future studies should include suitable true negative controls, such as water tanks without fish, to help disentangle potential reciprocal influences between fish and water microbiomes.

Impact of vertical and horizontal transmission

Vertical (parental) and horizontal (environmental) microbial transmission shape early microbial colonization. Paternal vertical transmission, initiated at mating and early male pregnancy, appeared to contribute to the initial offspring microbiota. Our experiment revealed an effect of paternal treatment on embryos during mid-pregnancy (d10), which then diminished, with no detection of the specific strain (at ASV level) in juveniles. While *Marinomonas* is commonly found in the brood pouch microbiota, it may not be an ideal candidate for tracking vertical transfer, as it was only very occasionally detected in isolated samples when examining the juvenile microbiota (Tanger et al. 2024; Wagner et al. 2024). Our analysis of microbial beta diversity indicates that the paternal treatment with *Marinomonas* affected the microbiome of embryos. At 7 days post-release (7 dpr), the observed influence further extends to the whole-body microbiota of juveniles. However, verifying transgenerational transfer would require the direct isolation of fluorescently tagged strains provided to the father. During the experiment, cultivation on kanamycin-containing agar plates and qPCR using primers targeting the fluorescent gene (GFP or mCherry) on the plasmid were attempted but not yet successful, requiring the development of new methods.

Temporal dynamics of early microbial colonization

The timing of microbial transmission, which we aimed to investigate by distinguishing between vertical and horizontal transfer treatments, plays a critical role in the development of the microbiome. Vertical transmission from the father, potentially beginning as early as during mating, provides the initial colonizers for the developing embryos. In contrast, horizontal transmission later in pregnancy introduces additional microbial species as the brood pouch becomes increasingly permeable. These distinct transmission routes likely influence the temporal dynamics of microbial composition, which we aimed to investigate with our experimental treatments.

A limitation of our setup is the artificial separation of vertical and horizontal transmission periods, which does not fully reflect natural processes, such as continuous vertical transmission throughout pregnancy or early horizontal transmission. Previous studies (Beemelmans et al. 2019; Tanger et al. 2024; Wagner et al. 2024) reported natural microbial shifts during pipefish

male pregnancy, particularly in late stages. Consistent with this, we observed temporal shifts in the microbiomes of the paternal brood pouch and embryos, particularly in controls, likely driven by environmental water microbial changes (Figure 2). These findings provide evidence that microbial communities undergo significant temporal shifts even without experimental treatments. In contrast, distinct treatment effects on alpha and beta diversity were observed (Figures 3, 5, 6, 7), indicating that our interventions influenced microbial dynamics beyond natural changes. By day 10, sterile embryos showed high counts of the experimentally added bacterial strains, demonstrating successful microbial transfer. This suggests that the treatments can accelerate or modify natural microbial colonization, potentially influencing host-microbe interactions and microbial community development.

Previous research on maternal and paternal transfer in this model system has suggested that the internal microbiome (represented here by sterile embryos) is primarily influenced by the maternal microbiome (Tanger et al. 2024). However, Beemelmanns et al. (2019) reported *Marinomonas* as being predominantly paternally associated, justifying its selection as a model for investigating paternal transfer in this study. The presence of these bacteria in sterile embryos, despite their absence in the paternal brood pouch, may suggest a higher flexibility in maternal and paternal microbial contributions than previously assumed. Without directly assessing the maternal egg microbiome, we can only speculate that the high *Marinomonas* counts have occurred via maternal provisioning. Methods that can precisely distinguish strains with GFP and mCherry molecular markers are currently under development and will facilitate more definitive conclusions. Finally, the persistent presence of experimental strains in control groups highlights the complexity of the natural microbiome. Despite introducing only two bacterial strains at specific time points, we were limited in tracking them, likely because existing microbial niches were already occupied, preventing the establishment of the fluorescently tagged bacteria without prior depletion with antibiotics as described in our previous work (Wagner et al. 2024).

Methodological limitations

A major challenge in this study was the limited number of animals available for experiments. Because sampling required sacrificing the fish and their embryos, we were only able to sample and sequence the microbiota once during pregnancy (d10). This restricted sampling schedule, resulting from the limited number of animals, may have caused us to miss important time points for initial microbial colonization and further constrained our ability to track the introduced bacterial strains over time. Furthermore, *S. typhle* is currently still being developed as a model organism

for host-microbe interaction studies. As the impact of environmental bacteria on their health and development is still largely unknown, it is challenging to raise and keep the fish under gnotobiotic conditions that would facilitate the detection of the target strains. Another layer of difficulty in drawing clear conclusions about transfer and colonization dynamics is the general complexity of microbial communities (He et al. 2020). Microbiomes are subject to natural variability, which may explain the lack of consistent patterns in some parts of the study, including instances where treatment effects were absent or less pronounced than expected (e.g., statistical trends ($0.05 > p > 0.10$)). Several factors may have contributed to these observations, including natural variability in microbial composition, enormous variations in the water environment over time, differences in microbial exposure during horizontal transfer, and the lack of antibiotic treatment prior to recolonization. Additionally, it was intended to screen the samples for the experimentally added fluorescent bacteria using three methods, out of which two (plate cultivation and qPCR) were not successful. The agar plates, which included kanamycin to selectively culture the fluorescent *Marinomonas* and *Ruegeria* strains, showed extensive overgrowth from strains with natural kanamycin resistance. This likely outcompeted the low-abundance fluorescent strains, impeding their detection. Based on our previous findings, kanamycin may not be the most effective choice for such applications. Future studies could benefit from integrating alternative antibiotic resistances, such as chloramphenicol, which has shown greater efficacy in depleting microbes that naturally occur in the pipefish and their environment (Wagner et al. 2024). The qPCR analysis initially revealed challenges with non-specific primer binding, likely due to the high proportion of fish DNA in the extracts, which masked the bacterial DNA, including the low-abundant fluorescent strains. To address this, we implemented an enzymatic digestion step to selectively degrade linear and larger circular DNA fragments, leaving only circular plasmid DNA intact. While this step strongly reduced background interference, it was insufficient to fully resolve the issue of non-specific primer binding, and thus the detection of the target strains. To further enhance detection specificity, the development of new primers or the application of additional molecular techniques could be considered. Methods such as *in situ* hybridization (Jensen 2014) or the use of hydrolysis probes like TaqMan (Nejati et al. 2020) may further improve specificity and enable reliable strain detection. Although we anticipated demonstrating a proof-of-concept for tracking microbial transfer during pregnancy and early microbial colonization using fluorescently tagged bacteria, these results underscore the need for continued optimization of molecular approaches before definitive conclusions can be drawn.

General remarks

The experiment provided initial insights into the temporal dynamics of microbial transmission during male pregnancy and early offspring microbial colonization, and demonstrated the challenges in establishing methods for visualizing and detecting these processes using micro and molecular biological techniques. Several limitations in the experimental design hindered our ability to draw final conclusions to support our hypotheses. The small sample size, the lack of antibiotic treatment before recolonization, and the selection of only two bacterial strains – *Marinomonas* and *Ruegeria* – out of the vast diversity in the natural pipefish microbiota limited the scope of the study. While these strains were chosen based on prior research, from the results of this study, we cannot claim that they are natural key players in microbial transfer. A broader bacterial community would be a more representative model, though fluorescent tagging of multiple strains would be technically challenging and could lead to increased competition between strains as indicated in our previous study (Wagner et al. 2024). Moreover, our attempts to detect fluorescently tagged strains in samples were not yet successful, underscoring the need for more refined molecular techniques that we are currently establishing to gain deeper insights into the experimental approach presented in this thesis chapter. As such, this experiment was initially designed as “proof-of-concept study” to demonstrate that we could isolate fluorescently labeled strains from adult and juvenile tissue, confirming microbial vertical and horizontal transfer. However, due to the challenges faced during the experiment the data remains currently difficult to interpret. Improved methods are necessary to conclusively identify vertical or horizontal microbial transfer.

This study should thus be regarded as a pilot project, offering valuable insights for future research. We learned that both maternal and paternal microbiomes may be more flexible than previously assumed, emphasizing the importance of careful strain selection for studying microbial transfer. Additionally, the ability of the natural microbiota to outcompete introduced strains highlights the need for effective depletion methods. While establishing a fully gnotobiotic system is challenging, as described in our previous work (Wagner et al. 2024), the focus should be on developing robust depletion techniques to overcome this limitation. The prevalence of kanamycin-resistant microbes in the marine environment also requires the use of well-evaluated antibiotic resistance genes for effective tracking through plate cultivation. Finally, the limited sampling time points and strict transfer timing in the experimental design may have resulted in missed critical stages of microbial transfer and colonization.

Chapter II

Future experiments should focus on addressing several key areas for improvement. First, preparatory lab work must establish reliable molecular detection methods to ensure accurate identification of the experimental strains. Ideally, multiple bacterial strains would be fluorescently tagged to better simulate natural microbial communities. However, competition assays will be essential to confirm that the experimentally modified strains are not outcompeted by wild-type bacteria. Additionally, including an antibiotic treatment prior to the experiment would help reduce the natural microbiota. Including true negative controls, such as tanks without fish, would establish a robust baseline and help mitigate bias from microbial shifts in the water. Expanding sampling time points, both during pregnancy and after birth, would offer a more comprehensive view of microbial dynamics. However, this presents a trade-off, as sacrificing more males for sampling reduces the number of replicates available for the experiment. Given the limitations of our experimental facilities and the limited fish we are allowed to use for an animal experiment due to animal welfare regulations, it may be more effective to focus one experiment primarily on closely monitoring critical time points during pregnancy, while later experiments could concentrate on tracking offspring development without disturbing the pregnancy. These refinements will improve the reliability of our findings and open the door for more detailed investigations of microbial transfer, with the potential to address the challenges identified in this study.

CONCLUSION

Our experimental approach demonstrates that early microbial colonization patterns before and after birth are highly diverse, dynamic, and tissue-specific. Vertical paternal transmission and horizontal environmental transmission are suggested to play distinct yet sometimes complementary roles in shaping the offspring microbiome. Treatment effects were observed in the paternal brood pouch tissue during pregnancy, the developing embryos, and the juveniles after birth, even in the absence of the experimentally added *Marinomonas* and *Ruegeria* strains. This suggests that minor microbial perturbations can trigger broader shifts in niche colonization and overall microbial composition. These findings may have potential applications in aquaculture, where manipulating microbial conditions could enhance offspring health by promoting beneficial microbes to occupy niches and outcompete harmful pathogens, ultimately improving survival rates and developmental outcomes in confined settings.

The unique reproductive strategy of male pregnancy in *Syngnathus typhle*, with temporally separated paternal and environmental microbial contributions, offers a valuable model for studying how distinct transmission routes interact to shape early microbial colonization. Using fluorescently tagged bacteria, this study tested a novel approach for visualizing microbial transfer, advancing our efforts to establish *S. typhle* as a model organism for host-microbe interactions. While the methods employed in this study provide valuable insights, they require further refinement to fully capture the complexity of microbial transfer. This study only represents a small fraction of the broader system, having focused on just two bacterial strains within a diverse microbial community. With improvements in detection methods, future research could enable a deeper understanding of the interplay between microbial transmission routes, environmental variability, and host-microbiota interactions during pregnancy and early development, positioning *S. typhle* as a powerful model for studying these dynamics.

ETHICS STATEMENT

The work was carried out in accordance with the German animal welfare law and with the ethical approval given by the *Ministerium für Landwirtschaft, ländliche Räume, Europa und Verbraucherschutz des Landes Schleswig-Holstein* (MLLEV-SH) (permit no. 63-7/18). No wild endangered species were used in this investigation.

5 SYNTHESIS

Microbiome research has expanded rapidly in recent decades, largely focusing on human systems due to their medical relevance (Turnbaugh et al. 2007; Gao et al. 2023). Studies on fish microbiota, though increasing, remain concentrated on a few economically significant species (Pérez-Pascual et al. 2021; Gómez de la Torre Canny et al. 2023; Luan et al. 2023). My research on microbial transfer in the sex role-reversed pipefish *Syngnathus typhle* combines the unique male pregnancy model with the less explored field of non-human microbiota, representing a pioneering effort to advance understanding of parental contributions to transgenerational microbial transfer beyond the mammalian model.

This thesis consists of two chapters presenting four experiments (Figure 1) that investigate the mechanisms and significance of paternal and horizontal microbial contributions during male pregnancy, using a combination of full-length 16S rRNA Sanger and V3/V4 MiSeq amplicon sequencing. To establish pipefish as a model system for microbiota research (Figure 1, grey box), I first focused on cultivating and **characterizing the sex-specific microbiota** (1) during different stages of male pregnancy (Chapter I). Reducing the diversity of the natural microbiota was another necessary step, for which I tested the **effectiveness of different antibiotics** (2) on the sex-specific microbiota (Chapter I). Building on this foundation, the first manipulation experiment investigated how altering the **paternal microbiota affected offspring development and early microbial colonization** (3). This involved treating the male brood pouch prior to mating, followed by long-term monitoring and frequent ribotyping of the juveniles after birth (Chapter I). To incorporate a temporal dimension, the second manipulation experiment involved treating the brood pouch and water to test the effects of **microbial introduction on vertical and horizontal transfer** (4), with a focus on timing and transmission pathways (Chapter II). Finally, a novel approach was developed and tested to **visualize microbial transfer** (5) and colonization dynamics through molecular markers (Chapter II).

