Cultivation and characterization of sex-specific microbiota in the broad-nosed pipefish *Syngnathus typhle*

- Master's Thesis in Biological Oceanography -

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List of abbreviations

ANOSIM	Analysis of similarities
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
EPJ	Early pregnancy juveniles
EPP	Early pregnancy brood pouch
ERC	European Research Council
ETEST	Epsilometer test for antibiotic susceptibility testing
FG	Female gonads
GFP	Green Fluorescent Protein
GTR	General Time-Reversible substitution model
HTS	High-throughput sequencing
IKMB	Institute of Clinical Molecular Biology, Kiel
LPJ	Late pregnancy juveniles
LPP	Late pregnancy brood pouch
MB	Marine Broth cultivation media
MCA	Multiple correspondence analysis
MIC	Minimum Inhibitory Concentration
ML	Maximum Likelihood
MLSA	Multi locus sequence analysis
NCBI	National Center for Biotechnology Information
NGS	Next-Generation sequencing
NP	Non-pregnant males
OD	Optical density
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PSU	Practical Salinity Unit
RAxML	Randomized Axelerated Maximum Likelihood
rRNA	Ribosomal ribonucleic acid
SILVA	A ribosomal RNA gene database project
SINA	SILVA Incremental Aligner
SW	Seawater
TCBS	Thiosulfate-citrate-bile salts-sucrose cultivation medium
WGS	Whole genome sequencing

Abstract

All living organisms are inhabited by a mutualistic microbial community, the microbiota. The microbiota boosts the host's immune system, which in turn regulates growth and composition of the microbial community. In viviparous animals, a major pathway for immune priming occurs via commensal bacteria that are transferred from parents to offspring during pregnancy, birth and breastfeeding. Although these processes are usually unique to the female body, evidence exists that fathers likewise are involved in the transfer of immunity and may influence the offspring microbiota. With its unique reproductive strategy, the broad-nosed pipefish *Syngnathus typhle* combines female egg production with male pregnancy and therefore allows separating sex from parental investment. Exploring the sex-specific parental transfer of microbiota permits to describe the initial microbial colonization and its potential interaction with trans-generational immune priming.

A previous NGS-based study suggested differences in the microbial communities of female gonads and male brood pouch. Using culture-based approaches, I isolated bacteria originating from six different sex-specific tissues of *S. typhle* and characterized them by Sanger sequencing of the complete 16S rRNA gene. In contrast to 3090 OTUs identified with the NGS-approach, only 92 bacterial species from 38 genera were culturable under laboratory conditions, however, differences between sexes and pregnancy stages in the microbial community composition remained detectable. In the next step, I plan to transfer this knowledge to live pipefish to perform controlled *in vivo* depletion and manipulation of sex-specific microbiota. I will therefore identify and screen a subset of bacterial strains that are representative for the specific tissues for their resistance against three different families of antibiotics *in vitro*.

Kurzfassung

Alle Organismen werden von einer mutualistischen Bakteriengemeinschaft, dem Mikrobiom, besiedelt. Das Mikrobiom stärkt das Immunsystem des Wirts, welches wiederum Wachstum und Zusammensetzung der Bakteriengemeinschaft reguliert. Bei lebensgebärenden Tieren geschieht das Priming des Immunsystems hauptsächlich durch kommensale Bakterien, die während der Schwangerschaft, Geburt und Stillzeit von den Eltern auf die Nachkommen übertragen werden. Obwohl diese Prozesse normalerweise nur im weiblichen Körper ablaufen, gibt es Hinweise darauf, dass Väter ebenfalls an der Übertragung der Immunsystems beteiligt sind und daher das Mikrobiom der Nachkommen möglicherweise beeinflussen können. Mit einer einzigartigen Fortpflanzungsstrategie kombiniert die Grasnadel *Syngnathus typhle* weibliche Eiproduktion mit männlicher Schwangerschaft und ermöglicht so eine Trennung von Geschlecht und elterliche Investition. Eine Untersuchung des geschlechtsspezifischen elterlichen Transfers des Mikrobioms trägt zur Beschreibung von mikrobieller Besiedlung und möglicher Wechselwirkungen mit dem generationsübergreifenden Priming des Immunsystems bei.

Eine frühere NGS-basierte Studie deutet auf Unterschiede in den Bakteriengemeinschaft weiblicher Gonaden und männlicher Bruttaschen hin. Mit kulturbasierten Ansätzen habe ich Bakterien aus sechs verschiedenen geschlechtsspezifischen Geweben von *S. typhle* isoliert und durch Sanger-Sequenzierung des gesamten 16S-rRNA-Gens charakterisiert. Im Gegensatz zu 3090 identifizierten OTUs mit der NGS-Methode, konnte ich unter Laborbedingungen nur 92 Bakterienarten aus 38 Gattungen kultivieren. Unterschiede in der mikrobiellen Zusammensetzung zwischen den Geschlechtern und Schwangerschaftsstadien blieben dabei jedoch nachweisbar. Im nächsten Schritt plane ich, dieses Wissen auf lebendige Grasnadeln zu übertragen, um eine kontrollierte *in vivo* Dezimierung und Manipulation des geschlechtsspezifischen Mikrobioms durchzuführen. Ich werde daher eine Untergruppe von Bakterienstämmen, die für die spezifischen Gewebe repräsentativ sind, identifizieren und auf ihre Resistenz gegen drei verschiedene Antibiotika-Familien *in vitro* untersuchen.

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Thanks for all your encouragement!

1 Introduction

From protists to humans, all animals and plants are inhabited by a large number of microbial organisms (Rosenberg and Zilber-Rosenberg 2016). These microorganisms include bacteria, viruses, fungi, archaea, and eukaryotes. In terms of ecological relationships, the microbes can range from pathogenic to commensalistic and mutualistic, depending on how they interact with their host (Braga et al. 2016). During the last decades, research mainly focused on the pathogenic, thus harmful, proportion of microorganisms, while vastly ignoring the non-pathogenic mutualistic microbial community (Lozupone et al. 2012). Only recently, cross-disciplinary research on host-microbe interactions has started to investigate these beneficial microbes, called the "microbiota". Mainly incited by the evidence that the microbiota exerts a comparably great impact on the host like a distinct organ, the symbiotic interactions gained increasing attention (Egerton et al. 2018). From an evolutionary perspective, symbiotic interactions were essential for the origin of eukaryotic cells. However, the underlying mechanisms that allowed the development of autopoietic units, i.e. units that are clearly separated from the environment while being capable of self-maintenance, remains mostly unclear (Margulis et al. 2006; Damiano and Luisi 2010). Nevertheless, the increasing appreciation for the importance of such symbioses further challenged our perception of self by indicating that all multicellular organisms are not individuals but rather metaorganisms (Rohwer et al. 2002; Bosch and McFall-Ngai 2011; Rees et al. 2018). The term "metaorganisms" was originally coined by Bell (1998) to describe any multicellular organism that exists through a constant co-evolution with its microbiota but nowadays refers to any multicellular host with its distinct community of associated microorganisms (Biagi et al. 2012). Specifically the gastrointestinal tract of vertebrates is a dynamic microbial ecosystem, which harbors an extensive variety of complex host-microbial relationships that usually interact in a stable homeostasis (Schaedler et al. 1965; Mazmanian and Lee 2014; Wang et al. 2018). Evidence was found that animal-bacterial interactions significantly shaped evolutionary processes at the point when microbes not only served as prey but moved towards providing digestible molecules in the host's digestive system. This process was driven by the development of tube-shaped, one-way passage guts (McFall-Ngai et al. 2013). Increasing effort in researching intestinal microbiota showed that, on the one hand, these relationships play a crucial role in supporting the health maintenance of the host, including development, digestion and protection against pathogens (Sommer and Bäckhed 2013; Romero et al. 2014; Esser et al. 2018; Wang et al. 2018). Additionally, the associated commensal microbes shape several metabolic pathways and exert trophic effects on the intestinal epithelial tissues (Montalto et al. 2009). On the other hand, recent studies indicate that many emerging diseases and allergic reactions nowadays are

potentially the outcome of disturbances and imbalances in the microbiome, caused by a failure of host control mechanisms (Ojetti et al. 2009; Esser et al. 2018) (Figure 1).

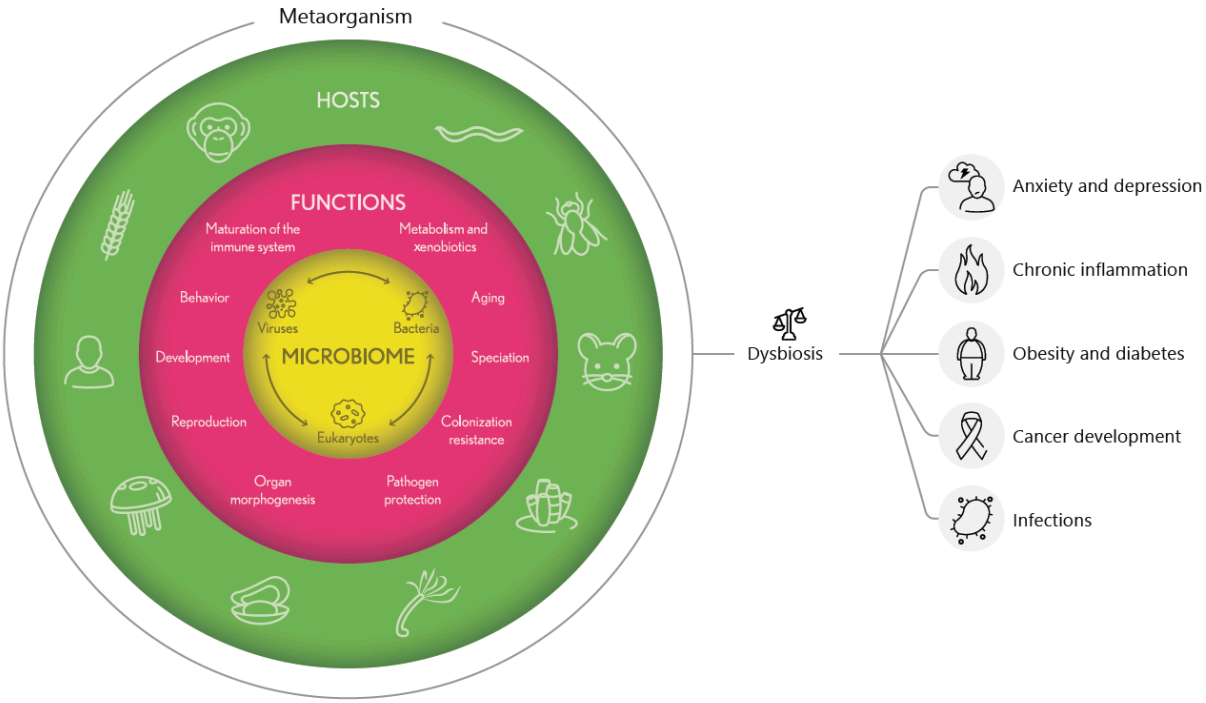


Figure 1: Functional interactions in metaorganisms. Esser et al. in Journal of Innate Immunity (2018)

In consideration of this, the constant interaction of the microbiota and the host immune defense is of great significance: The microbial community is recognized by the host's innate immune system which initiates an immune response against it, thus boosting the immune system. In contrast, the host's immune system regulates the presence, growth, and composition of the host-associated microorganisms, suggesting a strong interconnection between symbiont competition within the host ecosystem and host control over the symbionts (Hooper and Macpherson 2012; Thaiss et al. 2016; Foster et al. 2017). It is hypothesized that these complex interactions lead to a coevolution between the microbiota and a memory-based immune system (McFall-Ngai 2007; Lee and Mazmanian 2010). As most infections begin at the mucosal epithelia of an organism, these surfaces developed a mechanism to detect antigens while tolerating commensal microbes, the mucosal immune system (McGhee et al. 1992; MacDonald 2003). A set of innate and adaptive immune cells defend the host against pathogens while lymphocytes have developed to assist the immune system in differentiating between friends and enemies. Simultaneously, commensal microbes evolved a decreased pathogenicity which facilitates the colonization of the host's mucosal surfaces (Gomez et al. 2013). Together, the microbiota and the genome of the host

therefore form an effective immune regulatory system that has evolved to benefit from commensal bacteria rather than coding for these new beneficial traits itself (Gill et al. 2006).

In viviparous (live bearing) animals, this interconnected system of immunity boost and microbiota regulation was originally obtained from the environment. Between generations, it is then vertically translocated to the offspring during pregnancy (via the egg and placenta), birth (via direct contact with the mother's birth canal) and breastfeeding (via the breast milk) (Dunn et al. 2017; Pannaraj et al. 2017; van den Elsen et al. 2019). In most animals, all of these traits are usually united within the maternal body. For many years it was assumed that the development of the infants immune defense is initiated through microbial transfer from the mother (Hasselquist and Nilsson 2009; Zhang et al. 2013). However, several studies suggested, that the offspring microbial composition shows similarities to the one of the father (Yatsunenko et al. 2012; Schloss et al. 2014). Furthermore, evidence exists that fathers are likewise actively involved in transfer of immunity and this could potentially also hold true for the initial translocation of microbes, due to the close interconnection of the microbiota and the immune system (Roth et al. 2010, 2012b; Beemelmanns and Roth 2017). Therefore, the question arises, which role the microbiota takes during pregnancy and whether or not a sex-specific parental contribution influences the offspring microbiota. Besides, it is tempting to question, whether the microbiota impacts the immune system and if microbial transfer interlinks with trans-generational immune priming, the transfer of parental immunity to its offspring (Tetreau et al. 2019).

In order to better understand the connections between microbiota and its trans-generational transfer, one needs to acknowledge the evolution of pregnancy. In the course of vertebrate evolution, viviparity has evolved independently in over 150 lineages, driven by the selection for increased parental investment (Blackburn 2015). Viviparity requires a significantly higher investment (time, energy, resources) from the parent than oviparity (egg laying) (Clutton-Brock 1991). However, the protection of the embryo from external biotic (e.g. predation, parasitism) and abiotic effects (e.g. climate) demonstrates a major advantage over oviparity in a broad range of environmental conditions. The evolution of viviparity is an outstanding example for convergent evolution across vertebrates (Blackburn 1999, 2015; Lambert and Wiens 2013). Among the Osteichthyes (bony fishes), viviparity is found in more than 50 families, however, Syngnathidae, the family of seahorses, sea dragons and pipefish, are the only family in which male viviparity has developed (Crespi and Semeniuk 2004; Stölting and Wilson 2007). The unique reproductive strategy of seahorses and pipefish allows disentangling sex from parental investment: Females produce eggs that are transferred to the male through a small ovipositor. In the Nerophinae, eggs are glued to the ventral side of the male without receiving any further protection. In *Syngnathus*

and the Hippocampinae, however, eggs are transferred into sealed brood pouches. Embedded inside the brood pouch with its placenta-like structure, eggs are fertilized, protected and nourished for approximately five weeks until the male gives birth to the offspring (Azzarello 1991; Kvarnemo et al. 2011).

Figure 2: Broad-nosed pipefish *Syngnathus typhle*. Illustration by H. Gervais in Les Poissons (1877)

With the aim to unravel the importance of sex-specific parental contribution in regard to initial microbial colonization and the potential impact on the priming of the offspring immune defense, the sex-role reversed broad-nosed pipefish *Syngnathus typhle* serves as a perfect model organism. *S. typhle* is a syngnathid native to the seagrass meadows in shallow coastal waters of the Eastern Atlantic, Mediterranean and Baltic Sea (Malavasi et al. 2007) (Figure 2). Investigating the sex-specific microbial transfer of an organism in which egg production (transovarial microbial transfer) is separated from internal parental care (microbial transfer during pregnancy), further allows for an independent assessment of both, the mother's and the father's environmental experience and their joint impact on the offspring (Roth et al. 2011). This environmental experience, i.e. immunity acquired against previously encountered pathogen epitopes, plays a key role in boosting the immune response, as shown in a studies by Roth et al. (2012a) and Beemelmanns and Roth (2016), which revealed that the male pregnancy in *S. typhle* enables additional bacteria-specific immune priming of the next generation against locally abundant pathogens, e.g. bacteria of the genus *Vibrio spp*.

Growing appreciation for the diversity and importance of associated commensal microbes drives research further into exploring their exact functions and values as several underlying molecular mechanisms remain elusive. However, the majority of bacteria are considered to be "uncultivable" (Ekkers et al. 2012). It is estimated that less than 1-5% of the microbes visible under the microscope can be cultivated under laboratory conditions. This phenomenon, described as "the

great plate count anomaly", may be caused by several factors, including the cultivation media and its byproducts, the overall environmental conditions and lacking signals or interactions with other microorganisms and. Additionally, slow growth and the occurrence of rare species are frequently underestimated (Staley and Konopka 1985; Harwani 2012; Stewart 2012; Tanaka et al. 2014). With the emergence of novel cultivation techniques, studying the bacterial communities of the gut, the most common tissue for microbiota research, experienced a boost in the 1970s (Lagkouvardos et al. 2017). Most earlier studies were based on simple isolated culture experiments of fish gut bacteria (cf. Sakata et al. 1981) or microscopic characterizations of microbes, e.g. to reveal interactions between diet and microbe composition (Fishelson et al. 1985). Recent cultivation-based studies of fish microbiota showed that the percentage of total cultivable bacteria is usually low but may vary extremely between studies and species: estimates range from as low as 0.1% (Llewellyn et al. 2014; Wang et al. 2018) to up to 50% for characterization in rainbow trout (Spanggaard et al. 2000; Huber et al. 2004). By the turn of the millennium, advances in biotechnology gave rise to culture-independent high-throughput sequencing (HTS) methods, which facilitated fast characterizations of complex samples (Ercolini 2013). Due to the increasing time and cost efficiency of these methods, it was finally possible to describe the huge host-associated microbial diversity in gastro-intestinal systems, including those of fishes (Ghanbari et al. 2015). Although genomic approaches have revolutionized the field of microbiology by revealing a bigger picture of the entire microbiome network, culture-based approaches involving the physical bacterial strain remain a requirement for experiments assessing the characteristic features and functions of the microbiota (Clements et al. 2014). The more modern technologies promote to study the entire range of host-associated microbiota, the more challenging it appears to return to culture-dependent approaches.

Much is already known about the role of the intestinal microbiota. It is now time to move towards other tissues to broaden the understanding of the crucial processes and roles that the microbiota is involved in. In recent years, studies investigated the shift of skin microbiota in common snook (*Centropomus undecimalis*) and Atlantic salmon (*Salmo salar*) during their transitions between different environments (Lokesh and Kiron 2016; Tarnecki et al. 2019). Keller et al. (2018) further investigated the buccal microbiota in mouthbrooding cichlids. In contrast, studying the microbiota in the context of pregnancy evolution is a novel field of research. This is due to the long-standing assumption of the "sterile womb" hypothesis which implies that, at least in humans, uterus and placenta are sterile body cavities in a healthy pregnancy (Perez-Muñoz et al. 2017; Schoenmakers et al. 2019). Based on this, the presence of microbes during pregnancy was exclusively linked with infections, inflammations and other complications, often leading to preterm

births (Romero et al. 2007). However, this concept has been challenged in recent years by the characterization of a consistently present microbiota in the placenta (Aagaard et al. 2014), vagina (The Human Microbiome Project Consortium 2012; Wassenaar and Panigrahi 2014) and uterine cavity (Verstraelen et al. 2016) of healthy pregnant women. Furthermore, evidence exists that fetuses already develop their own distinct microbiota inside the womb (Younge et al. 2019). These findings now pave the way for studies that propagate investigating the role of microbes during pregnancy.

In a previous study conducted in my lab, Beemelmanns et al. (2019) examined the sex-specific composition of microbiota of *S. typhle*. Investigated were both sexes and four developmental stages of the male brood pouch (non-pregnant, early pregnancy, mid pregnancy and late pregnancy). Additionally, a sub-group of males and females were immune challenged by the injection of heat-killed bacteria. Microbial DNA samples were amplified for their 16S rRNA genes and characterized by next-generation sequencing (NGS). With this cultivation-independent technique, a total of 3090 OTUs were characterized which could demonstrate distinct differences in the microbial β-diversity of female gonads and male brood pouch. Regarding the microbial diversity over the course of pregnancy, no difference between non-pregnant, early and mid-pregnancy was detected. Only during late pregnancy, the microbial community experienced a major shift, resulting in a closer resemblance to the microbial composition of female gonads. This shift became particularly clear in those fish that had previously received an immune challenge.

Following up on this research, this master's thesis is the first phase of a study conducted within the ERC-funded project "Male pregnancy – Unravelling the coevolution of parental investment and immune defense", which aims at investigating the role of sex-specific microbiota in *Syngnathus typhle* (cf. 6 Future Perspectives). My aim is to illuminate the sex-specific transfer of microbiota in the pipefish *Syngnathus typhle*, its interactions with the immune system and to shed light on the function of the initial microbial colonizers. I hypothesize that both, maternal and paternal contributions significantly shape the offspring microbiota. For evaluation, the intended experimental microbial manipulation, however, requires having the targeted bacterial strains physically available. In accordance with the findings by Beemelmanns et al. (2019), I expect differences between male and female microbiota composition to be detectable even within the potentially small fraction of cultivable bacteria and that similar indicator species can be found in both, culture-independent and culture-dependent genotyping approaches. To address this, I isolated the microbiota from different sexes, pregnancy stages and tissues, followed by cultivation and genotyping by 16S rRNA Sanger sequencing. I aimed at investigating, which proportion of the pipefish-specific microbiota is cultivable. As the culture-independent approach indicated shifts

during late pregnancy, I further hypothesize that *S. typhle* juveniles develop their own distinct microbiota over the course of the pregnancy which differentiates from the brood pouch microbiota. I am interested in unravelling the phylogenetic relationship of the sex-specific bacterial community and how it develops during pregnancy. Ultimately, the desired outcome of this thesis was to obtain a set of cultivable, sex-specific bacteria available for controlled microbiota manipulation experiments which are the main task during the second phase of this research study.

The implementation of the experiments requires cross-disciplinary knowledge in the fields of microbiology, evolutionary ecology, immunology, fish maintenance and physiology. Results will provide insight into the initial microbial colonization of embryos by unraveling the paternal and maternal contribution to microbiota transfer and later assess the interaction with the maturation of the immune system. This research project is therefore a novel and exciting approach to investigate the importance of the sex-specific microbiota in the context of male pregnancy evolution.

2 Methods

2.1 Fish catching and handling

The broad-nosed pipefish *Syngnathus typhle* were caught in May and June 2019 by snorkeling using standard snorkeling gear (mask, snorkel, fins and wetsuit) and a hand net. The fish were collected in the bay of Orth, located in the south-west of the island of Fehmarn (54° 44’ N, 11° 04’ E) (Figure 3). Upon catching, animals were transferred into an isolated box and transported back to the laboratory facilities at GEOMAR, followed by an acclimation for several days in large aerated barrels inside a climate chamber. After acclimation, the fish were moved into the aquaria system, which consists of 100 L tanks, connected via a filtered Baltic Sea water recirculation system. Artificial seagrass was provided in each tank for shelter and protection. Salinity and water temperature were adjusted to Baltic summer conditions of 15 PSU and 18°C. The light cycle was set to 12h light and 12h dark. Tanks were cleaned on a daily basis and the fish were fed twice a day with laboratory hatched, enriched *Artemia* sp. and frozen *Mysis* spp.