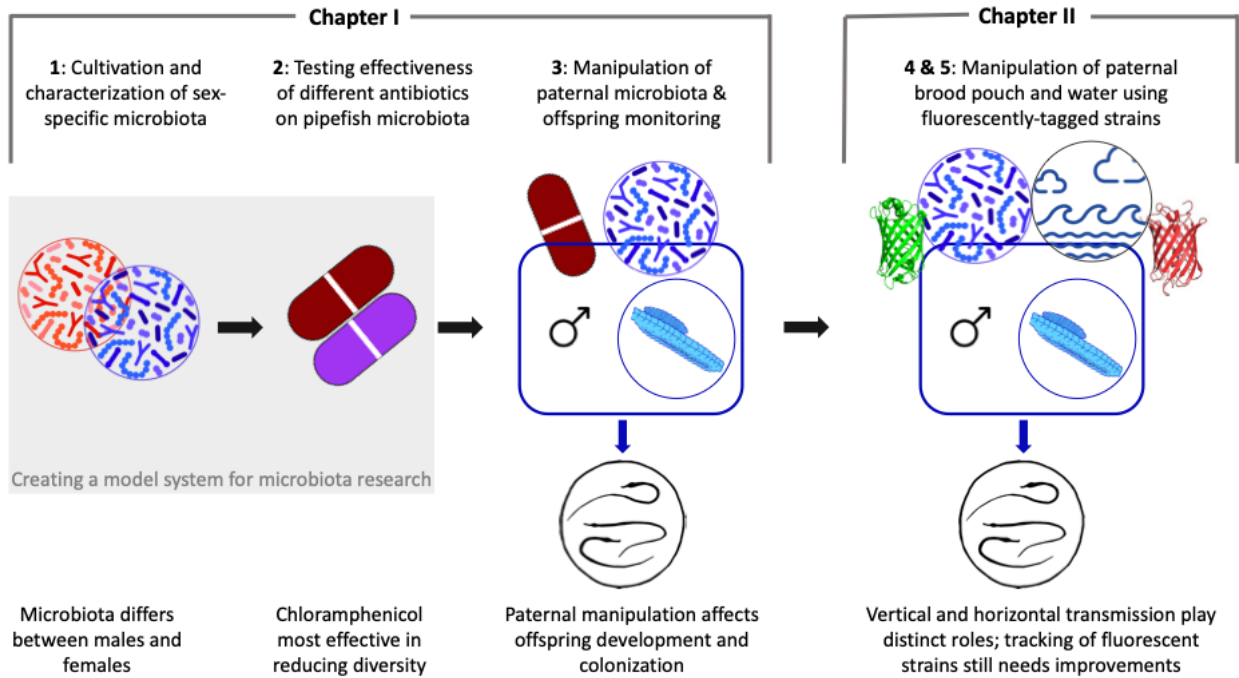


Figure 1: Graphical abstract of the thesis. Upper row shows main objectives of the experiments, bottom row highlights key findings. The grey box frames the two small experiments that aimed at establishing *Syngnathus typhle* as a model organism for microbiota studies. Image credits: Rebekka Leßke, Flaticon

5.1 The role of male pregnancy in microbial transfer and evolution

This thesis investigated paternal contributions to microbial transfer and it provided evidence that male pregnancy is an important route for fathers to transfer microbiota to their offspring. In my experiments, I focused on *Syngnathus typhle*, a local species with a semi-sealed brood pouch, which enables manipulation of its microbiota and easy assessment of pregnancy stages, as evaluated in **Chapter I** (Objective 3). The Syngnathidae family, however, comprises several species with diverse parental care strategies, making it an ideal system for exploring how these differing degrees in parental care influence microbial transfer. In *Nerophinae*, paternal investment is minimal, with eggs carried externally and fully exposed to the environment. In contrast, *Syngnathinae*, including *S. typhle*, have a semi-sealed brood pouch that changes from relatively sealed to more permeable. This physiological change gradually introduces environmental microbes which I tried to experimentally assess in **Chapter II** (Objective 4). *Hippocampinae* exhibit the highest investment, with a fully sealed pouch creating a controlled environment for embryonic development. Immunological studies (Parker 2022) suggest that even when the pouch opens before birth, environmental exposure is tightly regulated, highlighting the evolutionary interplay between parental care and microbial transmission across the family.

Synthesis

Male pregnancy in *S. typhle* may favor vertical microbial transmission as a stable mechanism for passing beneficial, co-evolved microbes to offspring, likely leading to a mild response from the innate immune system. Horizontal transmission, while riskier, could enhance resilience in dynamic habitats by introducing locally adapted microbes, despite stronger innate immune activation due to their novelty to the host. This dual strategy may allow *S. typhle* to balance microbial inheritance and environmental adaptation, especially in the Baltic Sea, where rapidly changing salinity and environmental stresses necessitate flexibility (Meier 2006; Andersson et al. 2015). While genetic adaptation and phenotypic plasticity are known to help *S. typhle* cope with environmental challenges (Goehlich et al. 2021), microbial transfer likely plays a complementary role.

The balance between vertical and horizontal transmission may help compensate for differences in immune system adaptations within Syngnathidae family. Both pipefishes (*Syngnathinae*) and seahorses (*Hippocampinae*) exhibit similar derived pregnancy traits, such as placenta-like structures, and downregulate genes in the major histocompatibility complex I (MHC I) pathway during early pregnancy to promote immunological tolerance (Parker et al. 2021). However, their immune systems differ: pipefishes have lost MHC II and CD4, while seahorses retain these genes but with a highly modified invariant chain (CD74) that may limit classical functionality (Roth et al. 2020). The complete loss of important adaptive immune genes in pipefishes may thus represent an evolutionary trade-off, where reduced adaptive immunity is counterbalanced by enhancing microbial priming. By contrast, seahorses, with a less permeable pouch and potentially more functional adaptive immune pathways, may rely on vertical transmission to minimize microbial exposure. These distinct strategies highlight evolutionary adaptations but require further experimental validation. Future studies could build upon the findings described in **Chapter II** and compare vertical and horizontal transmission along the gradient of parental investment in syngnathids, extending to free-spawning fish species, where internal pregnancy is absent, likely reducing vertical transmission but increasing horizontal exposure (Sylvain and Derome 2017; Diwan et al. 2023).

5.2 Sex or sex role?

Sex differences are a central focus in biological and medical research, influencing genetics, morphology, health, and disease. Sexual dimorphisms often arise from sex-biased gene expression, driving traits linked to sexual selection, antagonism, and parental investment (Grath and Parsch 2016; Pappert et al. 2024). Unsurprisingly, these effects are also reflected in the microbiota. Zhang et al. (2021) identified biological sex in humans as the primary driver of gut microbial variance in a study of 2338 adults, with women showing higher diversity and abundances of beneficial microbes. Peters et al. (2022) further supported these findings, reporting higher microbial diversity in women irrespective of their menopausal stage. However, disentangling true sex-specific effects in humans is challenging due to the influence of diet, lifestyle, and other environmental factors, underscoring once more the need for research beyond mammalian models.

The sex-role-reversed pipefish (*Syngnathus typhle*) offers a unique perspective on these dynamics. Prior research (Beemelmans et al. 2019; Tanger et al. 2024) revealed microbial differences between female gonads and the male brood pouch, supported by cultivation-based analyses (Objective 1). My first manipulation experiment (**Chapter I**) revealed that male pipefish exhibit significantly higher gut microbial diversity than females. While this strongly contrasts with patterns observed in conventional-sex species, it aligns with sexual immune dimorphisms reported for this system (Pappert et al. 2024). This raises critical questions: Does male microbial diversity stem from biological sex (= sex) or from being the pregnant sex (= sex role)? Does the act of parental care, including pregnancy, demand a more diverse microbiota, potentially to prime offspring via vertical microbial transfer? Higher microbial diversity in males may reflect a limited capacity to regulate microbiota due to resource allocation to parental care. Such diversity could benefit offspring development (**Chapter I**) or provide pathogen resistance during late pregnancy when environmental microbes infiltrate the brood pouch (**Chapter II**). Morphological factors, such as the proximity of the anus to the brood pouch opening (Zhang et al. 2020), may also regularly expose the brood pouch to gut microbes, selecting for a diverse microbiota to suppress harmful pathogens (Spragge et al. 2023).

In conventional female-pregnancy systems, males prioritize mating competition while females emphasize immune defense and longevity (Liker et al. 2015; Chapman et al. 2019; Schlupp 2021). These divergent strategies likely influence host-microbe interactions, as seen in *Drosophila* (Walters et al. 2020) or *Daphnia* (Akbar et al. 2020), though vertebrate studies remain scarce.

Despite advances made in this study, gaps persist in understanding how microbial diversity, parental investment, and morphology interact across Syngnathidae. My research provides an experimental foundation for studying paternal microbial transfer in a sex-role-reversed system. Expanding microbiota manipulation experiments, i.e., microbial depletion and recolonization, to species with conventional sex roles but similarly derived internal pregnancies is essential for uncovering broader evolutionary and functional dynamics of microbial transmission. Additionally, metagenomic or metatranscriptomic profiling of the brood pouch microbiota could identify functional traits relevant to male pregnancy and clarify whether the brood pouch microbiota is actively managed for offspring colonization or simply reflects environmental exposure. Differentiating between retained and transferred microbiota could shed light on mechanisms of vertical transfer, specifically extending results of **Chapter I**. If specific microbes are actively transferred, their functional roles (e.g., immune priming, pathogen resistance, or nutrient provision) could reveal how the paternal microbiome shapes offspring fitness. Comparing these functional traits in sex-role-reversed and conventional pregnancy systems could further uncover convergent or divergent evolutionary strategies in microbiota management, advancing our understanding of host-microbe co-evolution.

5.3 The interplay of vertical and horizontal transmission

Chapter I of my thesis provided first evidence for paternal microbial transfer during male pregnancy which was further elaborated in **Chapter II** which examined the intricate relationship between paternal (via male pregnancy) and horizontal (via the water) microbial transmission pathways (Objective 4). Both routes play significant roles in shaping microbial communities across various tissues (paternal: brood pouch tissue during early pregnancy, embryos, whole-body juveniles 7 days post-release; horizontal: brood pouch tissue during early pregnancy, whole-body juveniles 7 days post-release, juvenile gut at birth and 7 days post-release), highlighting their distinct contributions. However, the lack of significant interaction effects raises questions. One explanation could be that vertically transmitted microbes gain an early advantage, colonizing ecological niches during pregnancy and limiting opportunities for later-arriving environmental strains (Baquero et al. 2021). Alternatively, environmental microbes might complement or outcompete vertically inherited strains, particularly as the male brood pouch becomes increasingly permeable in late pregnancy. These processes could foster an *adaptive microbial landscape* shaped by the dynamic interplay between inheritance and environmental exposure (Org et al. 2015).

Synthesis

Does this dynamic confer advantages to the host, such as enhanced immune defense or metabolic flexibility? Pipefish, with their loss of immune genes, might minimize their reliance on the environment for a diverse and functional microbiota by inheriting a beneficial microbiota from the parent generation. This strategy could reduce – but not eliminate – the influence of environmental microbes. If so, does the host actively shape this microbial landscape as observed in laboratory mice (Liu et al. 2016; Benga et al. 2024), or is it still mainly determined by regional biotic and abiotic factors? Future research could address how microbial traits, host-mediated selective pressures, or environmental conditions like temperature and salinity drive this adaptive landscape. For example, how does the functional diversity or metabolic capacity of environmental microbes influence their colonization success? Additionally, how do physiological changes in the brood pouch during pregnancy affect the balance between vertical and horizontal transmission? To explore these dynamics, we collected *S. typhle* from the more saline (~26 PSU) Limfjord region (Denmark) and exposed them to changing salinities in a comparative experiment with *S. typhle* from Kiel Bight (~18 PSU). This dataset provides an opportunity to address some of the questions raised, particularly regarding how environmental factors, such as salinity, influence microbial transfer. These findings could complement our findings that environmental microbes significantly affect microbial colonization in late pregnancy (**Chapter II**) and offer further insights into the interplay between host-driven processes and environmental conditions in shaping the microbial landscape.

5.4 Creating a gnotobiotic pipefish

Microbes are ubiquitous and indispensable for life on earth. Studying the role of specific bacteria, particularly through the manipulation approaches described in this thesis (specifically **Chapter I**), requires full control over the natural microbiota of the model organism and its environment. In **Chapter I**, I evaluated the effectiveness of antibiotics as a simple yet potentially efficient method for depleting the sex-specific microbiota of *Syngnathus typhle*. Chloramphenicol proved particularly effective in this case, significantly reducing overall microbial diversity and selectively targeting strains associated with the paternal brood pouch microbiota, when applied for at least eight days (Objective 2). During the first manipulation experiment (Objective 3), this treatment successfully induced shifts in microbiota composition, both in the fathers and in the offspring after birth. In **Chapter II**, however, no antibiotic treatment was applied to minimize stress on the fish. In retrospect, skipping this step may have been a limitation, as recolonization treatment effects were sometimes weak, possibly due to introducing bacterial strains into a stable, unaltered microbiome (see 5.3 *The interplay of vertical and horizontal transmission*). Despite of being a

simple method, it is important to note that antibiotic treatment alone is insufficient for achieving full control over the diverse microbiota of adult fish. Antibiotics reduce but do not eliminate all microbial strains, particularly antibiotic-resistant bacteria or those embedded in biofilms. Persistent resident microbes often remain in protected niches, such as mucus layers or skin, where antibiotics have limited efficacy (Grice and Segre 2011; Fleming and Rumbaugh 2018). Moreover, fish in non-sterile environments are rapidly recolonized by microbes from water, food, or other sources, impeding the distinction between natural and manipulated microbiomes. Antibiotic-induced disruptions generally alter but do not reset the microbiome, leaving remnants of the original microbial community intact (Ramirez et al. 2020; Patangia et al. 2022).

To achieve full control, the establishment of a true gnotobiotic model system with a well-defined or absent microbiome (Coates 1973) is essential, requiring specialized equipment and frequent methodological testing. For aquatic organisms, such systems are established for zebrafish (Figure 2A, Pham et al. 2008) and sponge (Schmittmann et al. 2022) models, while they remain largely unexplored for other species. Typically, these systems begin with surface-sterilized eggs to prevent microbial carryover from the earliest time point possible. Iodine-based sterilization, as described by (Tanger et al. 2024) and implemented for treating embryos in **Chapter II**, or bleach treatments (Martins et al. 2016) are commonly used. Additional infrastructure including sterile tanks, autoclaved water, and filtered air supplies must ensure aseptic handling is maintained following strict protocols (Figure 2). Feed also needs to be sterile, posing a challenge for pipefish, which preferentially consume live mysids or *Artemia*, both of which harbor significant microbial loads (Puente et al. 1992; Tkavc et al. 2011). Since autoclaving compromises the viability and potentially the nutritional value of live feed, alternative approaches must be developed. Bleach-sterilized *Artemia* have been successfully used but are limited to smaller life stages. For adult pipefish, larger live *Artemia* or treated frozen mysids may be required, though these options still pose challenges for maintaining continuous sterility. Finally, it has to be considered that a stable gnotobiotic system also requires regular monitoring of water and fish samples using culture-based or molecular methods, such as qPCR or 16S rRNA sequencing.