Figure 3: Locations for field sampling of *S. typhle* (green) and laboratory facilities (red). Map data ©2019 GeoBasis-DE/BKG, Google

2.2 Sex-specific microbiota isolation and cultivation

Microbiota samples were isolated from six different groups: two pregnancy stages, their corresponding juveniles, non-pregnant males and females of *Syngnathus typhle*:

1) Females: gonads (**FG**)
2) Non-pregnant males: Placenta-like tissue of the developing brood pouch (**NP**)
3) Early pregnant males: Placenta-like tissue of the brood pouch (**EPP**)
4) Early pregnant males: Whole juveniles (**EPJ**)
5) Late pregnant males: Placenta-like tissue of the brood pouch (**LPP**)
6) Late pregnant males: Whole juveniles (**LPJ**)

In total, 65 fish were used for the study: 20 females, 17 non-pregnant-, 16 early pregnant and 12 late pregnant males. Pipefish were killed by an overdose (500 mg/L) of the anesthetic tricaine methanesulfonate (MS-222), followed by length and weight measurements. The gonads of female pipefish were completely dissected. The brood pouch of male pipefish was opened, and the placenta-like tissue was extracted with sterile forceps, scalpels and surgical scissors. Using 100 µl sterile phosphate buffered saline (PBS), the tissue samples were flushed into 2 ml Eppendorf tubes and homogenized using a sterile pestle. To achieve a high number of cultivable bacterial strains, two different culture media that are commonly used for isolation and cultivation of marine bacteria were used: Marine Broth (MB) and thiosulfate-citrate-bile salts-sucrose agar (TCBS), both provided as pre-mixed instant medium.

(1) Marine Broth (MB)

Instant medium (Difco 2216)	37.4 g
Agar	15 g
ddH_20	1 l

Main components: peptone, yeast, magnesium chloride and sodium chloride

(2) Thiosulfate-citrate-bile salts-sucrose agar (TCBS)

Instant medium	35.2 g
Agar	1 g
ddH_20	400 ml

Main components: sucrose, dipeptone, sodium thiosulfate and sodium citrate, and sodium chloride

Marine Broth is considered as a general culture media for heterotrophic marine bacteria (Høvik Hansen and Sørheim 1991). In contrast, TCBS is commonly used for selectively cultivating gram-negative bacteria of the genus *Vibrio* spp. (Nicholls et al. 1976). The homogenized tissue was equally distributed and spread over the two agar plates using a sterile pipette tip. Afterwards, the agar plates were incubated in a dark incubator at 25°C for 4 days.

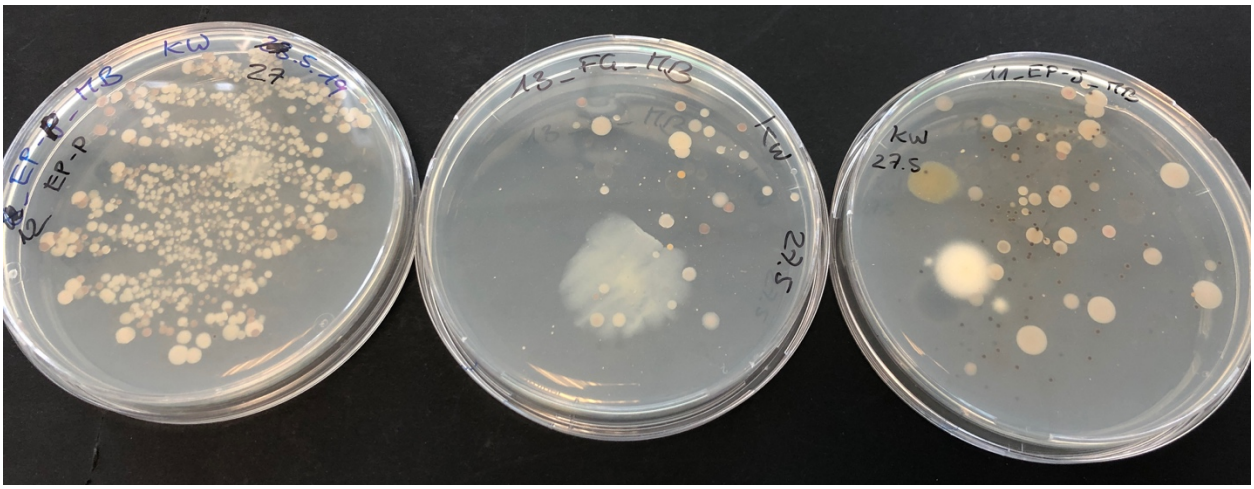


Figure 4: Cultivation of *S. typhle* sex-specific microbiota on Marine Broth (MB) agar. Displayed are isolated bacteria from the placenta-like tissue during early pregnancy (left), from the female gonads (middle) and the juveniles during early pregnancy (right). The bright white spot on the right plate is an unidentified fungus.

After incubation, the plates were visually examined (Figure 4). With the aim to capture a broad diversity of bacterial strains, as many morphologically different colonies as possible were selected for genotyping, summing up to a total of 385 colonies across all stages, including sea water (SW) and PBS controls. Each colony was described (size, color, texture) and assigned to a serial number. Purification was done by streaking part of the bacterial colony onto agar plates of the same culture medium that were previously divided into eights (Figure 5). After another 4 days incubation period, the purified bacterial colonies were scraped off the agar plate using a sterile toothpick.

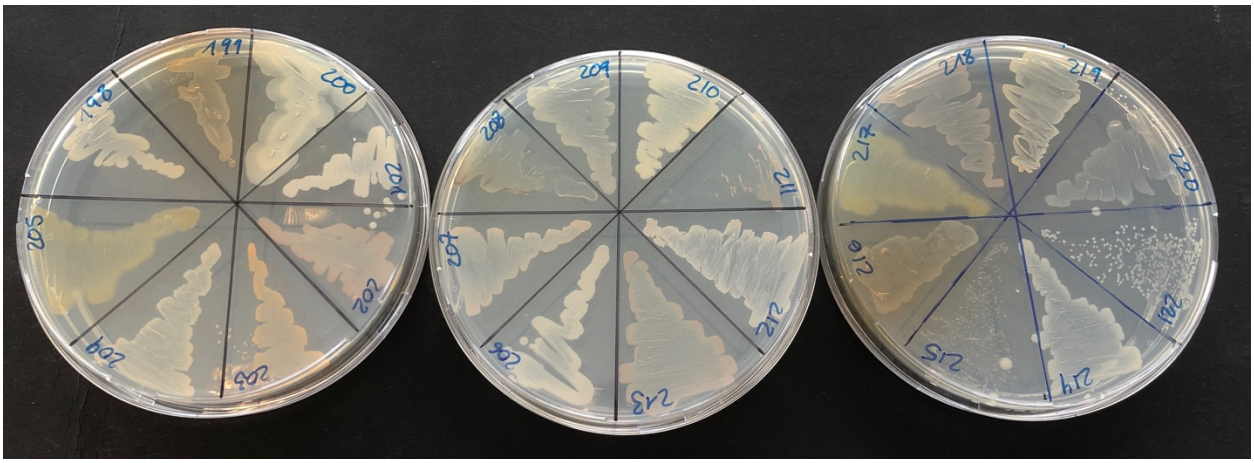


Figure 5: Purification of bacterial colonies on Marine Broth (MB) agar. The number corresponds to the assigned bacterial serial number.

Half of the colony was washed off with 50 µl PCR-grade water into 0.2 ml PCR tubes. The bacterial cells were boiled in a thermocycler at 99°C for 15 minutes to break up the cell walls, diluted 1:10 with PCR-grade water and stored at -20°C for colony PCR. The other half of the colony was washed off with 800 µl liquid Marine Broth medium into 2 ml Eppendorf tubes and placed on a shaking rack at 180 rpm and 25°C for 3 days. After incubation, 800 µl of a 50:50 MB-medium + glycerol mixture was added to the liquid cultures (25% final glycerol content), which were then transferred to a cryogenic freezer for preservation at -80°C.

2.3 Amplification and 16S rRNA genotyping

Characterization of the sex-specific microbiota was done based on PCR amplification of the 16S ribosomal RNA gene. The 16S rRNA gene is a ~1500 bp gene which consists of ten conserved and nine highly variable regions. It is present in all bacterial species to code for the RNA component of the 30S subunit of the bacterial ribosome (Figure 6). In a 30 µl reaction, 16S rRNA

genes of all microbiota samples were amplified using the 'Hot Start' *Taq*-DNA-Polymerase (VWR International), following the manufacturer's protocol. Negative controls containing only PCR-grade water were included in all runs. Aiming for the highest possible taxonomic accuracy, two primers were selected to amplify nearly the complete length of the 16S rRNA gene: 27F (5' → 3': AGAGTTTGATCMTGGCTCAG, spanning positions 8-27) and 1492R (5' → 3': ACCTTGTTACGACTT, spanning positions 1492-1506) (Lane 1991). The colony PCR was carried with the following settings: 95°C for 1 min, 30 cycles of 95°C for 30 s, followed by 50°C for 30 s and 72°C for 1:30 min with a final elongation at 72°C for 7 min and an indefinite hold at 10°C. PCR products were visually evaluated by gel electrophoresis (2% agarose gel, GelRed nucleic acid stain, 100bp DNA ladder (Invitrogen/Thermo Fisher Scientific)).
Purification and Sanger sequencing of PCR products using both primers were carried out by the Institute of Clinical Molecular Biology (IKMB) at the University of Kiel, Germany.

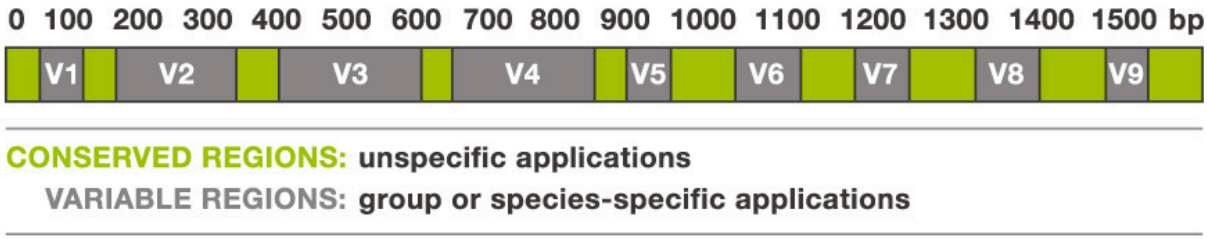


Figure 6: The full-length 16S rRNA gene with its nine variable regions (V1-V9) distributed throughout the highly conserved 16S sequence. Graphic retrieved from biology.stackexchange.com

2.4 Sequence processing and data analysis

Raw sequences were aligned and edited using the CodonCode Aligner software V8.0.2. (http://www.codoncode.com/aligner). Primer and other low-quality sequences were trimmed from the end of the contigs. A consensus sequence was exported and submitted to NCBI's BLAST - Basic Local Alignment Search Tool for taxonomic identification within the 16S ribosomal RNA sequences database using the Megablast algorithm (Altschul et al. 1997). For phylogenetic analyses, the consensus sequences were processed with the SINA aligner v1.2.11 (Pruesse et al. 2012). The software aligned the sequences using identical parameters and reference alignments employed for creating the SILVA alignment databases SEED (Release 137). SEED is a public dataset providing taxonomy for bacteria, archaea and eukaryota domains, based on phylogenetic trees for the rRNA genes (Quast et al. 2013; Yilmaz et al. 2014; Glöckner et al. 2017). In the generated output, all sequences that did not reach an identity of at least 70% to one

of the SEED sequences were omitted. Statistical phylogeny was estimated using maximum likelihood (ML). Maximum likelihood trees based on a General Time-Reversible (GTR) substitution model (Tavaré 1986) were computed with the aligned sequences, not including neighbor sequences. The phylogenetic trees were built using the RAxML (Randomized Axelerated Maximum Likelihood) program, due to its accuracy in returning good likelihood scores (Liu et al. 2011; Stamatakis 2014). Visualization and editing of the trees was performed using the software FigTree v1.4.4 (Rambaut 2018).