However, establishing a gnotobiotic system specifically for *S. typhle* presents unique challenges. As a marine species, pipefish depend on environmental marine microbes, the roles of which are not yet fully understood. Unlike the well-established gnotobiotic zebrafish model, pipefish require highly specific conditions to mate, which naturally occur only during a brief annual time window (Jones et al. 1999). Recreating these conditions involves precise control of water quality, food, and environmental factors. The internal pregnancy of *S. typhle* adds further complexity, as

Synthesis

embryos would need to develop in nutrient solutions under sterile conditions instead of the paternal brood pouch. Additionally, in our breeding facilities, pipefish take at least seven months to reach sexual maturity, requiring them to be raised in sterile conditions for extended periods without their natural diet or potentially critical environmental influences. Despite these challenges, our research group has taken important steps toward developing a gnotobiotic system. These include using artificial seawater with lower microbial loads, adapting protocols to treat larger amounts of *Artemia* with bleach, and designing experimental tanks with filtered air supply, which have been tested in recent experiments (Figure 2B). These S2-compliant tanks were suitable to harbor the fish during mating and pregnancy, however, they were not designed for long-term raising and husbandry. While these measures represent progress, achieving a fully functional gnotobiotic system would therefore require further methodological and infrastructural advancements, alongside a careful assessment of its feasibility given the biological and reproductive needs of *S. typhle* and other members of the Syngnathidae family.

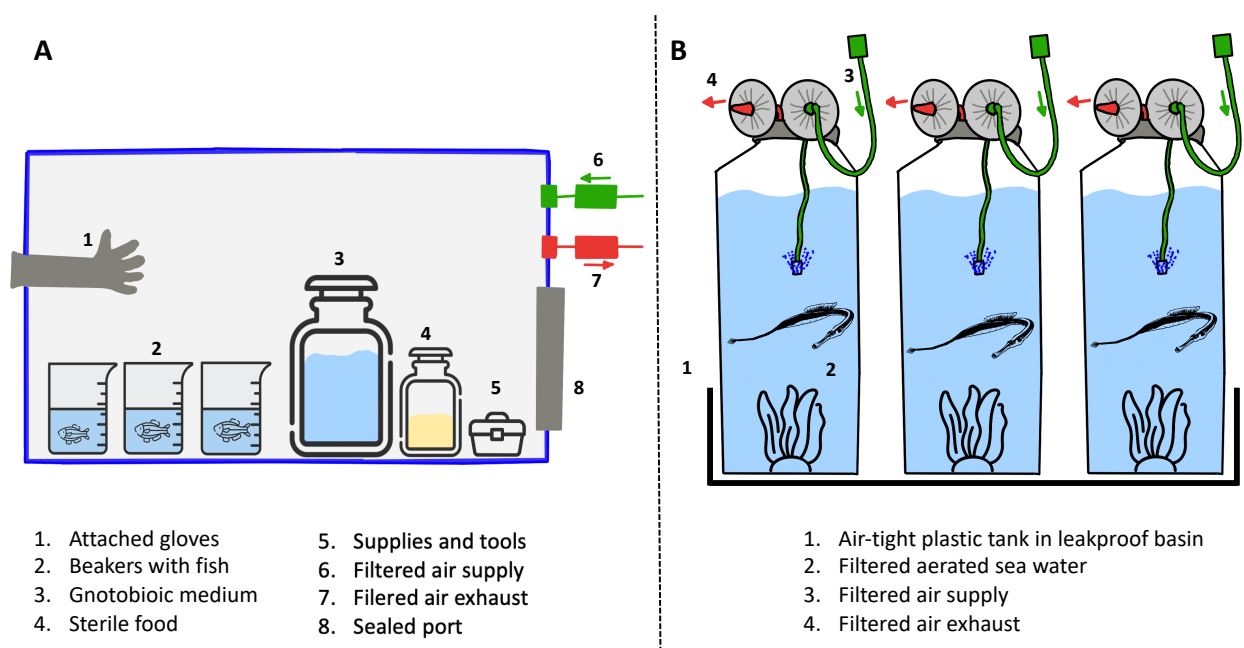


Figure 2: Different approaches to develop gnotobiotic systems for aquatic organisms. (A) Schematic representation of a sealed isolator equipped for gnotobiotic zebrafish husbandry, adapted from Pham et al. 2008 (Nat. Protoc). (B) Newly designed S2-compliant air-tight tanks used for recent *S. typhle* microbial transfer experiments in our research group. Image credits: Alba Filella, Flaticon

5.5 The big picture

This thesis has unraveled pathways of microbial transfer from fathers and the environment to offspring during male pregnancy in sex role-reversed pipefish, emphasizing the importance of sex role. These processes, however, must be considered within a broader context. The intricate link between the immune system and microbiota, extensively described in humans, appears to be equally relevant to *Syngnathus typhle*. Here, not only the microbiota but also elements of the immune system are passed to the next generation via transgenerational immune priming (TGIP). TGIP describes how parental immune experiences, such as pathogen exposure, enhance offspring resistance through phenotypic plasticity (Roth et al. 2018). This phenomenon has been demonstrated in Syngnathidae (Roth et al. 2012; Beemelmans and Roth 2016a, b), even spanning multiple generations (Beemelmans and Roth 2017). A recent study (Schneider et al. 2024) bridged the gap between TGIP and microbiota, providing experimental evidence that TGIP not only enhances immune defense but also influences offspring microbial communities.

It is reasonable to assume that *S. typhle* has evolved a multipronged strategy to optimize offspring health and development, with immune priming and shaping of microbial communities complemented by limited environmental influences as two key pathways. Additional pathways may include the evolution of the brood pouch with its placenta-like tissue and internal pregnancy itself. The intimate association between fathers and embryos during male pregnancy provides a direct mechanism for priming offspring microbiota, as demonstrated in this study and could be seen as an incubator – a controlled environment where offspring are protected and specifically prepared for their release to the external environment. However, other pathways – such as genetic, hormonal, or behavioral mechanisms – likely contribute and remain to be discovered (Figure 3). The question remains: why has such a complex, multifaceted approach evolved? While the loss of key immune genes in *S. typhle* (Roth et al. 2020) might intuitively suggest immunosuppression, recent evidence indicates that these fishes have developed compensatory mechanisms to maintain effective immune functions (Parker et al. 2023), enabling these fish to allocate resources to other traits, such as reproductive investment or mate choice, rather than maintaining costly, high-level immune defenses. Alternatively, increased flexibility in phenotypic plasticity may allow these fish to adapt to fluctuating environments. This flexibility could explain the colonization success of *S. typhle* and closely related species, such as *S. rostellatus*, in the Baltic Sea, where other species like cod and herring struggle under rapid environmental changes (Andersson et al. 2023). However, the resilience of *Nerophis ophidion*, a syngnathid lacking internal pregnancy but thriving in the same habitats, challenges this hypothesis and highlights the

Synthesis

importance of comparative studies within this family. Future research should further explore the diverse pathways of microbial transfer, including their functional implications for host immunity and development. This unique system, with potentially multifaceted strategies for preparing offspring despite the loss of immune genes, highlights the urgent need to shift focus toward the host side. Only by unraveling the host's role in these interactions can we achieve a full understanding of how microbial transmission shapes adaptive host-microbe interactions and its role in the evolution of male pregnancy.

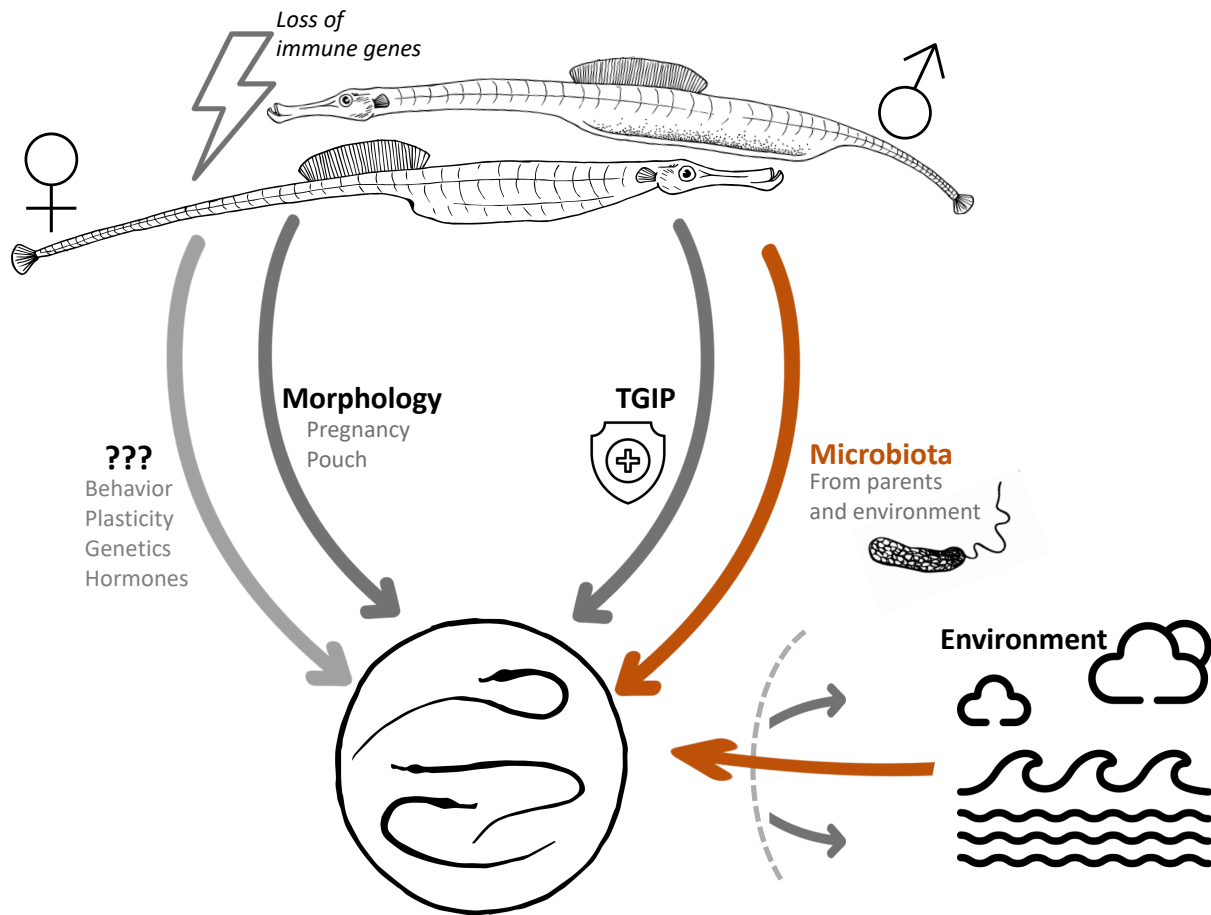


Figure 3: A multipronged approach of parents to shape offspring immunity and microbiota in order to optimize health and development? This thesis has unraveled paternal and environmental influences on offspring microbial colonization, complementing previous research on trans-generational immune priming in these fish. But there are potentially many more pathways for parents to prepare their offspring that are yet to be fully discovered. Image credits: Rebekka Leßke, Alba Filella, Flaticon

5.6 Concluding remarks

In my thesis, I examined how distinct microbial transmission routes influence early microbial colonization and development in a sex-role-reversed model system. This research provides evidence that fathers actively shape the microbiota of their offspring during male pregnancy, offering a novel perspective on microbial transfer mechanisms in contrast to classic (mammalian) model systems where sex-specific contributions are difficult to disentangle.

My findings demonstrate that the introduction of specific microbes can shorten pregnancy and improve offspring survival, while environmental microbes entering the brood pouch during late pregnancy also influence early microbial colonization in juveniles. A key contribution of this thesis was developing methods to establish pipefish as a model system, providing the necessary foundation for microbial manipulation experiments in the context of male pregnancy.

While microbial manipulation in this system presents challenges due to complex host-microbe-environment interactions, my experimental approaches consistently impacted microbial composition and early colonization. Unexpected results suggest potential underlying mechanisms, such as parental immune adaptations that influence offspring microbiota. These outcomes underscore the importance of considering host factors in future studies to fully understand microbial transfer dynamics and the evolutionary implications of male pregnancy.

6 FUTURE PERSPECTIVES

The unique male pregnancy in syngnathids provides an exceptional framework for exploring transgenerational microbial transfer and early microbial colonization. This thesis has begun to unravel paternal and environmental contributions to these processes, offering a foundation for future studies. However, further work is needed to disentangle the interplay of factors shaping microbial colonization. Since completing the experimental phase of this thesis, our research on microbial transfer has advanced significantly. The relocation of the Marine Evolutionary Biology group to Kiel University provided access to modernized infrastructure, facilitating key methodological improvements. The construction of an S2-level climate chamber enabled microbial transfer experiments during the 2023 and 2024 mating seasons, incorporating chloramphenicol-resistant fluorescent strains and refined *Artemia* decontamination protocols. These improvements reduced opportunistic contamination and enhanced the feasibility of tracking microbial strains. Building on this foundation, future efforts will expand the analysis of microbial transfer across both sexes. This will build on the successful implementation of feed-based vectors (i.e., *Artemia* and live mysids) for fluorescently tagged strains. The goal is to equally study both maternal and paternal microbial contributions, ultimately aiming to fully disentangle sex-specific contributions to microbial provisioning.

While this thesis has primarily focused on the microbial aspect of host-microbe interactions, future research should delve deeper into the host's physiological and molecular responses. Gene expression studies, such as RNAseq, can shed light on immune and stress pathways modulated during microbial colonization and pregnancy (Frost et al. 2023). Single-cell transcriptomics could reveal cell-type-specific responses within reproductive tissues (Li et al. 2020), while metabolomic and proteomic approaches could identify molecular pathways linking microbial presence to host traits like pregnancy duration and offspring fitness (Starr et al. 2018). Comparative analyses across syngnathids with varying parental care strategies, coupled with advanced imaging techniques (e.g., Campisciano and Biffi 2022; Mondragón-Palomino et al. 2022), could provide evolutionary insights into the co-evolution of host-microbiota interactions and male pregnancy. Together, these approaches provide a framework for exploring the evolutionary and functional dimensions of host-microbiota interactions. By advancing our understanding of microbial transmission and host adaptation, this thesis contributes to broader questions in evolutionary biology, including the evolution of male pregnancy and other forms of reproductive investment. Insights from this work have the potential to inform our understanding of microbial dynamics across species and contribute to the broader field of host-microbiota co-evolution.

AUTHOR CONTRIBUTIONS

Chapter I

Experiment 1: **KSW** and OR conceived the study. **KSW** collected the samples and processed them in the laboratory with help from SMM. **KSW** processed and analyzed microbial Sanger sequencing data.

Experiment 2: **KSW** and OR conceived the study. **KSW**, SMM and OR collected samples at different time points during the experiment. **KSW** processed the samples in the lab with help from SMM. **KSW** processed and analyzed microbial 16S rRNA sequencing data.

Experiment 3: **KSW**, FS and OR conceived the study. **KSW**, FS, SMM and OR collected samples at differing stages during pregnancy and while raising the offspring. FS recorded and analyzed life-history parameters. **KSW** processed the samples in the lab. **KSW** processed and analyzed microbial 16S rRNA sequencing data.

KSW wrote the manuscript, synthesizing findings from all three experiments, with input and editing from OR.

Chapter II

RL, SMM and OR conceived the study. NFH provided advice and assistance for the fluorescent tagging of bacterial strains. RL, SMM and OR collected samples at differing stages during pregnancy and after birth. SMM processed samples in the lab. **KSW** processed and analyzed microbial 16S rRNA sequencing data. **KSW** wrote the manuscript, with input and editing from OR.

EIDESSTATTLICHE ERKLÄRUNG

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

**DADS MATTER –
PATERNAL TRANSFER OF MICROBIOTA THROUGH MALE PREGNANCY IN THE SEX
ROLE-REVERSED PIPEFISH *SYNGNATHUS TYPHLE***

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.

Zusätzlich erkläre ich hiermit, dass mir in der Vergangenheit kein akademischer Grad entzogen wurde.

Kapitel I wurde mit Olivia Roth als Co-Author als Vorabdruck auf *bioRxiv* hochgeladen.

Kim-Sara Wagner

Kiel, 07.01.2025

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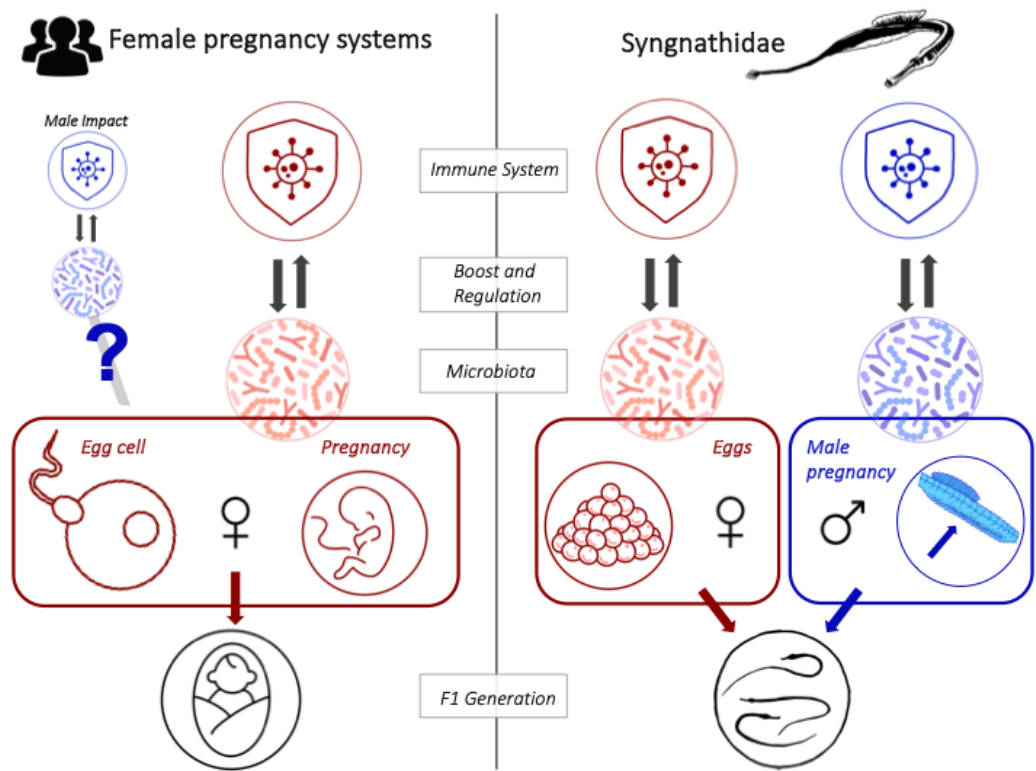
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APPENDICES

Appendix – Chapter I



Supplementary Figure 1: Immune system and microbiota transfer across generations: Comparison of classic female pregnancy systems (left) with unique male pregnancy in the pipefish *Syngnathus typhle* (right) with a focus on the interaction of the microbiota and the immune system.

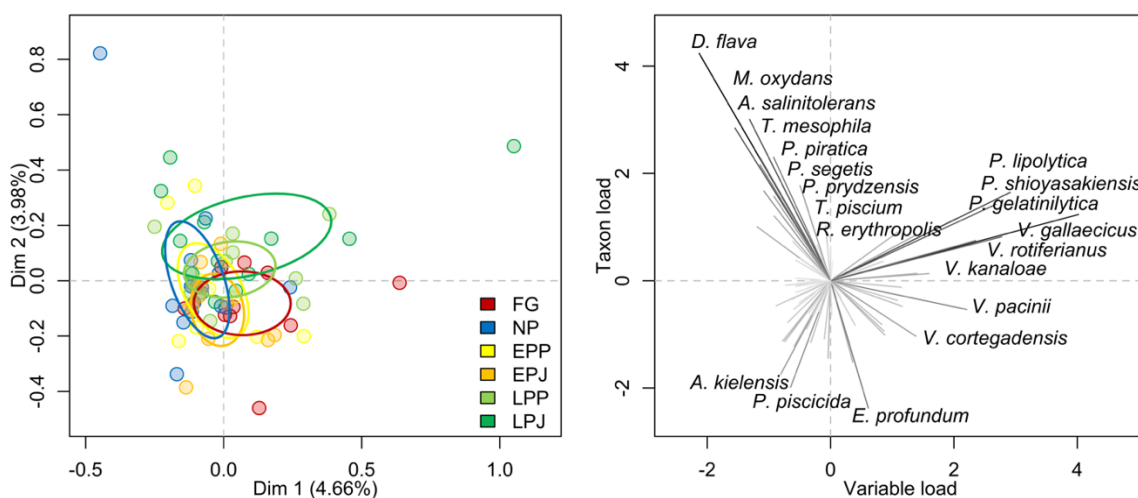
MiSeq Run	47	48	63	64	65	79
Experiment	Experiment 2		Experiment 3			
Samples	377	377	289	384	377	30
Min. reads	22	22	13	7	16	57639
Median	28517.0	21022.0	29912.0	27566.5	23589.0	135136.5
Mean	30871.65	21747.52	30129.50	28620.82	26122.26	149190.37
Max. reads	102427	55732	83458	115401	137303	344546
Total reads	11638610	8198814	8707426	10990395	9848093	4475711
Rep seqs	10688		23841			

Supplementary Table 1: QIIME2 sequence processing statistics. Samples were randomly distributed over all sequencing runs. DADA2 workflow was applied separately for each run before merging all runs into a master data set.

Experiment 1: Cultivation and characterization of sex-specific microbiota

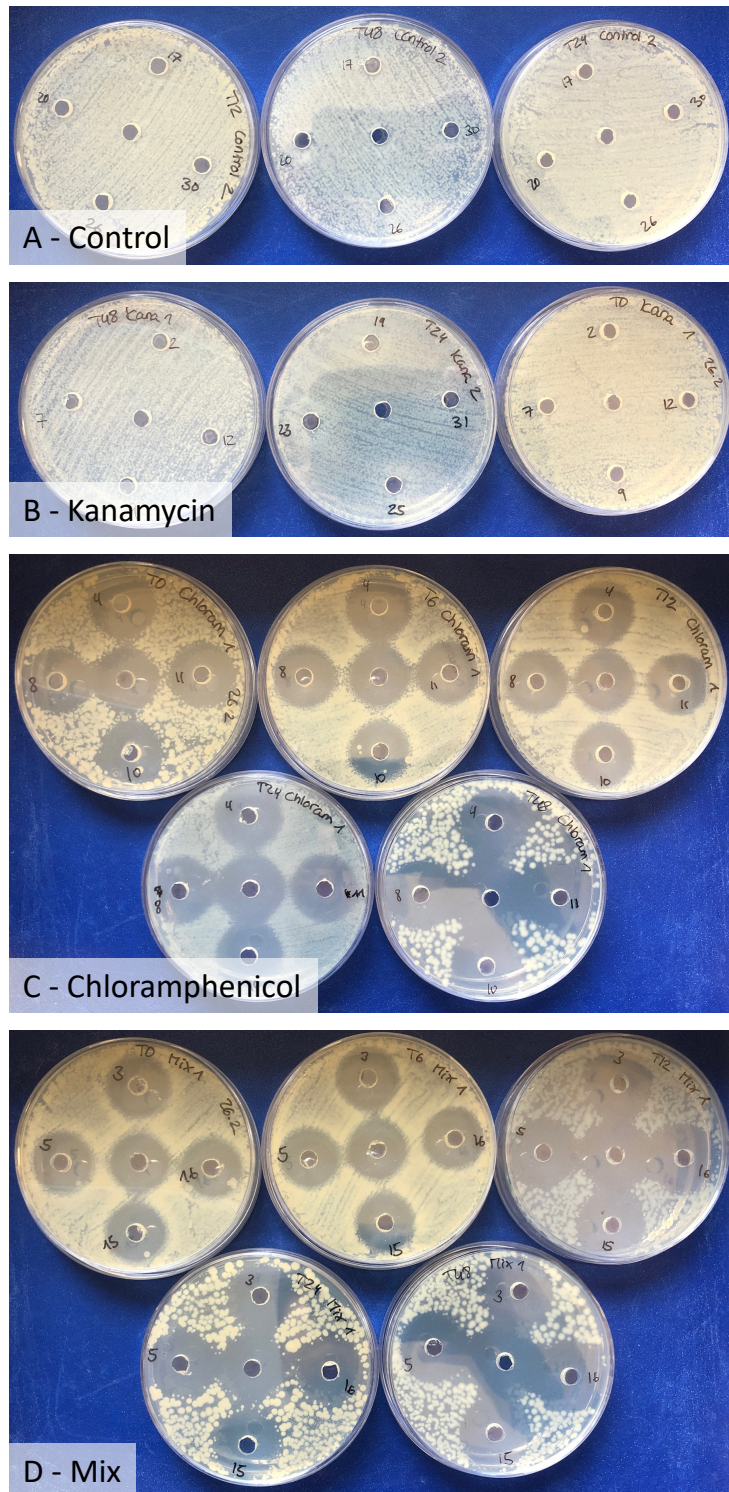
Pairs	Df	SumsOfSqs	F.Model	R2	p.value	p.adjusted
FG - NP	1	0.6658655	1.4589270	0.05126430	0.035	0.1050000
FG - EPP	1	0.5352662	1.1910171	0.05367113	0.176	0.3016667
FG - EPJ	1	0.4758822	1.0656973	0.04829656	0.369	0.4612500
FG - LPP	1	0.5355834	1.1764959	0.04866279	0.167	0.3016667
FG - LPJ	1	0.7346852	1.6352001	0.06637657	0.004	0.0200000
NP - EPP	1	0.3875046	0.8660220	0.03482752	0.689	0.7382143
NP - EPJ	1	0.5451620	1.2252517	0.04857243	0.181	0.3016667
NP - LPP	1	0.5849061	1.2918955	0.04733623	0.087	0.2175000
NP - LPJ	1	0.9318041	2.0822734	0.07414903	0.001	0.0150000
EPP - EPJ	1	0.3080231	0.7114310	0.03802120	0.891	0.8910000
EPP - LPP	1	0.4249862	0.9564965	0.04564200	0.540	0.6230769
EPP - LPJ	1	0.8262708	1.8886978	0.08628644	0.002	0.0150000
EPJ - LPP	1	0.4849914	1.0990119	0.05208831	0.328	0.4612500
EPJ - LPJ	1	0.7400999	1.7034783	0.07848872	0.006	0.0225000
LPP - LPJ	1	0.4841525	1.0888442	0.04715889	0.354	0.4612500

Supplementary Table 2: Pairwise adonis comparing sexes and/or stages of pregnancy in *S. typhle*. Abbreviations: FG = female gonads, NP = non pregnant, EPP = early pregnant pouch, EPJ = early pregnant juveniles, LPP = late pregnant pouch, LPJ = late pregnant juveniles.