2.5 Statistical analyses

Data analysis, statistical testing and graphic visualization were performed using a presence-absence matrix of 79 samples across all stages (FG, NP, EPP, EPJ, LPP, LPJ and control) versus 98 bacterial species in the R software environment v.3.5.3 (R Core Team 2019). Microbiota composition by developmental stage for the 12 most abundant bacterial genera was visualized with the data visualization packages ggplot2 and ggpubr (Wickham 2009; Kassambara 2019). Moreover, 29 significant bacterial species were chosen to demonstrate a potential change in composition at a higher resolution. To account for potential differences in using a general and a species-specific cultivation media, all plots were generated twice: one containing the data from MB agar only and one showing the combination of both, MB and TCBS. To statistically test whether there is a significant difference between the microbiota found in the six different groups, an analysis of similarities (ANOSIM) between all stages and within each stage individually was performed using the Community Ecology Package vegan v2.5-4 (Oksanen et al. 2019). ANOSIM test statistics use a ranked dissimilarity matrix and were calculated with the semimetric Bray-Curtis dissimilarity index based on 999 permutations. A multiple correspondence analysis (MCA) using the FactoMineR package in R was applied to detect associations between the developmental stages, reveal variables that strongly contribute in explaining variations in the data set and represent the outcome in a low-dimensional Euclidean space (Lê et al. 2008). An MCA biplot that visualizes a pattern of the developmental stages (scores) with a factor map displaying the 20 most important taxa (loadings) was created for the first three dimensions. In total, the first three dimensions explained 12.28 of the total variances. To visualize a potential clustering pattern of the developmental stages, data ellipses were drawn for each stage using the Companion to Applied Regression (car) package (Weisberg and Fox 2019).

3 Results

In total, 767 16S rRNA sequences were retained from Sanger sequencing. After assembly and quality control, 376 consensus sequences were exported for analysis. From 79 tissue samples across all stages (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)), I could identify 92 bacterial species from 38 genera. This corresponds to 2.97% of the total number of OTUs identified in the cultivation-independent sequencing approach by Beemelmanns et al. (2019). Additionally, another six bacterial species from four genera were isolated from the seawater in which the fish were kept. A PBS negative control taken on day 3 of microbiota sampling indicated minor contaminations which were identified as *Rhodococcus quingshengii* and *Tenibaculum mesophilum*. One negative control used in the PCR showed a band on the agarose gel which was identified as *Marinomonas rhizomae*. Whereas all tissue samples from the late pregnancy stages (LPP, LPJ) contained cultivable bacteria (100%), less than two third (65%) of tissue samples from the female gonads (FG) contained bacteria that were cultivable under laboratory conditions (Table 1).

Table 1: Overview of sampling and microbiota cultivation. Shown are six groups sampled from *S. typhle* and two control groups

Sex/Stage	Samples	Cultivated	Proportion [%]	Bacterial species identified
Female gonads (**FG**)	20	13	65	47
Non-pregnant (**NP**)	17	16	94	55
Early pregnancy pouch (**EPP**)	16	11	69	49
Early pregnancy juveniles (**EPJ**)	15	10	67	41
Late pregnancy pouch (**LPP**)	12	12	100	51
Late pregnancy juveniles (**LPJ**)	12	12	100	51
Seawater (**SW**)	4	4	100	22
PBS control (**PBS**)	4	1	25	2

The analysis of similarities (ANOSIM) between and within all stages revealed returned a significant p-value < 0.05. However, an R value of 0.067 indicated that the similarity within all stages was not lower than between the stages, thus no clear effect across the sampling groups was detected (Figure 7). In contrast to all other tested groups, the group of bacteria isolated from the non-pregnant (NP) brood pouch exclusively displayed a lower dissimilarity index and within-group median.

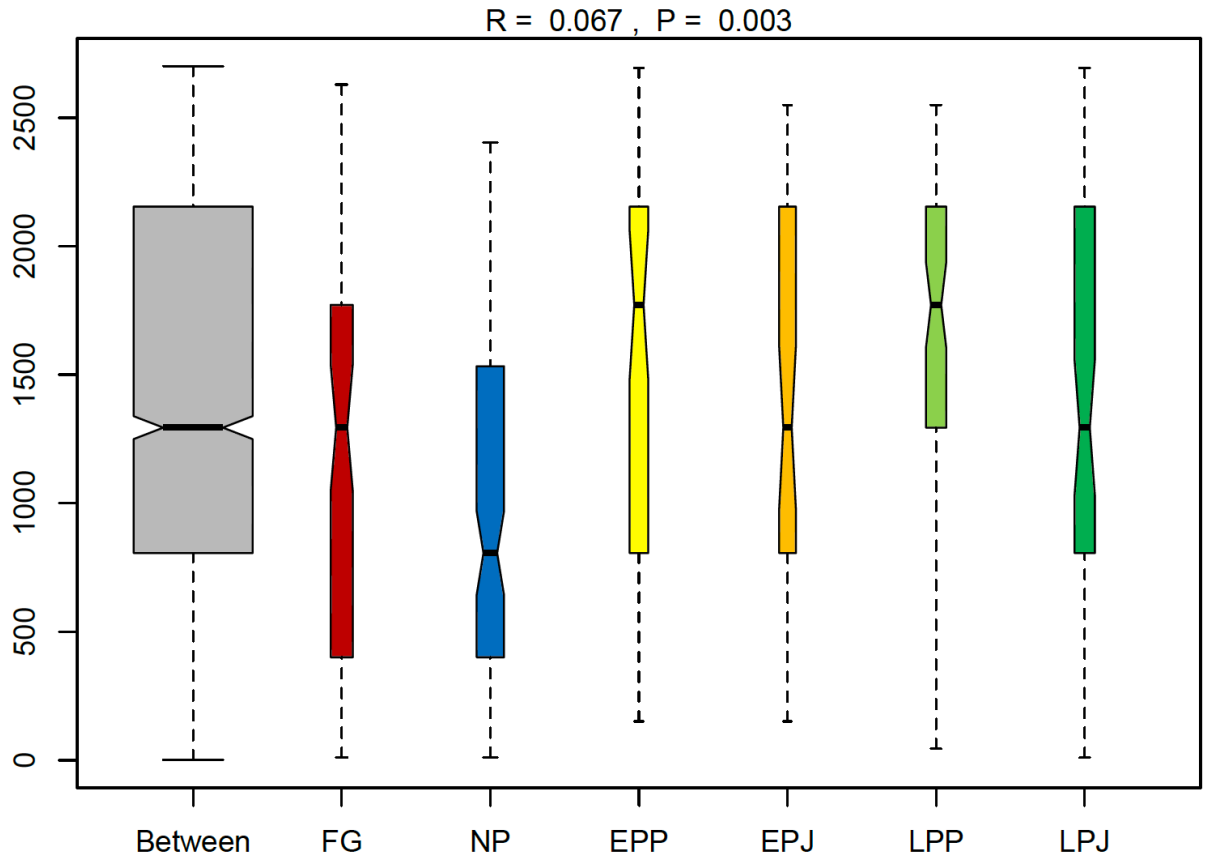


Figure 7: Analysis of similarities (ANOSIM) between and within the tested groups: Female gonads (FG), non-pregnant males (NP), early pregnancy brood pouch (EPP), early pregnancy juveniles (EPJ), late pregnancy brood pouch (LPP) and late pregnancy juveniles (LPJ). The color coding refers to the different groups as shown in Table 1. The width of the bars represents the sample size, the black bar represents the within-group median and the dashed lines represent the standard error. An R value close to 0 suggests dissimilarity between groups.

Stage-specific microbiota composition was displayed by the 12 most abundant bacterial genera based on the presence-absence matrix (Supplemental Table 1). In terms of absolute abundances, bacteria of the genus *Vibrio* were most often found, followed by *Shewanella* spp. and *Pseudoalteromonas* spp. (Figure 8). Excluding the *Vibrio*-selective TCBS culture media from the data set did not cause any major differences despite from the absolute number of isolated *Vibrio*

strains which strongly decreased from 118 to 52 isolated strains. As the aim was to capture and cultivate as many bacterial strains as possible, results from both media, Marine Broth (MB) and TCBS, were included in the statistical analysis.

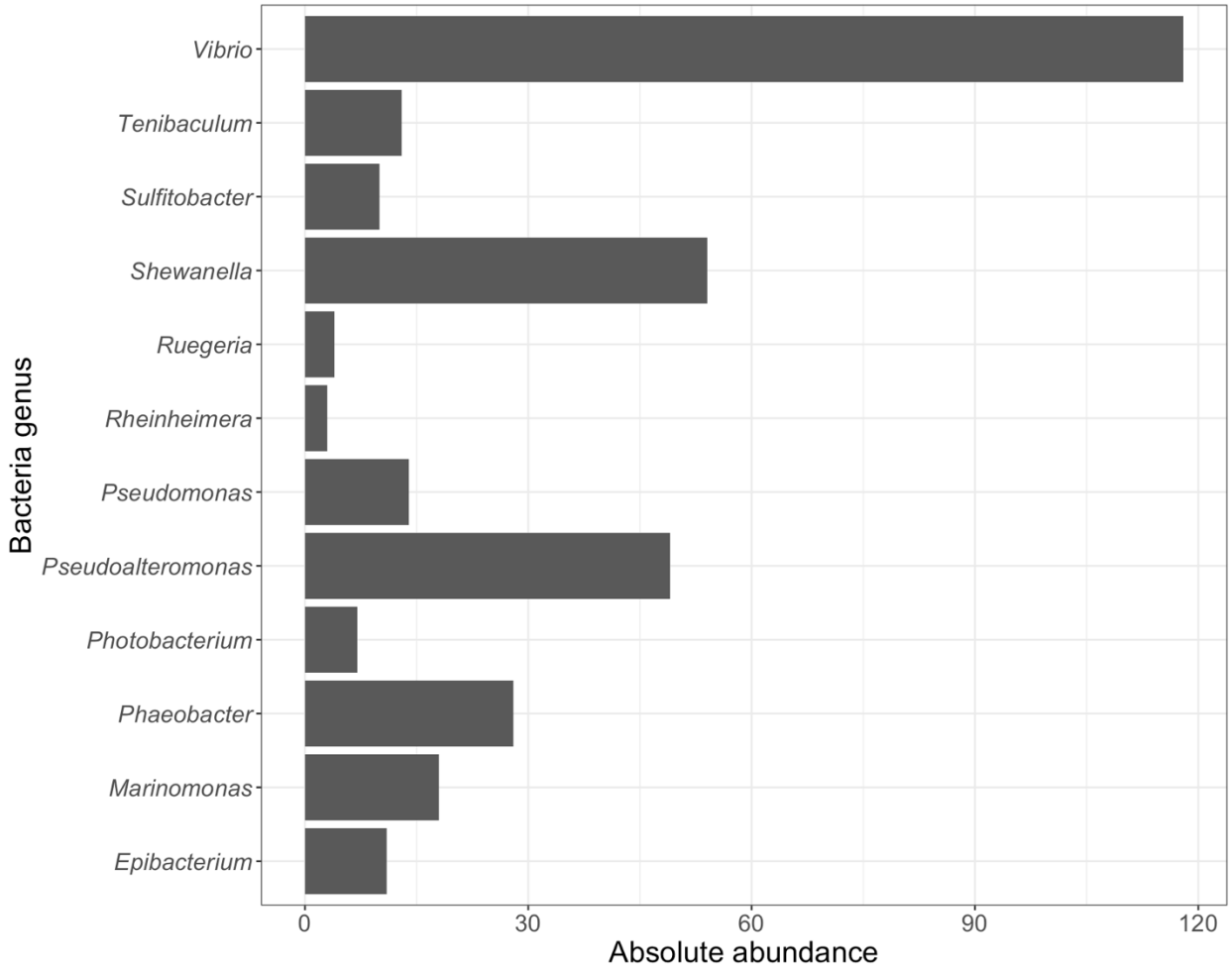


Figure 8: The 12 most abundant bacterial genera isolated from sex-specific tissue of *S. typhle*. Values are absolute.

For assessment of relative abundances, the 12 bacterial genera were grouped according to the sex and tissue they were isolated from (Figure 9). *Vibrio* spp., *Shewanella* spp. and *Pseudoalteromonas* spp. were relatively equally distributed across all groups and were also isolated from seawater (SW). Bacteria of the genus *Pseudomonas*, *Marinomonas* and *Epibacterium* were found in male pipefish and in the seawater (SW) but could not be found in female pipefish, i.e. they were absent in the female gonads (FG). *Pseudomonas* and *Marinomonas* were strongly prevalent during late pregnancy (LPP, LPJ), *Epibacterium* was more prevalent in the non-pregnant (NP) males. *Ruegeria* spp. was the only bacterial genus exclusively

isolated from the tissue of male pipefish but not found in seawater (SW). It was particularly abundant in the non-pregnant (NP) brood pouch.

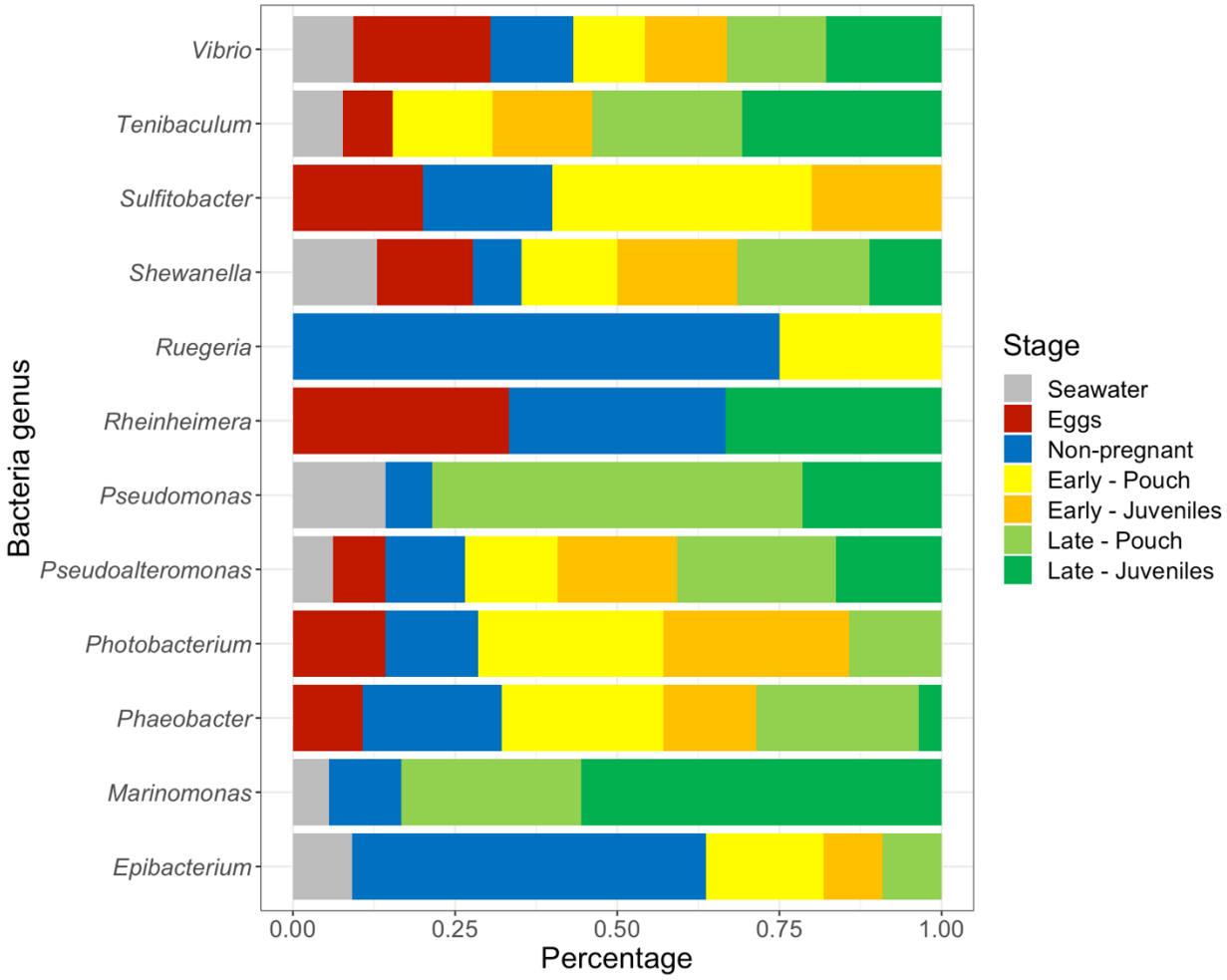


Figure 9: Bar chart displaying the 12 most abundant bacterial genera isolated from *S. typhle*. Shown are relative values and the respective sex/stage from which the bacterial genus was isolated from. The color coding refers to the different groups as shown in Table 1.

As a representation at genus level may lead to inaccuracies in the interpretation, the resolution was further increased to species level. From the 92 identified strains, 29 bacterial species showed certain specificities such as (a) universal presence across all stages, (b) stage-specifically grouping or (c) stage, respectively sex specificity presence and absence (Supplemental Table 2). Therefore, the 29 bacterial species were also grouped according to the sex and tissue they were isolated from and visualized in a relative abundance plot (Figure 10).

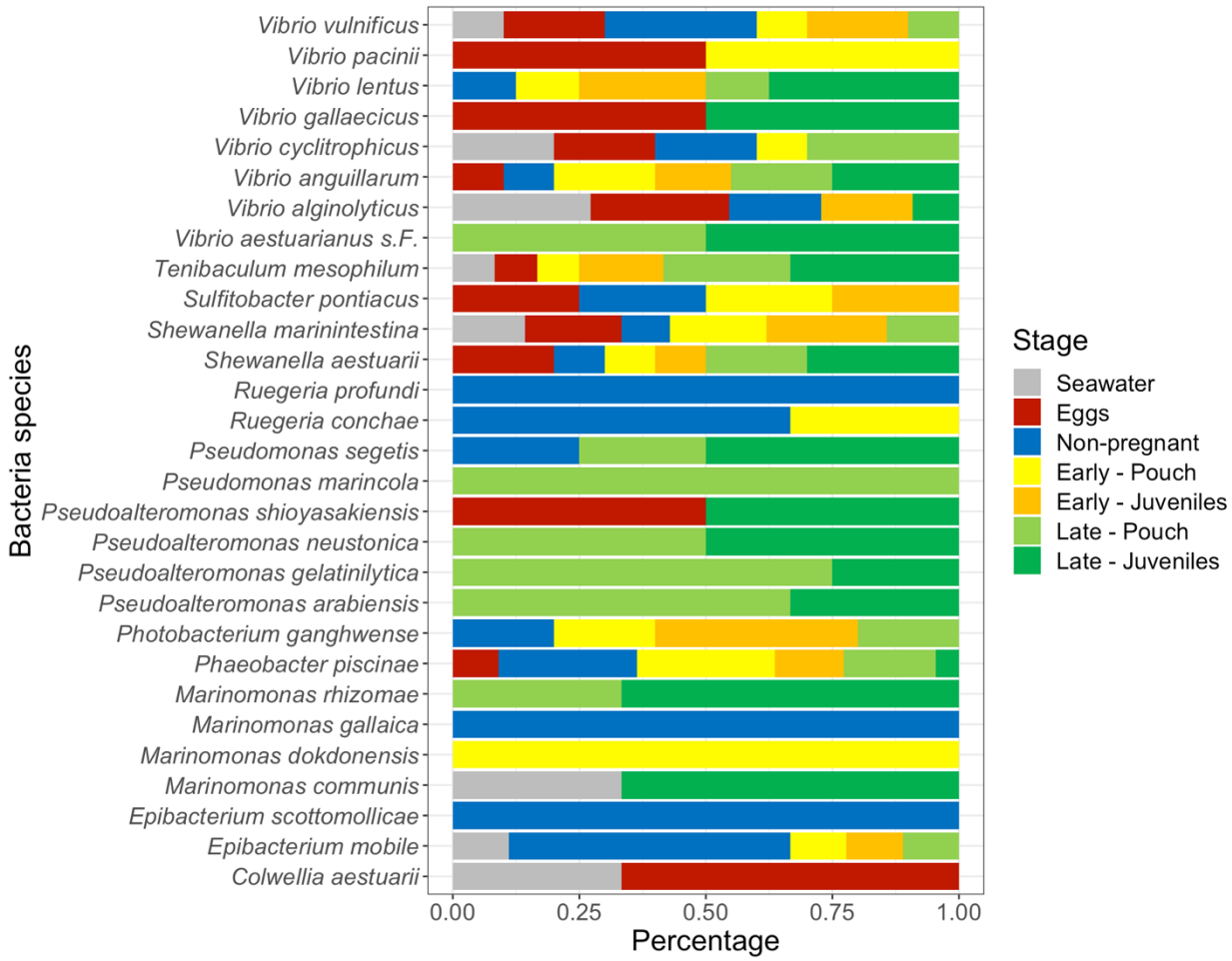


Figure 10: Bar chart of 29 bacterial species isolated from *S. typhle* that show (a) universal presence across all stages, (b) stage-specifically grouping or (c) stage, respectively sex specificity presence and absence. Shown are relative values, grouped by developmental stage.

The species-specific resolution enabled a classification of the bacterial species into three categories: (a) bacterial species that are lost during early pregnancy (*Ruegeria conchae*, *Vibrio pacinii*, *Sulfitobacter pontiacus*), (b) bacterial species that are only found during late pregnancy, hence stage specific (*Marinomonas rhizomae, Pseudoalteromonas arabiensis*, *Pseudoalteromonas gelatinilytica*, *Pseudoalteromonas neustonica*, *Pseudomonas marincola* and *Vibrio aesturianus subs. Francensis*) and (c) bacterial species that skip entire developmental stages and are only found in female gonads and in the juveniles during late pregnancy (e.g. *Vibrio gallaecicus*, *Pseudoalteromonas shioyasakiensis*).

Based on this classification, important species for paternal and maternal contribution to microbiota transfer could be identified. For paternal contribution, four bacterial genera were identified which comprise a total of 10 species. For maternal contribution, a higher resolution at species level was needed to identify four bacterial species transferred exclusively by the mother (Table 2).