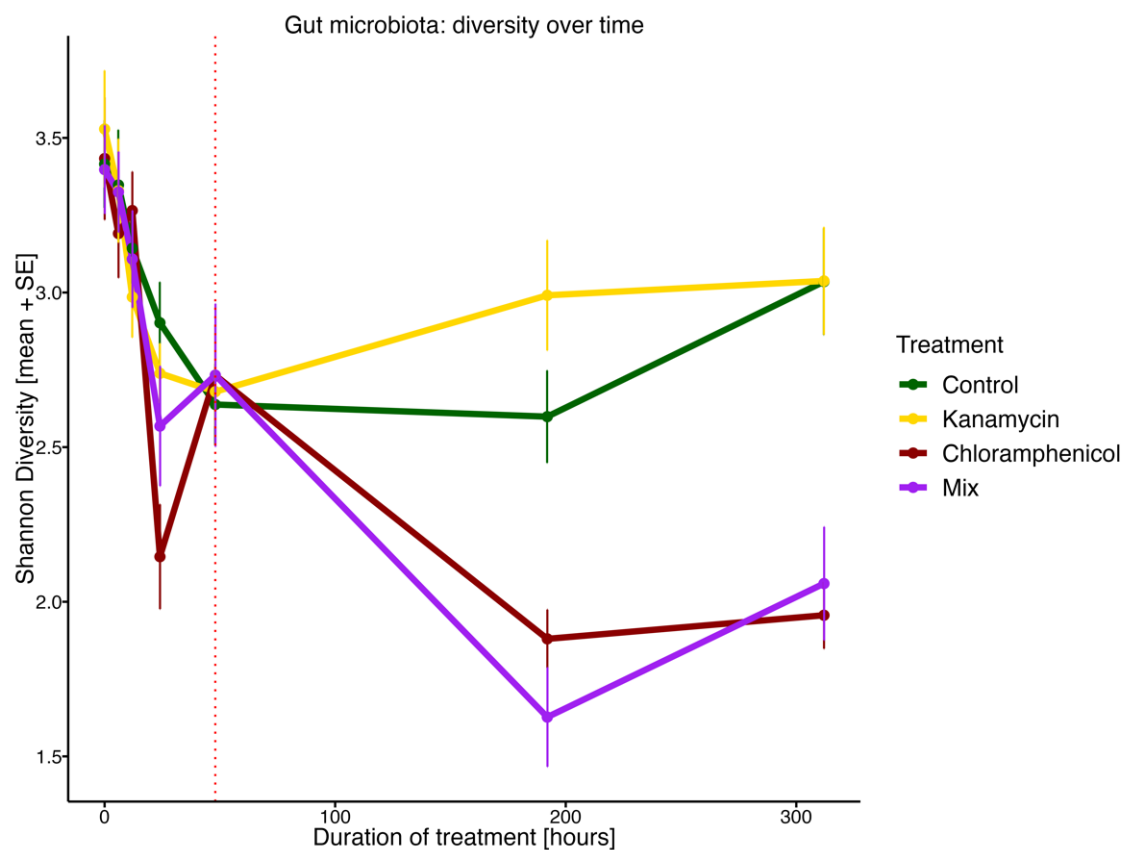
Supplementary Figure 2: Multiple correspondence analysis (MCA) of six groups for dimensions 1 and 2 (A) and factor map displaying the loadings retained by the isolated bacterial species (B). Ellipses include 40% of data of the respective group. Colors represent sex and pregnancy stages as described above.

Experiment 2: Effectiveness of antibiotics for natural pipefish microbiota depletion

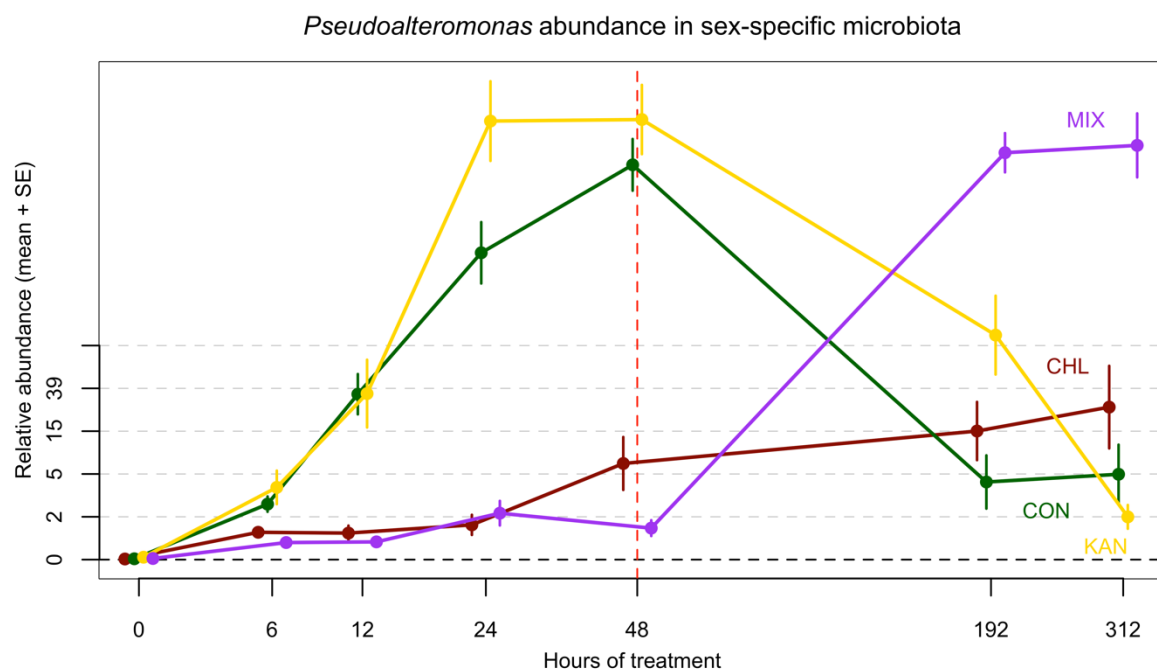


Supplementary Figure 3: Well diffusion test to test for antibiotics in the water. Each well number corresponds to its respective tank. The center well was used for a control sample. Pictures were taken after 12 hours of incubation. Control (A) and Kanamycin (B) show no zones of inhibition, whereas treatments containing Chloramphenicol (C and D) show clear zones. Pictures show plates from beginning of the experiment (T0), after 24 hours (T24) and 48 hours (T48). For C and D additionally time points T6 and T12.

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Supplementary Figure 4: Alpha diversity changes over time under the influence of different antibiotic treatments in the gut microbiota of *S. typhle*. Colors represent treatment groups and data points display mean diversity \pm standard error.

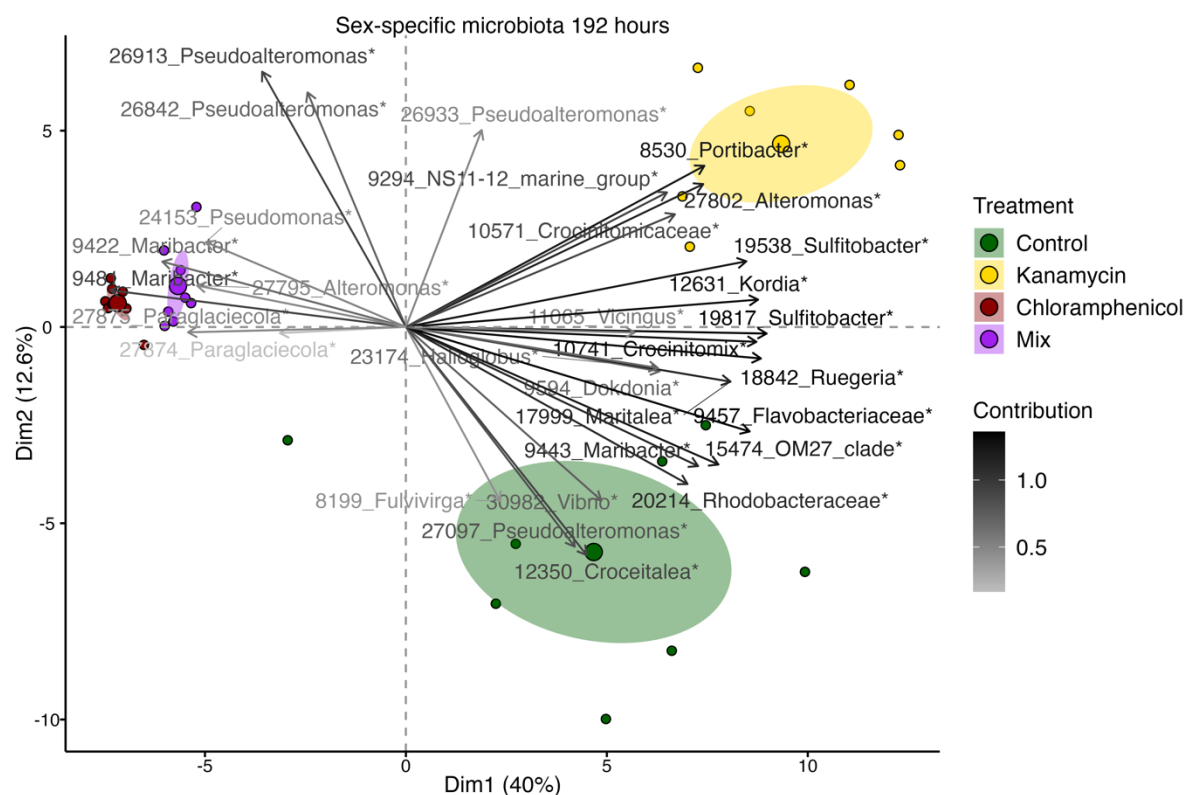


Supplementary Figure 5: *Pseudoalteromonas* was initially selected as a paternal indicator species within the spike community but could not be depleted by the selected antibiotics and therefore excluded for further experiments.

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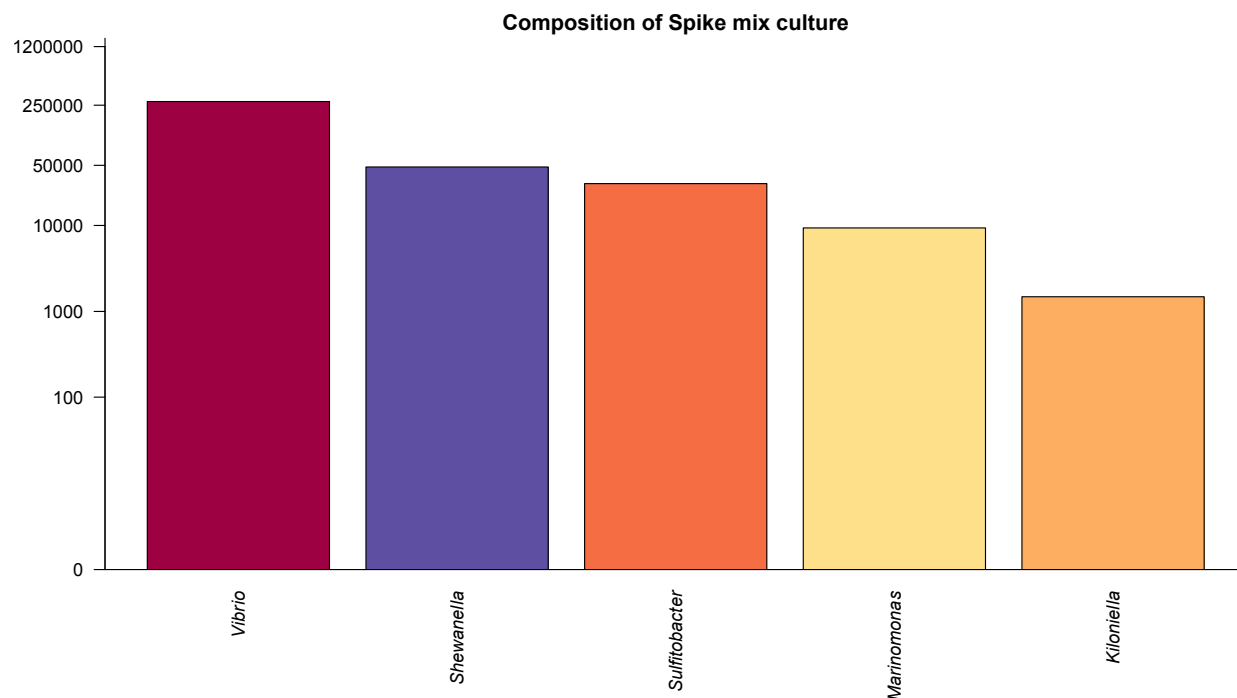
	Df	SumOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Treatment	3	7.885	2.6282	13.308	0.06948	0.001 ***
Time	1	7.962	7.9623	40.317	0.07016	0.001 ***
Individual	52	33.077	0.6361	3.221	0.29146	0.001 ***
Treatment:Time	3	8.87	2.9568	14.972	0.07816	0.001 ***
Residuals	282	55.692	0.1975		0.49074	
Total	341	113.486			1	

Supplementary Table 3: Repeated measures PERMANOVA reveals significant impact of time, treatment and individual on microbial beta-diversity of the sex-specific microbiota



Supplementary Figure 6: PCA Biplot of the sex-specific microbiota under the influence of different antibiotics at 192 hours of treatment. Ellipses display a 95% confidence interval around the group's mean. Colors represent treatment groups. Factor map displays the ASV ID and the genus name, respectively family name if genus could not be identified.

Experiment 3: Manipulating paternal sex-specific microbiota to unravel impact on the offspring



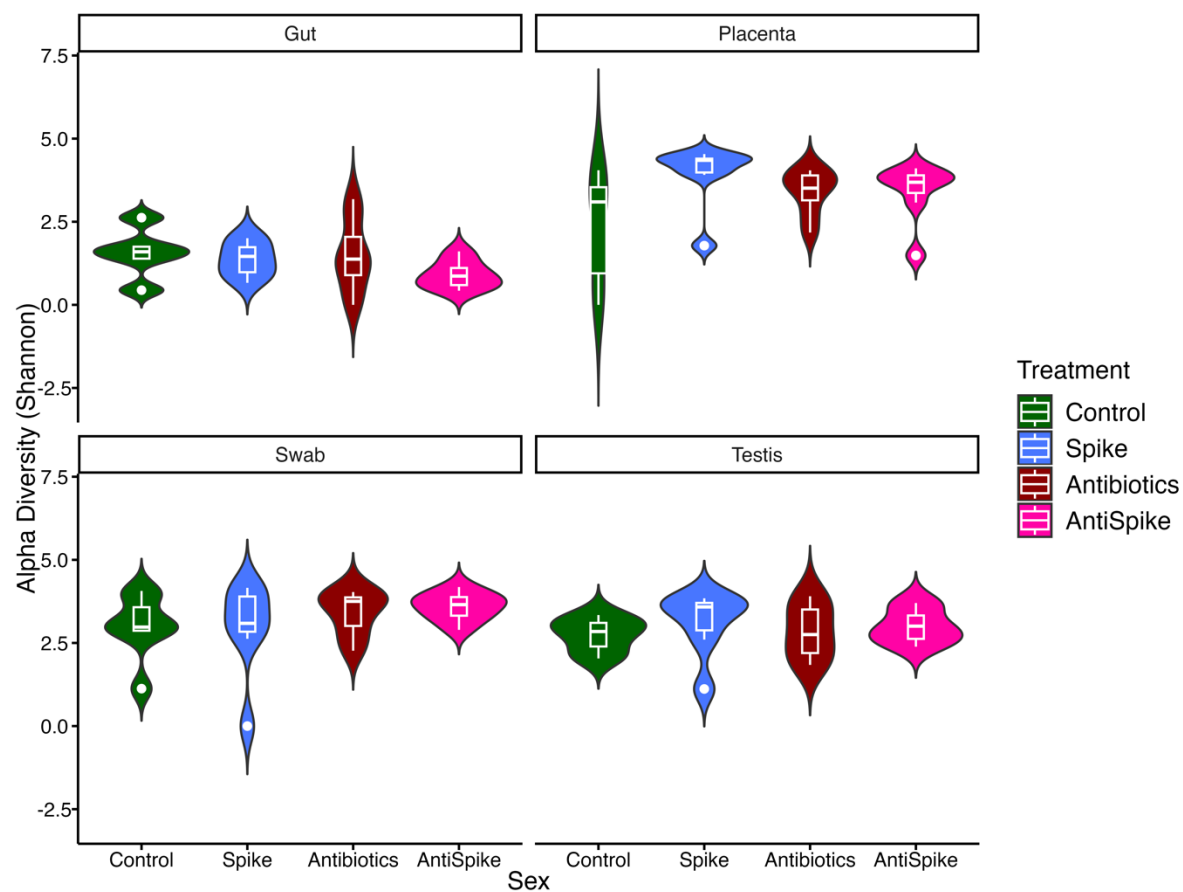
Supplementary Figure 7: Composition of spike community mixed culture that was used in 30x concentration for treating the male brood pouch. Bars represent sequencing counts of selected candidate strains within the culture.