Table 2: Important genera and species for paternal and maternal contribution to microbiota transfer in *S. typhle*

Paternal contribution	Maternal contribution
***Epibacterium* spp.**	*Pseudoalteromonas shioyasakiensis*
– *E. mobile*	*Tenibaculum mesophilum*
– *E. scottomollicae*	*Vibrio gallaecicus*
	Vibrio pacinii
***Marinomonas* spp.**	
– *M. communis*	
– *M. dokdonensis*	
– *M. gallaica*	
– *M. rhizomae*	
***Pseudomonas* spp.**	
– *P. marincola*	
– *P. segetis*	
***Ruegeria* spp.**	
– *R. conchae*	
– *R. profundi*	

For assessing bacterial community shifts between sexes and developmental stages, the phylogeny of isolated bacterial species was calculated based on maximum likelihood and displayed in a phylogenetic tree.

The baseline of sex-specific microbiota in *S. typhle* was displayed by combining bacterial species isolated from the female gonads (FG) with bacterial species isolated from the non-pregnant male brood pouch (NP). This represents the original status of microbial composition for both sexes before mating takes place (Figure 11). Both, female gonads and non-pregnant brood pouch showed a distinct clustering of the microbial community. *Vibrio* spp. and *Shewanella* spp. were predominantly prevalent in the female gonads, whereas *Phaeobacter* spp., and *Epibacterium* spp. were strongly abundant in the non-pregnant brood pouch.

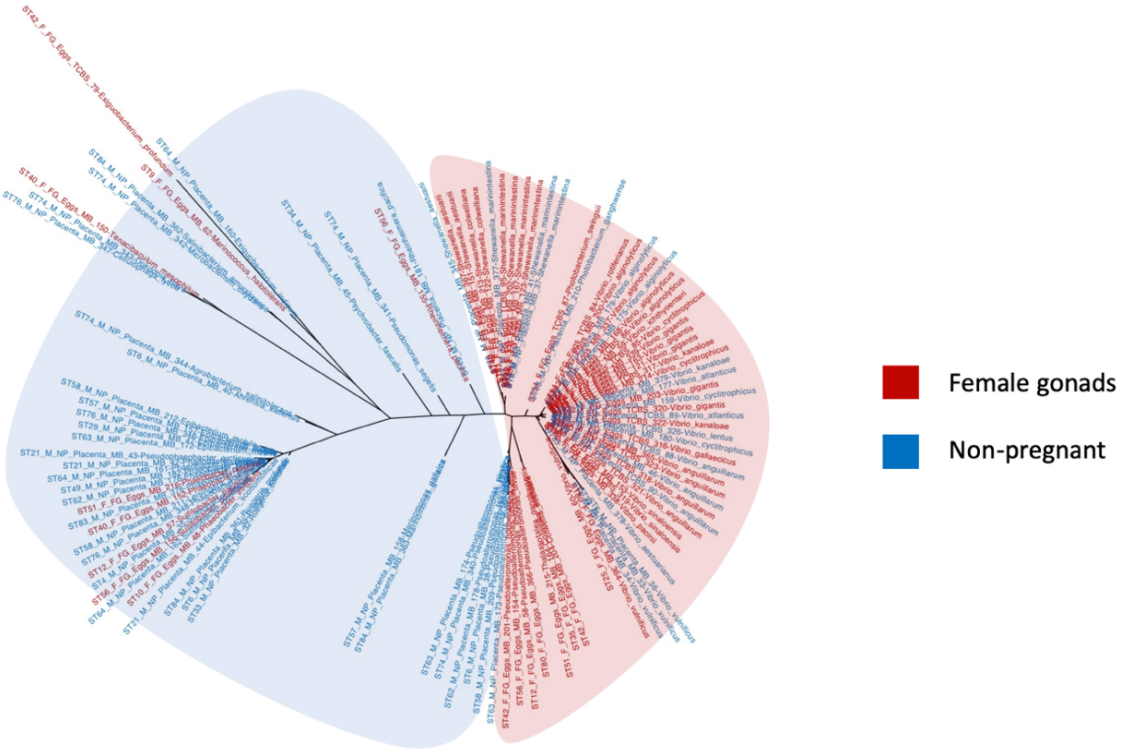


Figure 11: Phylogeny based on maximum likelihood (RAxML). Sex-specific microbiota in *S. typhle*: Bacterial species isolated from the placenta-like tissue of non-pregnant (NP) males (blue) and bacterial species isolated from the female gonads (red). Colored areas visualize the clusters.

Shifts occurring over the course of pregnancy were visualized by combining the bacterial species isolated from the female gonads (FG) with those associated to the early pregnancy stage (EPP, EPJ). In this stage, the initial clustered pattern was no longer prevalent but replaced by a rather mixed pattern in which bacterial strains associated with the female gonads were present throughout the phylogenetic tree together with bacterial strains associated with the early pregnancy stage (Figure 12).

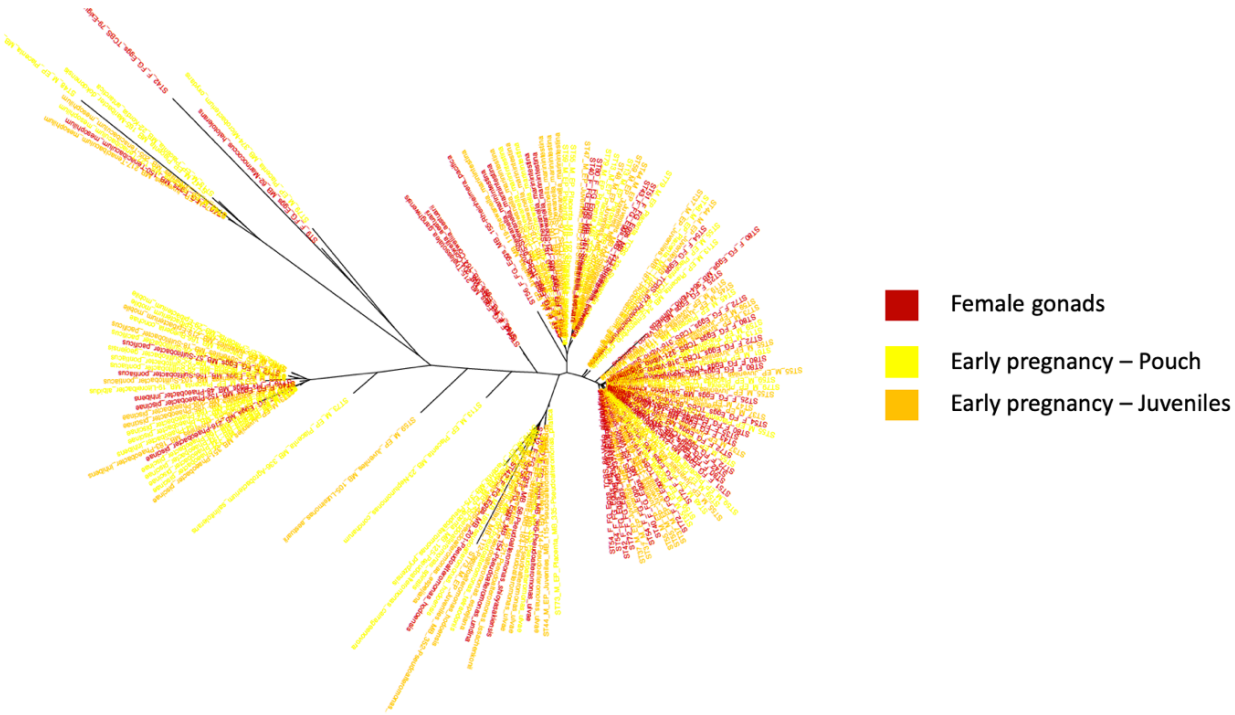


Figure 12: Phylogeny based on maximum likelihood (RAxML). Bacterial community structure in *S. typhle* during early pregnancy: Bacterial species isolated from the placenta-like tissue of early pregnancy (EPP) males (yellow), early pregnancy juveniles (EPJ) (orange) and bacterial species isolated from the female gonads (red).

In contrast, a clustered pattern reappeared during the late pregnancy stage (Figure 13). Distinct areas of the phylogenetic tree were associated with bacterial species isolated from the late pregnancy stages (LPP, LPJ) represented by the genera *Tenibaculum* spp., *Rhodococcus* sp., *Marinomonas* spp. and *Pseudomonas* spp. In the female gonads (FG), *Vibrio* spp. remained the prevalent bacterial genus. However, clustering was not as distinct as shown in the original status of microbial composition (Figure 11).

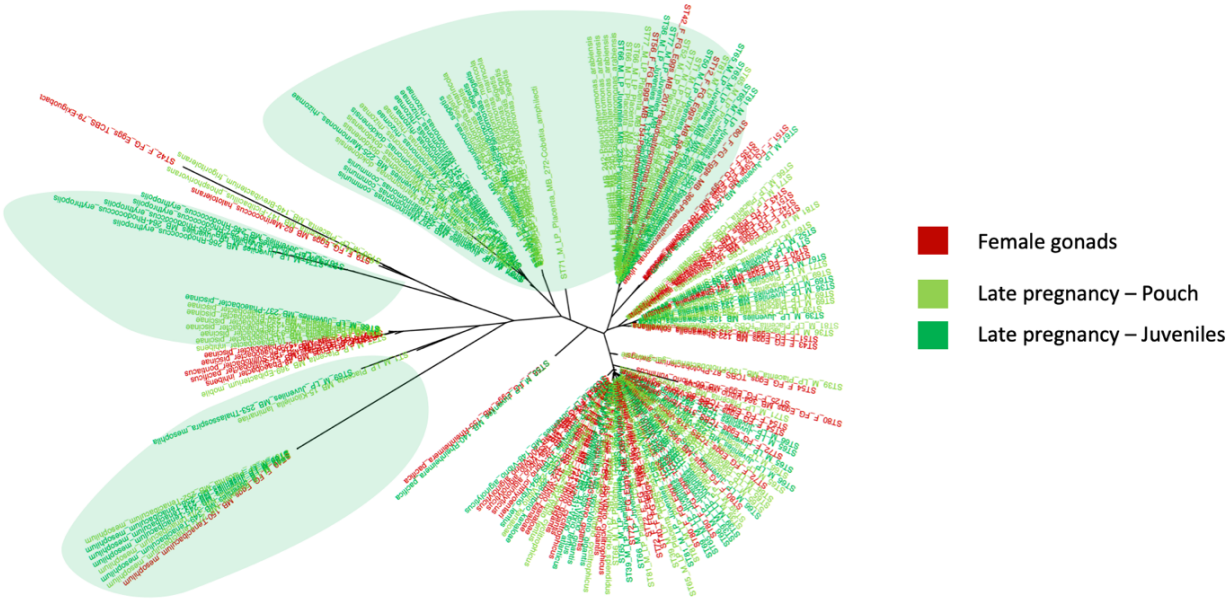


Figure 13: Phylogeny based on maximum likelihood (RAxML). Bacterial community structure in *S. typhle* during late pregnancy: Bacterial species isolated from the placenta-like tissue of late pregnancy (EPP) males (light green), late pregnancy juveniles (EPJ) (dark green) and bacterial species isolated from the female gonads (red). Colored areas visualize the clusters.

A multiple correspondence analysis (MCA) was performed to visualize associations between *S. typhle* developmental stages and to identify bacterial species that drive the observed patterns. The explained variance by the first three dimensions was 4.66% (Dim 1), 3.98%. (Dim 2) and 3.64% (Dim3), summing up to a total of 12.28%. Between dimensions 4 and 72, no further significant drop in explained variance was given (Supplemental Figure 2). Interpretation is therefore based on the first three dimensions, shown in combined plots for dimension 1 - dimension 2 (Figure 14), dimension 1 – dimension 3 (Figure 15) and dimension 2 – dimension 3 (Figure 16). The MCA (Figure 14A, Figure 15A, Figure 16A) visualized a clustering pattern of the six different groups (FG, NP, EPP, EPJ, LPP, LPJ), whereas the factor map (Figure 14B, Figure 15B, Figure 16B) displayed the contribution of variance given by each of the isolated bacterial

species (loadings in %). Visualization and interpretation of the loadings, however, were limited to the 20 bacterial species with the highest contribution to the observed pattern. As an extension to the factor maps, the 20 bacterial species which contributed the most to the different patterns seen in the three dimensions were sorted according to their level of contribution (in %) and plotted in descending order from highest contribution to lowest contribution (Figure 17). Dimensions 1 and 2 explained a total of 8.64% of the variation in the data. Although all stages showed overlapping areas, indications for a pattern exist: 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 the non-pregnant (NP) males and the early pregnancy stage (EPP, EPJ) showed the strongest overlap (Figure 14A). Overall, this pattern was mostly driven by *Vibrio gallaecicus* and *Dokdonia flava* in dimension 1 (Figure 17A) and *Dokdonia flava* and *Exiguobacterium profundum* in dimension 2 (Figure 17B). In the female gonads (FG), bacteria of the genus *Vibrio* were overrepresented, whereas the microbiota of the late pregnancy brood pouch (LPP) appeared to be shaped by bacteria of the genus *Pseudoalteromonas* (Figure 14B).

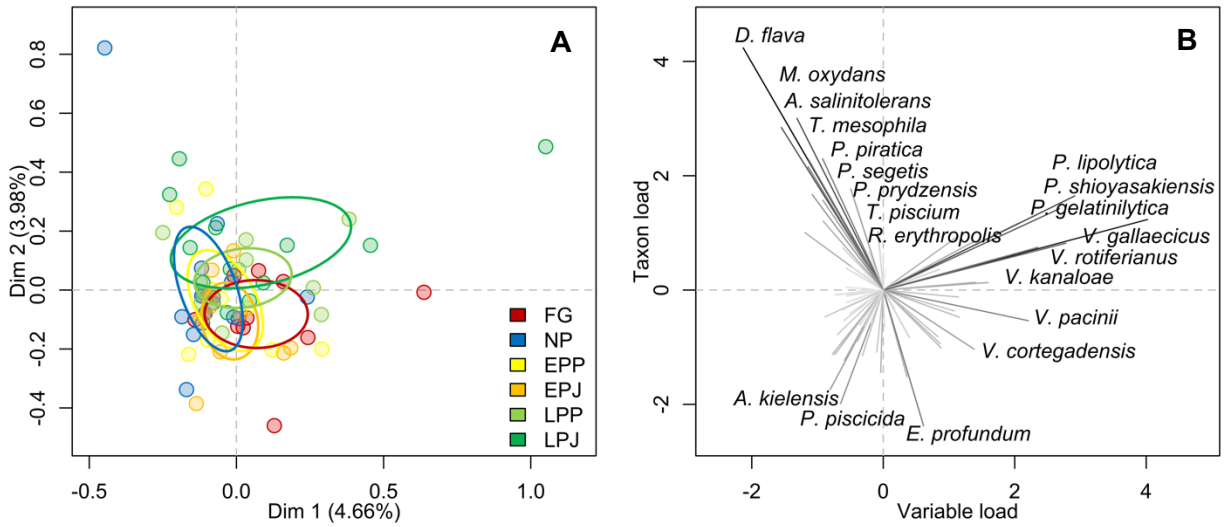


Figure 14: Multiple correspondence analysis (MCA) of six groups for dimensions 1 and 2 (A) and factor map displaying the loadings retained by the isolated bacterial species (B). Ellipses include 30% of data of the respective group.

Dimensions 2 and 3 explained a total of 7.62% of variation in the data (Figure 15A). The microbiota of late pregnancy juveniles (LPJ) was driven away from the other stages in both dimensions, however, the remaining five developmental stages showed strong overlaps in their centers. The same pattern was shown when visualizing the combined effect of dimension 1 and

3, which explained a total of 8.3% of variation (Figure 16A). In dimension 3, the observed pattern was mostly driven by *Dokdonia flava* and *Thalassotalea piscium* (Figure 17C). Whereas the first two dimensions provided evidence for high abundances of *Pseudoalteromonas* spp. during late pregnancy, the same stage appeared to be shaped by numerous bacteria of the genus *Marinomonas*, *Thalassospira*, *Thalassotalea*, *Rhodococcus* and *Pseudoalteromonas* in dimensions 2 and 3 (Figure 15B) and dimensions 1 and 3 (Figure 16B).

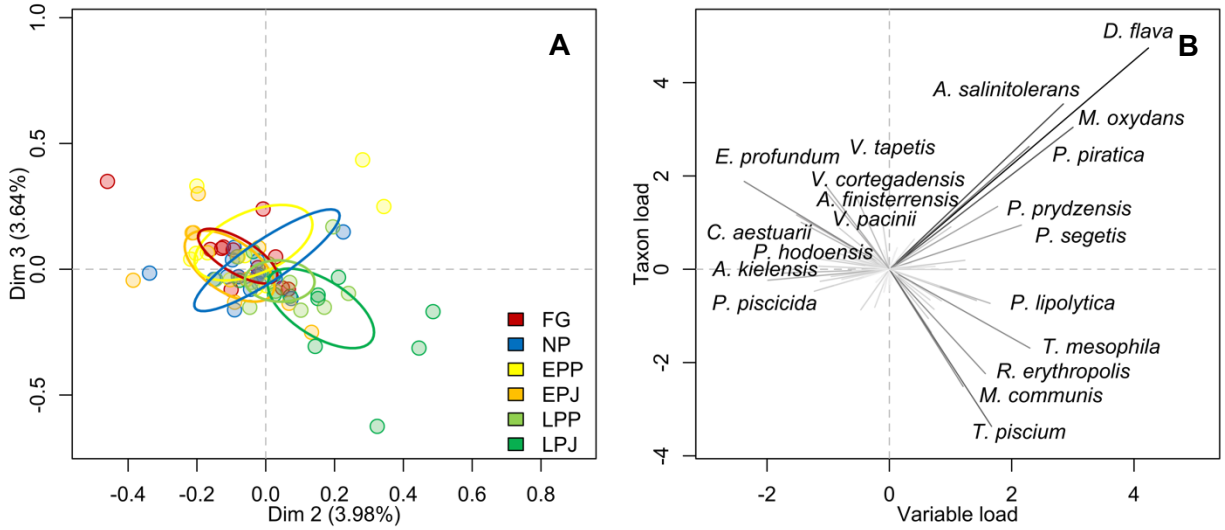


Figure 15: Multiple correspondence analysis (MCA) of six groups for dimensions 2 and 3 (A) and factor map displaying the loadings retained by the isolated bacterial species (B). Ellipses include 30% of data of the respective group.

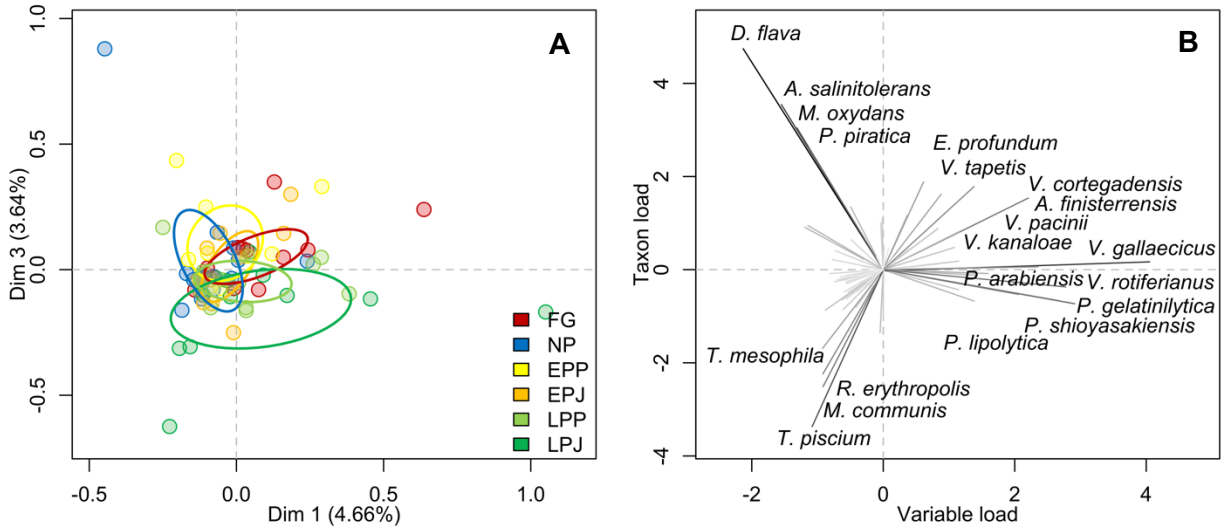


Figure 16: Multiple correspondence analysis (MCA) of six groups for dimensions 1 and 3 (A) and factor map displaying the loadings retained by the isolated bacterial species (B). Ellipses include 30% of data of the respective group.

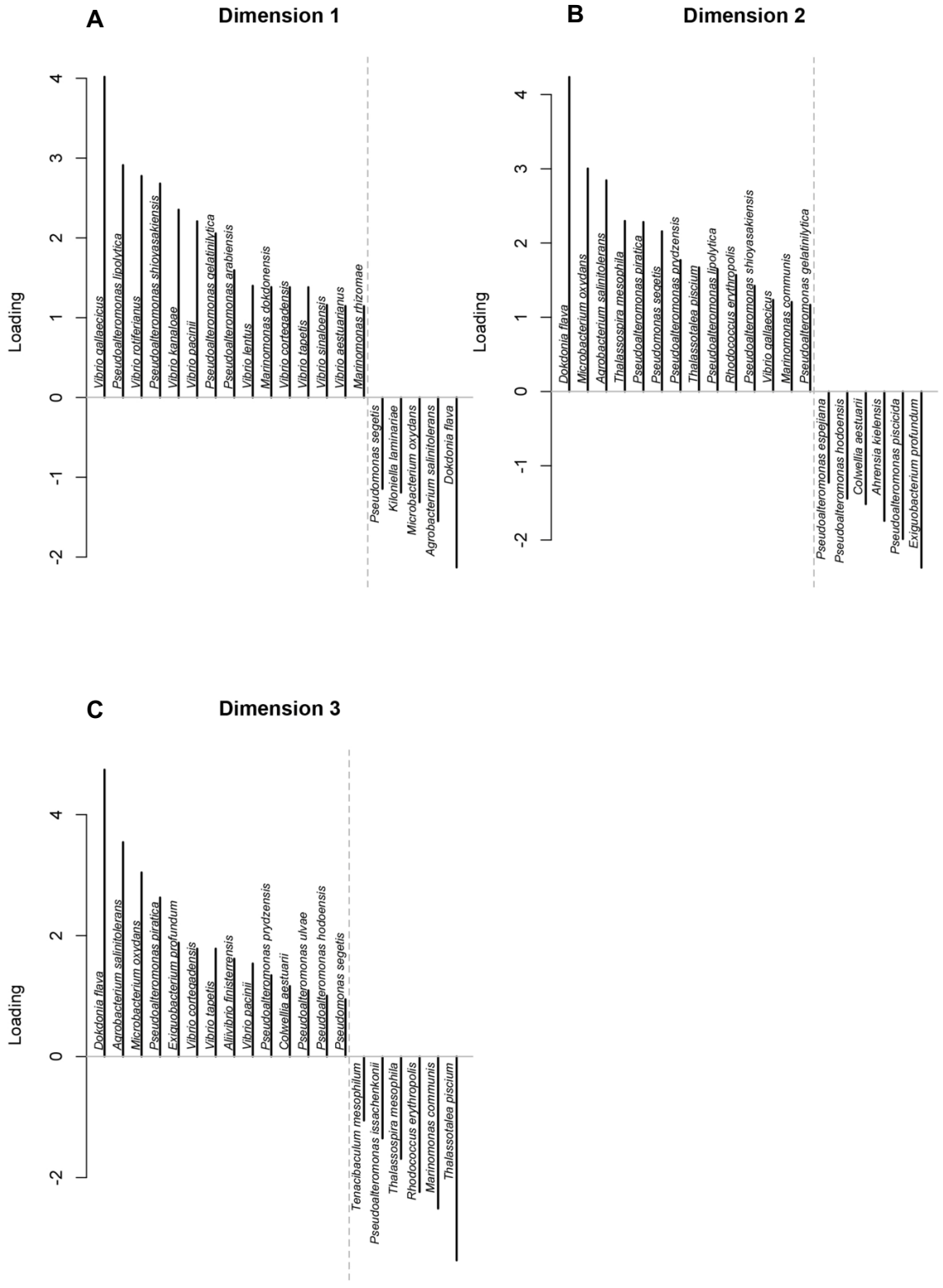


Figure 17: Contribution of variance explained by the bacterial species. Shown are the 20 species with the strongest contribution for to the different patterns shown in dimension 1 (A), dimension 2 (B) and dimension 3 (C). Species are sorted according to their level of contribution (in %) and plotted in descending order from highest contribution to lowest contribution.