Experiment 3 (A) – Adults

Treatment	Filling of brood pouch (% + SD)	Gestation length (mean days + SD)	Number of offspring (mean + SD)
All fish	86.76 ± 18.7	29.18 ± 1.62	31.76 ± 15.06
Spike_Treat	85.3 ± 21.76	28.47 ± 0.8	28.88 ± 14.69
Anti_Treat	90.63 ± 15.48	29.38 ± 1.63	33.63 ± 14.41
Control	91.67 ± 12.5	29.78 ± 1.86	36.11 ± 19.77
Spike	75 ± 25	28.22 ± 0.97	24.11 ± 7.85
Antibiotics	84.38 ± 18.6	30 ± 2.14	33 ± 9.12
AntiSpike	96.86 ± 8.84	28.75 ± 0.46	34.25 ± 18.99

Supplementary Table 4: Pregnancy statistic for all treatment groups. Spike_Treat includes fish of the treatment groups Spike and AntiSpike and Anti_Treat includes fish of the groups Antibiotics and AntiSpike.

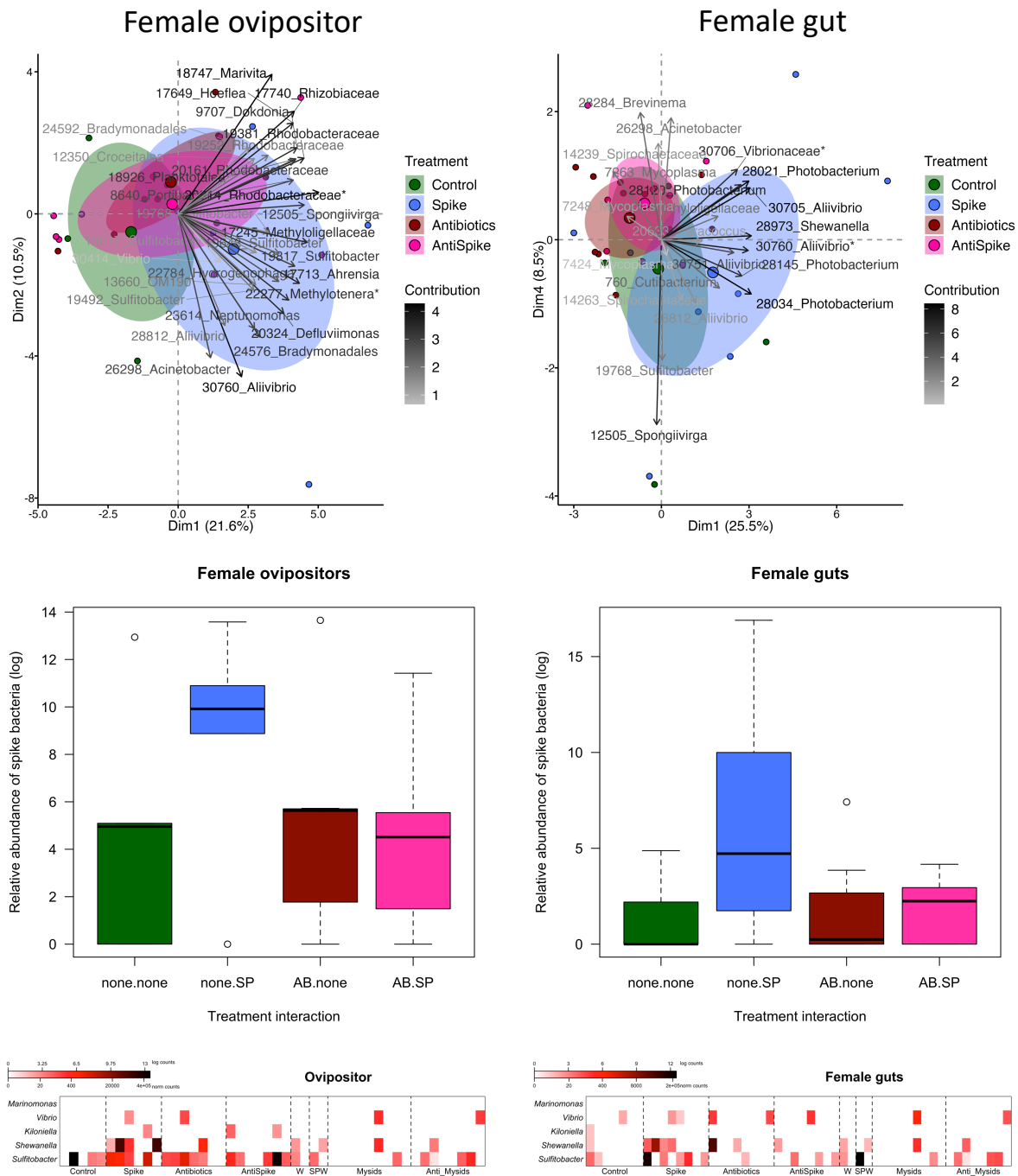
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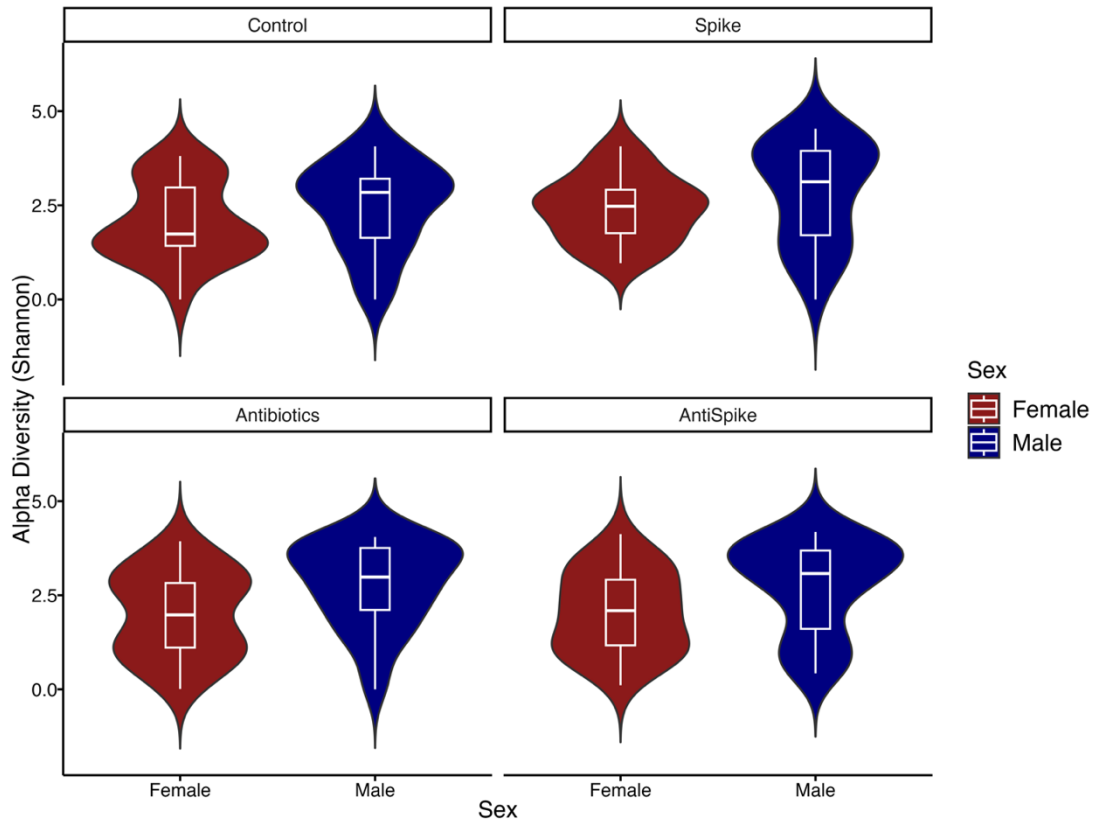
Supplementary Figure 8: Violin and boxplots showing alpha diversity of male organs under the influence of different treatments. Panels indicate from which organ the microbiota was ribotyped.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Organ	3	69.02	23.005	28.424	3.32e-13 ***
Treatment	3	3.69	1.230	1.520	0.214
Organ:Treatment	9	10.41	1.156	1.429	0.187
Residuals	95	76.89	0.809		

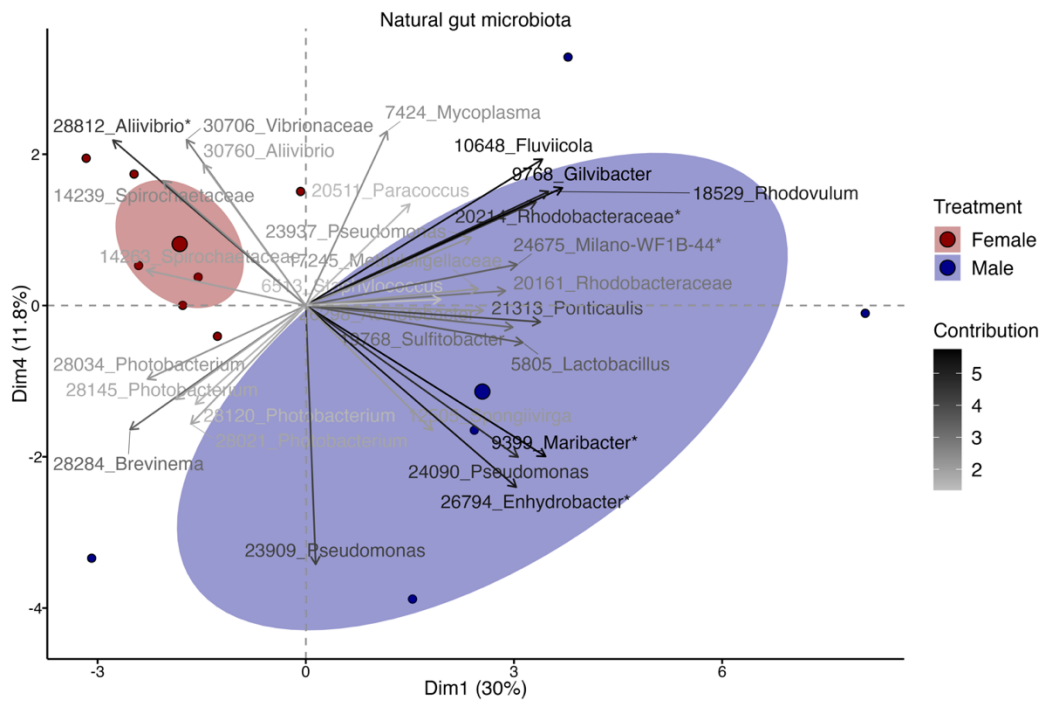
Supplementary Table 5: Two-way ANOVA comparing male organs under the influence of different treatments.



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Supplementary Figure 10: Violin and boxplot showing an alpha diversity comparison of females (red) and males (blue) separated by treatment group.



Supplementary Figure 11: The natural gut microbiota of male and female pipefish (Control group only) indicates a strong sexual dimorphism. Ellipses indicate a confidence interval around the group's mean. Factor map shows ASV ID and genus.

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	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Sex	1	18.76	18.761	14.539	0.000181 ***
Treatment	3	3.43	1.142	0.885	0.449662
Sex:Treatment	3	1.45	0.485	0.376	0.770559
Residuals	206	265.82	1.290		

Supplementary Table 6: Two-way ANOVA results suggest a sexual dimorphism within the alpha diversity of male and female pipefish

	Df	Pillai	Approx. F	Num Df	Den Df	Pr(>F)
(Intercept)	1	0.00000	0.00000	6	5	1.00000
Sex	1	0.89181	6.8689	6	5	0.02583 *
Residuals	10					

Supplementary Table 7: One-way ANOVA results suggest a sexual dimorphism in the gut microbiota of pipefish (beta-diversity). Here, only the gut tissue of untreated males and females (Control group) was analyzed.

Experiment 3 (B) – Juveniles

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	54	18	0.333	0.802
Time	1	16961	16961	313.608	<2e-16 ***
Treatment:Time	3	238	79	1.466	0.242
Residuals	32	1731	54		

Supplementary Table 8: Juvenile weight development is affected by time but not by treatment

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	7	2	1.150	0.3441
Time	1	4968	4968	2380.310	<2e-16 ***
Treatment:Time	3	19	6	2.991	0.0454 *
Residuals	32	67	2		

*Supplementary Table 9: Juvenile size development is strongly affected by time and time*treatment interaction but not by treatment alone*

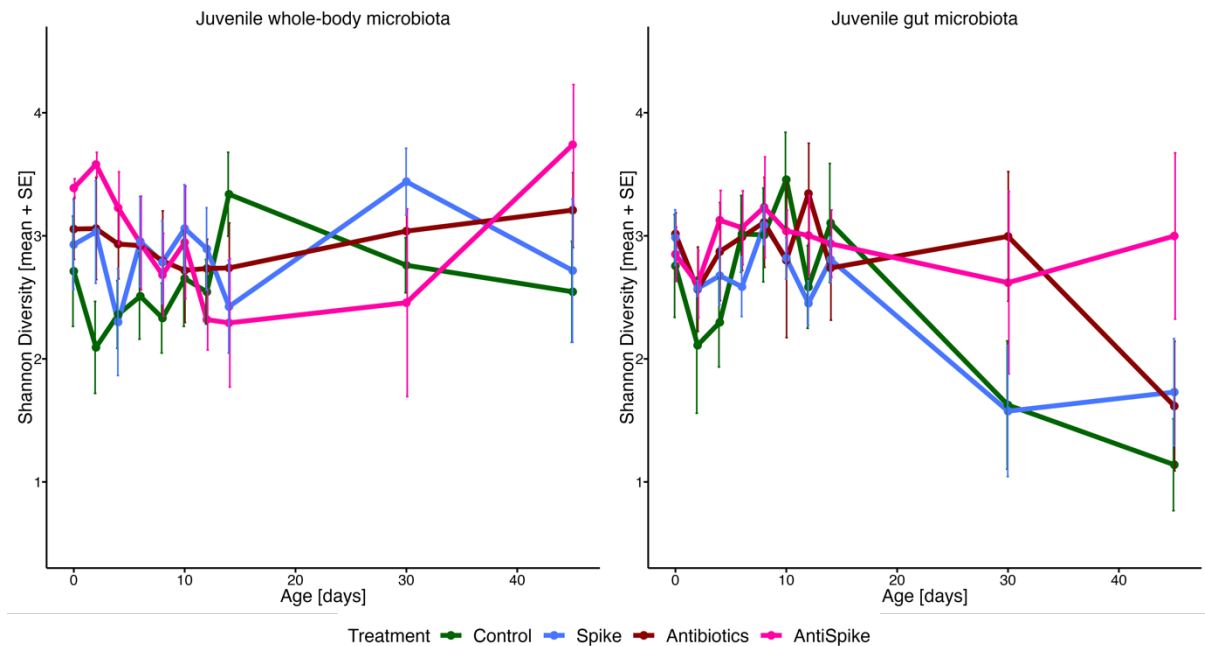
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	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	3.31	1.10	22.95	1.85e-14 ***
Time	1	124.10	124.10	2583.85	<2e-16 ***
Treatment:Time	3	2.21	0.74	15.37	8.03e-10 ***
Residuals	1234	59.27	0.05		

Supplementary Table 10: Juvenile survival is strongly affected by treatment, time and their interaction

Contrast	Estimate	SE	df	t.ratio	p.value
Antibiotics-AntiSpike	0.0230	0.0184	1234	1.251	0.5945
Antibiotics-Control	0.0451	0.0162	1234	2.792	0.0273 *
Antibiotics-Spike	-0.0942	0.0175	1234	-5.398	<.0001 ***
AntiSpike-Control	0.0221	0.0184	1234	1.198	0.6283
AntiSpike-Spike	-0.1172	0.0196	1234	-5.992	<.0001 ***
Control-Spike	-0.1393	0.0175	1234	-7.983	<.0001 ***

Supplementary Table 11: Contrasts (pairwise, adjusted by TukeyHSD) reveal that offspring of spike-treated fathers have a significantly higher survival.



Supplementary Figure 12: Alpha diversity (Shannon index) of juvenile whole body (A) and gut (B) microbiota from birth until 45 days post release. Colors indicate paternal treatment groups. Data points represent mean diversity ± standard error (SE).

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	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	2.878693	0.128019	22.486	<2e-16 ***
AntiSpike	0.026886	0.163318	0.165	0.8694
Control	-0.335738	0.161476	-2.079	0.0386 *
Spike	-0.060166	0.164025	-0.367	0.7141
TimePoint	0.002756	0.004850	0.568	0.5704

Supplementary Table 12: Whole-body microbiota: ANCOVA testing the influence of treatment and/or time on microbiota composition (alpha-diversity)

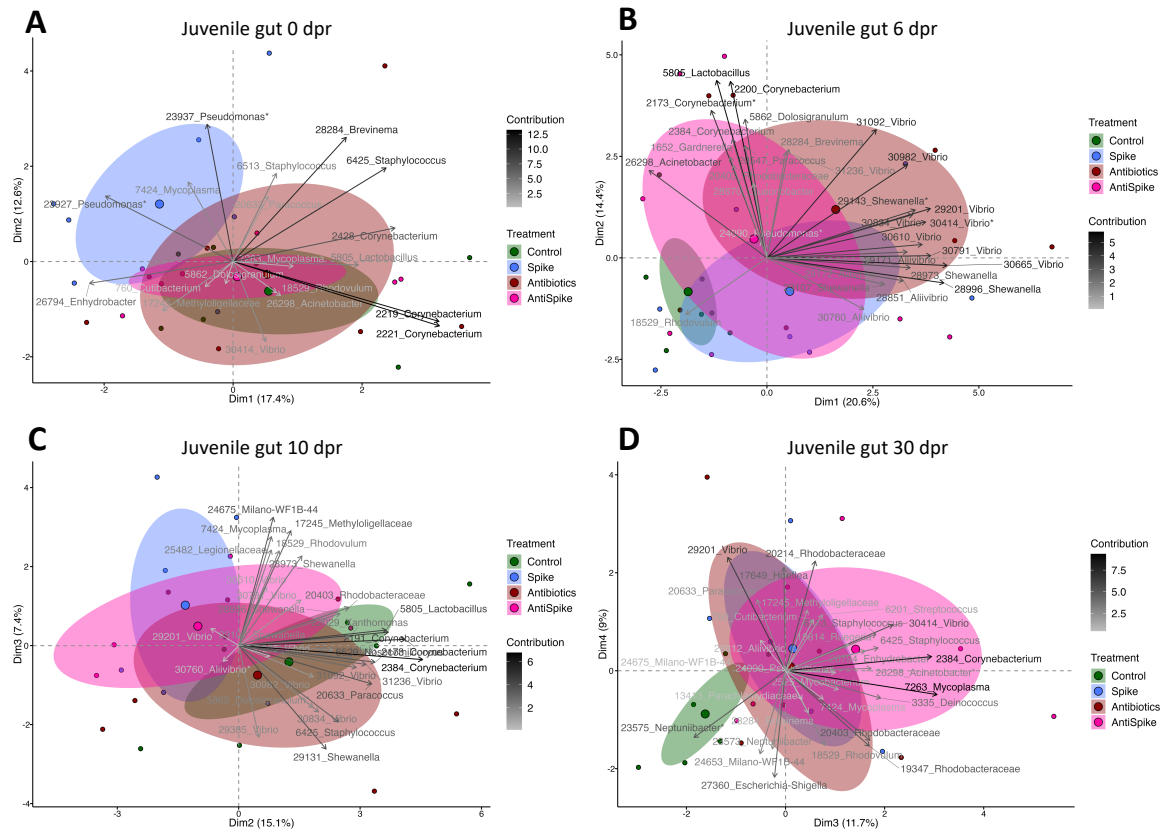
	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	3.092842	0.133810	23.114	< 2e-16 ***
AntiSpike	0.127106	0.170633	0.745	0.457
Control	-0.269765	0.169415	-1.592	0.113
Spike	-0.246375	0.171374	-1.438	0.152
TimePoint	-0.022126	0.004995	-4.430	1.4e-05 ***

Supplementary Table 13: Gut microbiota: ANCOVA testing the influence of treatment and/or time on microbiota composition (alpha-diversity)

dpr	0	2	4	6	8	10	12	14	30	45
AB	0.386	0.052	0.043	0.039	0.636	0.025	0.094	0.717	0.025	0.501
SP	0.204	0.013	0.035	0.006	0.302	0.017	0.299	0.015	0.009	0.308
AB:SP	0.826	0.943	0.261	0.267	0.602	0.883	0.084	0.039	0.251	0.411

Supplementary Table 14: Beta-diversity: ANOVA p-values evaluating the MANOVA model on the principal components (PCs) of the external whole-body microbiome of juveniles at each time point. Comparison of Antibiotics (AB) treatment groups (Antibiotics and AntiSpike) and Spike (SP) treatment groups (Spike and AntiSpike).

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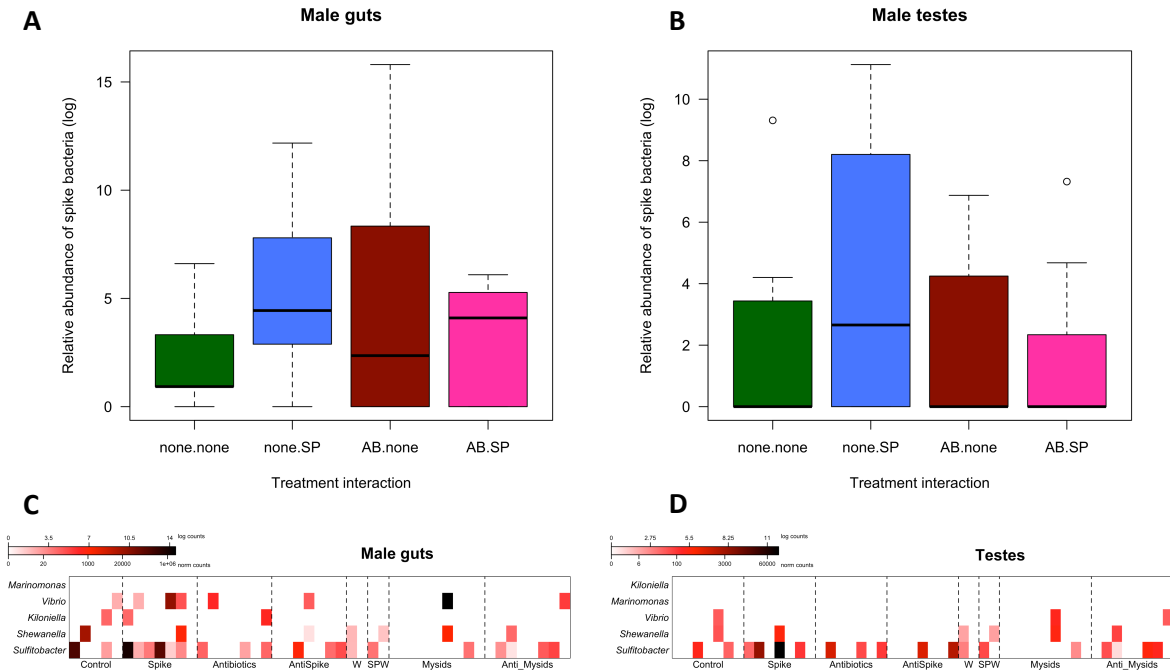


Supplementary Figure 13: Internal (gut) microbiota composition of juveniles from birth (A), 6 days (B), 10 days (C) up to 30 days (D) after birth. Colors represent paternal treatment. Ellipses show a 95% confidence interval around the group's mean. Factor map shows ASV ID and genus name respectively family name if genus could not be identified.

dpr	0	2	4	6	8	10	12	14	30	45
AB	0.646	0.399	0.435	0.185	0.245	0.875	0.135	0.923	0.332	0.218
SP	0.325	0.467	0.488	0.941	0.591	0.291	0.044	0.924	0.366	0.558
AB:SP	0.471	0.290	0.244	0.336	0.422	0.975	0.481	0.348	0.609	0.707

Supplementary Table 15: Beta diversity: ANOVA p-values evaluating the MANOVA model on the principal components (PCs) of the internal sterile gut microbiome of juveniles at each time point. Comparison of Antibiotics (AB) treatment groups (Antibiotics and AntiSpike) and Spike (SP) treatment groups (Spike and AntiSpike).

Experiment 3 (C) – Spike community



Supplementary Figure 14: Relative abundance of spike community strains in the male gut (A) and the male testes (B). Heatmaps showing counts of individual strains for gut (C) and testes (D).

	Placenta-like tissue	Brood pouch swabs	Male gut	Male testes	Ovipositor	Female gut
All strains	0.021	0.044	0.217	0.637	0.250	0.080
<i>Kiloniella</i>	0.433	0.321	0.532	<i>n/a</i>	0.368	0.392
<i>Marinomonas</i>	<i>n/a</i>	0.392	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>
<i>Sulfotobacter</i>	0.091	0.760	0.817	0.542	0.286	0.082
<i>Vibrio</i>	0.258	0.240	0.219	0.392	0.392	0.708
<i>Shewanella</i>	0.392	0.018	0.392	0.392	0.179	0.430

Supplementary Table 16: Kruskal-Wallis H test *p*-values indicate impact of spike community strains on adult tissues of *S. typhle*. Data is based on normalized counts.

List of Supplementary Spreadsheets

1. **1_Exp1_statistics.xlsx**: Statistical tests for experiment 1
2. **2_Exp2_Antibiotics_stats.xlsx**: Alpha diversity tests for experiment 2
3. **3_Exp2_beta_sex-specific_microbiome_stats.xlsx**: ISA and test statistics for sex-specific microbiota in experiment 2
4. **4_Exp3_Life-History.xlsx**: Life-history parameters recorded during experiment 3
5. **5_Exp3_beta_adult_microbiome_stats.xlsx**: ISA and test statistics for adult microbiota in experiment 3
6. **6_Exp3_beta_WholeJuv_microbiome_stats.xlsx**: ISA and test statistics for whole-body juvenile microbiota in experiment 3
7. **7_Exp3_beta_SteGut_microbiome_stats.xlsx**: ISA and test statistics for juvenile gut microbiota in experiment 3
8. **8_Exp3_Spike_screening_stats.xlsx**: Test statistics for spike screening on genus level

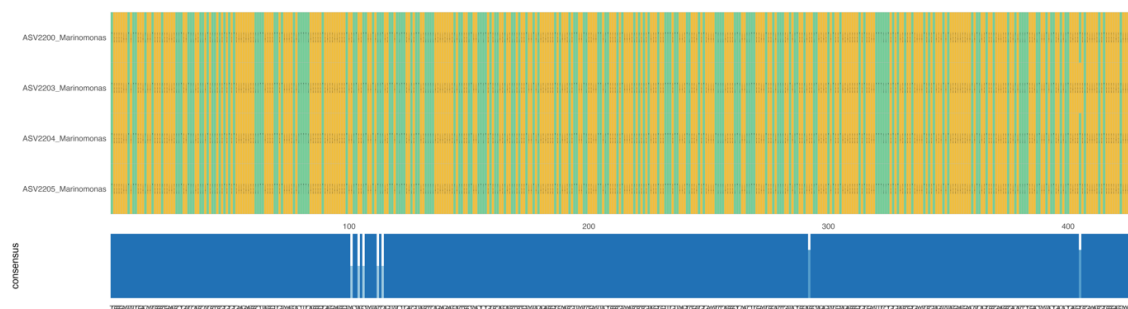
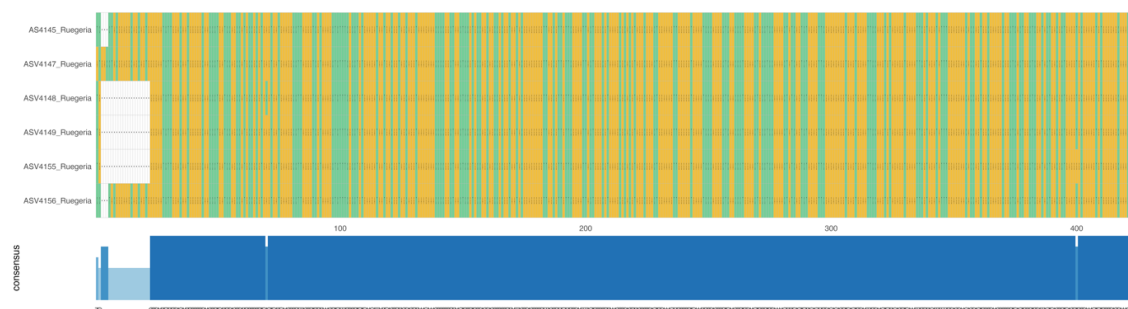
Appendix – Chapter II

Target Gene	Primer Direction	Sequence (5' → 3')
GFP	Forward	TCCATGGCCAACACTTGTCA
GFP	Reverse	ACGTGTCTTTGAGTTTCCCGTC
mCherry	Forward	GCGCCTACAACGTCAACATC
mCherry	Reverse	TTTACTACAGCTCGTCCATGCC

Supplementary Table 1: Primers used for qPCR detection of GFP and mCherry genes

Spike strain	Tagging	Transfer	Assigned ASV IDs
<i>Marinomonas</i>	GFP	Paternal (mating)	ASV2200, ASV2203, ASV2204, ASV2205
<i>Ruegeria</i>	mCherry	Horizontal (day 21)	ASV4145, ASV4147, ASV4148, ASV4149, ASV4155, ASV4156

Supplementary Table 2: Information about the two spike strains used in this experiment

Supplementary Figure 1: Multiple Sequence Alignment (MSA) of ASVs assigned to the *Marinomonas* spike community (Paternal transfer). ASV IDs are based on the consensus sequence of the 16S rRNA V3/V4 hypervariable regions (~430 bp).Supplementary Figure 2: Multiple Sequence Alignment (MSA) of ASVs assigned to the *Ruegeria* spike community (Horizontal transfer). ASV IDs are based on the consensus sequence of the 16S rRNA V3/V4 hypervariable regions (~430 bp).

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Number of samples	200
Minimum number of reads	24
Median	25465.0
Mean	24598.241706161138
Standard error	888.6669
Maximum number of reads	61037
Total reads	5190229
Number of representative sequences	4508

Supplementary Table 3: QIIME2 sequence processing statistics.

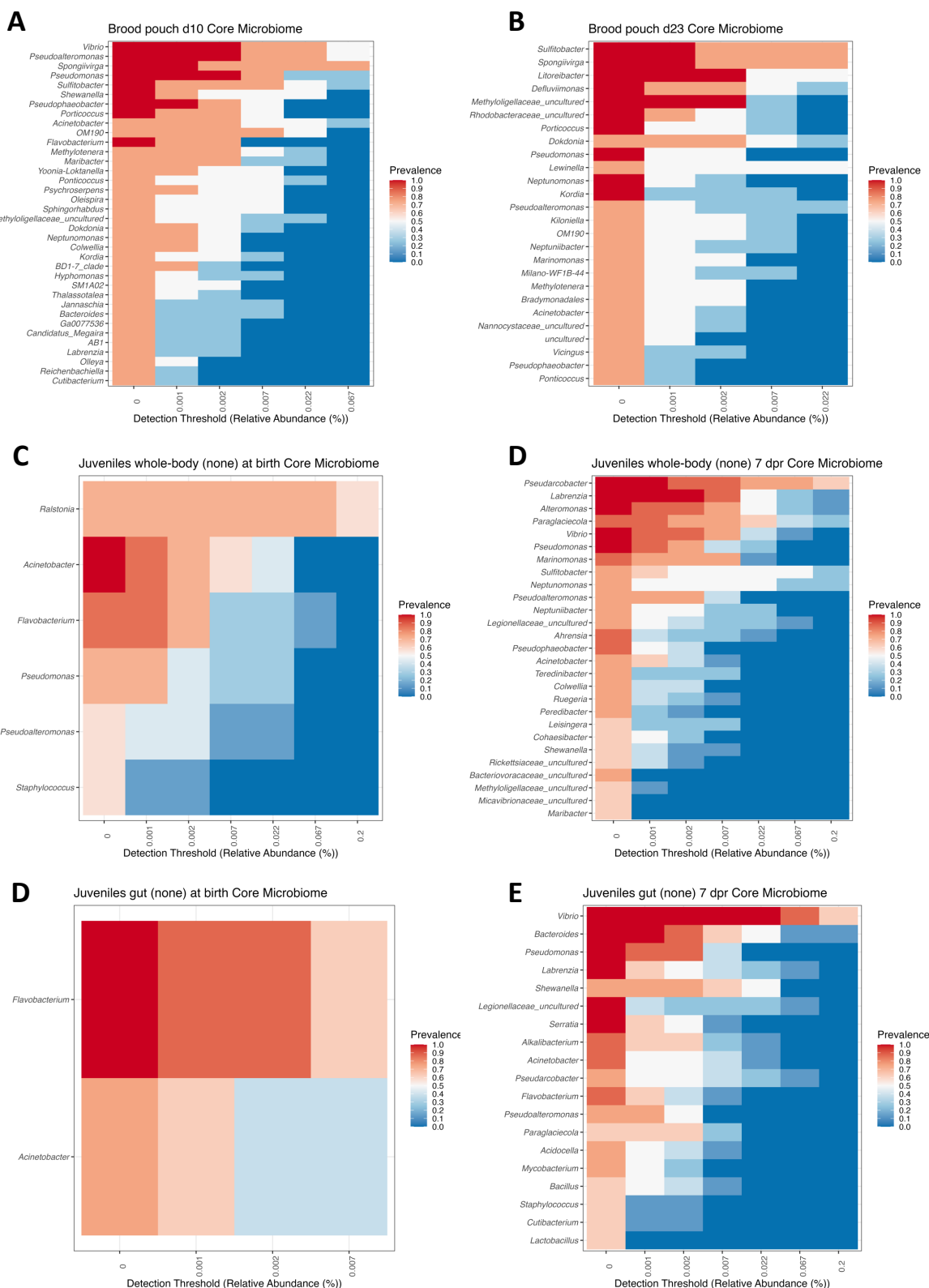
Tissue	Day	Top 5 core microbiota (genus level) – control group
Brood pouch	10	<ul style="list-style-type: none"> - <i>Vibrio</i> - <i>Pseudoalteromonas</i> - <i>Spongiivirga</i> - <i>Pseudomonas</i> - <i>Sulfitobacter</i>
	23	<ul style="list-style-type: none"> - <i>Sulfitobacter</i> - <i>Spongiivirga</i> - <i>Litoreibacter</i> - <i>Devluviimonas</i> - <i>Methyloligellaceae_uncultured</i>
Embryo	10	<ul style="list-style-type: none"> - <i>Vibrio</i> - <i>Pseudoalteromonas</i> - <i>Pseudomonas</i> - <i>Spongiivirga</i> - <i>Bacteroides</i>
Embryo sterile	10	<ul style="list-style-type: none"> - <i>Acinetobacter</i> - <i>Bacteroides</i> - <i>Pseudomonas</i> - <i>Vibrio</i> - <i>Flavobacterium</i>
Juvenile whole-body	23 (0 dpr)	<ul style="list-style-type: none"> - <i>Acinetobacter</i> - <i>Flavobacterium</i> - <i>Pseudomonas</i> - <i>Pseudoalteromonas</i> - <i>Staphylococcus</i>

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	30 (7 dpr)	<ul style="list-style-type: none"> - <i>Pseudoarcobacter</i> - <i>Labrenzia</i> - <i>Alteromonas</i> - <i>Paraglaciecola</i> - <i>Vibrio</i>
Juvenile gut	23 (0 dpr)	<ul style="list-style-type: none"> - <i>Flavobacterium</i> - <i>Acinetobacter</i>
	30 (7 dpr)	<ul style="list-style-type: none"> - <i>Vibrio</i> - <i>Bacteroides</i> - <i>Pseudomonas</i> - <i>Labrenzia</i> - <i>Shewanella</i>
Water	10	<ul style="list-style-type: none"> - <i>NS3a_marine_group</i> - <i>Flavobacterium</i> - <i>RS62_marine_group</i> - <i>SAR92_clade</i> - <i>Pseudoalteromonas</i>
	23	<ul style="list-style-type: none"> - <i>Glaciecola</i> - <i>Pseudorhodobacter</i> - <i>Celeribacter</i> - <i>Oleispira</i> - <i>Neptuniibacter</i>

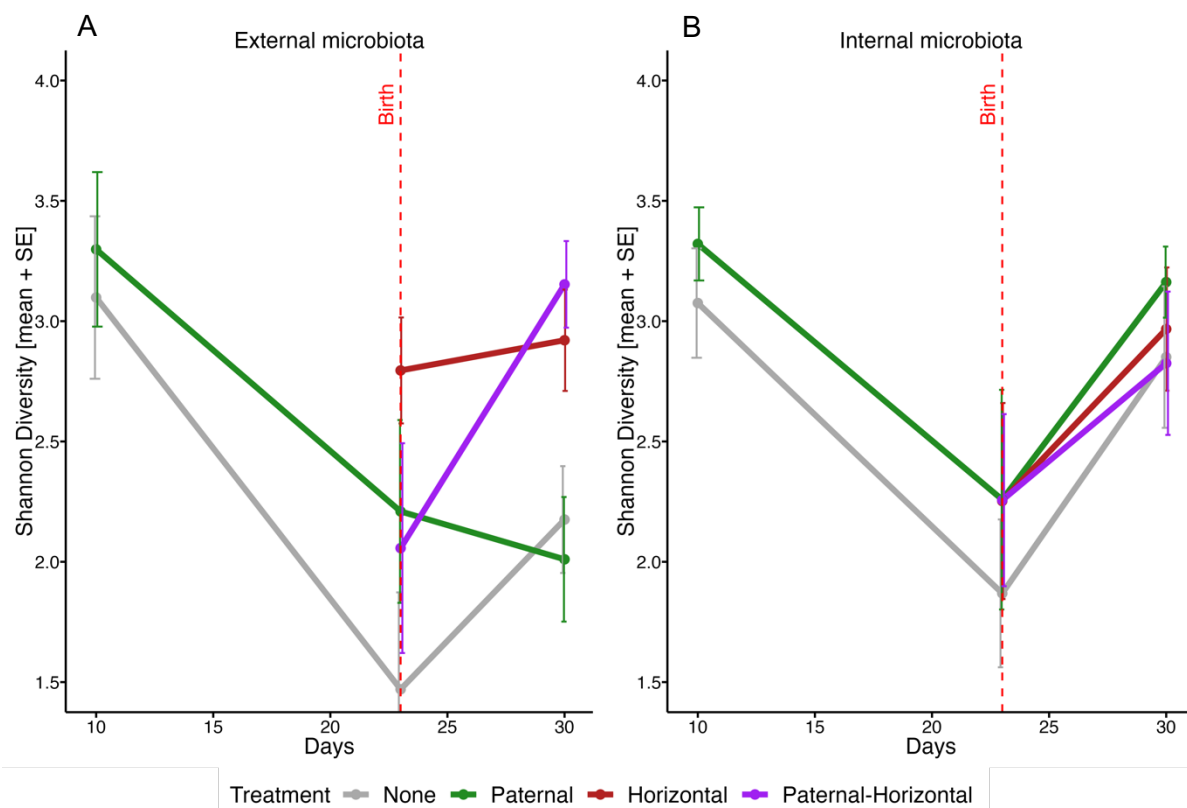
Supplementary Table 4: Core microbiota of all tissues (untreated control group). Taxa were selected on ASV level (0.0001 abundance, 0.5 prevalence filter) and collapsed at genus level. Displayed are top 5 genera based on their total abundance across all samples of the respective group. Full list of core microbiota displayed in Supplementary Spreadsheet 2.

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Supplementary Figure 3: Heatmaps displaying core microbiota (genus level) of the untreated control groups for male brood pouch early (A) and late (B) pregnancy, whole-body juveniles at birth (C) and 7 dpr (D) as well as juvenile guts at birth (D) and 7 dpr (E). The detection threshold (relative abundance) is shown on the x-axis, with taxa listed on the y-axis. Prevalence values are indicated by the color gradient, with red representing higher prevalence (up to 1.0) and blue representing lower prevalence (down to 0).

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Supplementary Figure 4: Alpha diversity of external (A) and internal (B) microbiota during pregnancy (d10) until 7 days post-release (d30). Data points show mean Shannon diversity (+ SE). Colors represent treatment groups

List of Supplementary Spreadsheets

1. **SuppSpreadsheet1_Spike_Strains.xlsx**: ASVs and sequences of experimentally added spike strains
2. **SuppSpreadsheet2_Core_Microbiota.xlsx**: List of core microbiota ASVs with taxonomic classification
3. **SuppSpreadsheet3_beta_pregnancy_microbiome.xlsx**: ISA and test statistics for brood pouch and embryos
4. **SuppSpreadsheet4_beta_post-release_microbiome.xlsx**: ISA and test statistics for juveniles after birth
5. **SuppSpreadsheet5_alpha_diversity.xlsx**: alpha diversity test statistics (ANCOVA) of juveniles
6. **SuppSpreadsheet6_spike_screening.xlsx**: Test statistics (GLS) for spike screening on ASV level

“Every one of us is a zoo in our own right – a colony enclosed within a single body.

A multi-species collective.

An entire world.”

Ed Yong, I Contain Multitudes:
The Microbes Within Us and a Grander View of Life