4 Discussion

In order to obtain a set of cultivable bacteria that can be used for controlled microbiota manipulation in further studies, I applied a descriptive and explorative approach for the cultivation and characterization of sex-specific microbiota. The bacteria were isolated from reproductively relevant tissues of the broad-nosed pipefish *Syngnathus typhle*. The unique male pregnancy in *S. typhle* permits to separately investigate paternal and maternal contribution to microbiota transfer, track changes over the course of pregnancy and to describe the initial microbial colonization of the offspring. The bacteria originated from two different pregnancy stages, their corresponding juveniles, non-pregnant males and females of *S. typhle* and were grown on two different cultivation media (Marine Broth and TCBS). Using 16S rRNA Sanger sequencing, the cultivation-dependent technique resulted in the identification of 92 bacterial species, which accounts for approximately 3% of the species identified through the culture-independent approach by Beemelmanns et al. (2019). Even though more than 97% of the pipefish microbiota thus remain uncultivable in this setting, similar patterns of microbiota composition and composition changes were detectable.

Patterns and shifts in microbiota composition

The genera *Vibrio*, *Shewanella* and *Pseudoalteromonas* were the highest abundant cultivable bacteria (Figure *8*). *Vibrio* spp. is a predominant bacterial genus in aquatic environments, associated with several commensal and pathogenic interactions with marine organisms (Oliver et al. 2013; Romalde et al. 2014). *Shewanella* spp. are common components of fish microbiota, often associated with the microbial community of the reproductive system and the digestive tract of fish larvae (Hau and Gralnick 2007; Navarrete et al. 2009). Bacteria of the genus *Pseudoalteromonas* are likewise frequently found in association with marine organisms and are known to produce antimicrobial compounds that are active against biofilm formation of other microbial fish pathogens (Holmström and Kjelleberg 1999; Klein et al. 2011; Rodrigues et al. 2014). In accordance with the high absolute abundances (Figure 8), these three bacterial genera were found across all sexes and developmental stages, whereas many other taxa were exclusively found in males, female gonads or in the juveniles during late pregnancy (Figure 9). This indicates that I may have captured species important for paternal and maternal contribution (Table 2). For maternal contribution, I identified *Pseudoalteromonas shioyasakiensis*, *Tenibaculum mesophilum*, *Vibrio gallaecicus* and *Vibrio pacinii* as candidates for indicator species. For paternal contribution, I was able to characterize entire bacterial genera that are potential indicators: *Epibacterium* spp., *Marinomonas* spp., *Pseudomonas* spp. and *Ruegeria*

spp. *Marinomonas* spp. are commonly associated with the initial colonization of embryos (Romero et al. 2014), *Pseudomonas* spp. are a dominant commensal taxa within the fish microbiota which can protect its host against other pathogens (Kissoyan et al. 2019) and *Ruegeria* spp. occur in high abundances on cod larvae (Fjellheim et al. 2012). Especially the identification of *Marinomonas* spp., *Ruegeria* spp. and *Tenibaculum mesophilum* were of particular interest as these taxa were also found to be indicator species in the culture-independent approach (Beemelmanns et al. 2019), hence support my suggestion to regard them as important species for sex-dependent microbial transfer.

Driven by the progress in microbiota research, increasing evidence exists that the microbiota does not exist in a static state but constantly undergoes substantial changes (O'Toole and Claesson 2010; Claesson et al. 2011). A major objective of this research study was therefore to not only identify patterns of microbial composition but also reveal potential changes over the course of pregnancy. The phylogenetic analysis and the global pattern produced by the multiple correspondence analysis (MCA) propose exactly these shifts in the microbial community composition. To interpret the proposed changes, one needs to acknowledge the processes that occur in the body during pregnancy: Pregnancy imposes several major physiological and metabolic changes on the organism. These changes are suggested to be impacted by and likewise exert an impact on the microbiota composition (DiGiulio et al. 2015; Nuriel-Ohayon et al. 2016). In humans it was shown that both, the gut and vaginal microbiota composition are altered drastically from the second trimester onwards (Koren et al. 2012). This may thus correspond to the observed shifts in *S. typhle*. The phylogenetic analysis (Figure 12, Figure 13) further strengthened these visible shifts in the clustering patterns, which were represented by a transition from a well-mixed pattern during early pregnancy to a pattern with distinct clusters during late pregnancy. However, as the main intention of the phylogenetic analysis was to put the identified bacterial species in relation and show detectable changes over the course of pregnancy. Whether or not these clusters are also of biological relevance needs to be further investigated. However, the clusters represent an accumulation of closely related species. Therefore, I can speculate that a cluster with a mixed pattern generally indicates a larger diversity of taxa found within a specific sex or developmental stage. The MCA and the phylogenetic analysis further indicated that the most important shift in microbiota composition occurs during late pregnancy. The late pregnancy in pipefish is characterized by important structural changes: the larvae have used up their yolk sac and opened their mouth which gives way to the initial microbial colonization of the gut. It goes in line with the isolation of *Marinomonas rhizomae, Pseudoalteromonas arabiensis, Pseudoalteromonas gelatinilytica, Pseudoalteromonas neustonica, Pseudomonas marincola and*

Vibrio aesturianus subs. Francensis, which are unique to the late pregnancy stage (Figure 10). This could support the hypothesis that major restructuring of the microbial community takes place during juvenile development, leading to the onset of their own distinct microbiota (Edwards et al. 2017; Younge et al. 2019). Furthermore, as the larvae grow, the brood pouch skin becomes chapped and increasingly permeable which allows bacteria from the surrounding environment to penetrate the brood pouch (Beemelmanns et al. 2019). This can be regarded as a mechanism to adjust juveniles to the environment they will encounter after birth. In this stage I found *Marinomonas communis* and *Pseudoalteromonas phenolica* (Figure 10). These strains were only found in late pregnancy juveniles and in seawater, thus support the assumption that colonization may also occur through the water. However, I cannot differentiate at this point, whether a bacterial species originated from the juvenile gut itself or its skin surface that is in direct contact with the placenta-like tissue of the brood pouch. In contrast to the bacteria unique to the late pregnancy stage, I also found bacteria of the genus *Sulfitobacter* and *Pseudoalteromonas* that were present in all developmental stages except the late pregnancy stage (Figure 10). It is therefore tempting to speculate that these bacteria are exclusively harbored for the early development of the juveniles, thus are outcompeted by the changing brood pouch conditions towards the end of the pregnancy.

My analyses are based on less than 3% of the strains identified through culture-independent sequencing (Beemelmanns et al. 2019) but it is striking that patterns and changes in microbiota composition remain similar. However, particularly the changes shown in the MCA need to be regarded with caution. This is because the dimensions contribute little to the explained variation and the ellipses themselves only include 30% of the data, hence do not represent proper confidence intervals.

Cultivation as a necessary but restricting factor

Although an analysis of similarities (ANOSIM) revealed no distinct differences between the tested groups (sex, developmental stage), the visualization of my data displayed similarities to the patterns generated through the culture-independent sequencing approach (Figure 14). Yet, these patterns seen in the multiple correspondence analysis (MCA) were not as distinct, therefore one should rather consider them as trends for a pattern. This points out one of the challenges I encountered in my experimental approach. Cultivation is a necessary step to acquire bacterial strains for manipulation experiments, but it also acts as an extreme filter. A cultivable proportion of 3% falls within the range of 1-5% commonly given in literature (Rappé and Giovannoni 2003; Harwani 2012; Epstein 2013). This reduction in cultivable species number can be explained by

the great plate count anomaly, the observation that most bacterial species are uncultivable under laboratory conditions (Stewart 2012). Overall, I was able to characterize between 41 and 55 species per tissue type. However, from some tissues it was difficult to isolate microbes. Particularly from the female gonads (FG) or the early pregnancy stage (EPP, EPJ) I could only cultivate bacteria from two third of the samples, although Beemelmanns et al. (2019) provided evidence that especially the gonads are the tissue with both, the highest species richness and diversity. As none of the bacteria which I isolated from the gonads were represented in the 50 most abundant OTUs of the culture-independent approach, this may indicate that the most abundant bacteria in this tissue are uncultivable. The low number of cultivable samples from the early pregnancy stage can be explained by the non-standardized amount of tissue that was sampled: After the eggs have nested inside the placenta-like tissue, they are particularly fragile and difficult to dissect. As some intact eggs were needed for another experiment, I often took the microbiota sample from a single egg. This has potentially introduced a bias in my data which needs to be accounted for in future studies, e.g. by setting a fixed weight of tissue that needs to be sampled. Regarding the diversity of species identified during this experiment, 48 out of the total 92 identified species were only found once among all isolated samples (Supplemental Table 1). This gives reason to question whether these specific species generally occur in low abundances or if they are difficult to cultivate and were captured by coincidence. My methods provide clear answers to my research questions. However, given that one third of the identified taxa were only found once, a few uncertainties in regard to the planned manipulation experiments arise. These questions can be solved in future studies by metagenomics and whole genome sequencing (WGS) through which it is possible to infer the function of uncultivable bacteria. For this explorative research, I used Marine Broth which represents the major mineral composition of sea water and is thus suitable for capturing a wide range of marine bacteria. In order to acquire a more detailed insight into the diversity of a highly abundant bacterial genus in marine environments, I additionally used the *Vibrio*-selective TCBS cultivation medium. The methods I chose aimed at cultivating a broad spectrum of bacteria to select strains suitable for manipulation. Future selection of cultivation media could aim at specifically matching the nutrient requirements of the indicator species identified through the culture-independent approach. However, the total number of cultivable bacteria will always remain low, thus it is a question of investing more time and money in the process of cultivating the bacterial species.

Advantages and limitations of 16S rRNA genotyping

16S rRNA gene sequencing is one of the most common approaches for a rapid and accurate identification of bacteria. During the experiment, I have included negative controls and checked the identified taxa for plausibility (e.g. the identified bacteria should relate to marine environments or to commensal microbes), to prevent possible contamination effects. The full 16S rRNA gene spans approximately 1500 bp and contains nine highly variable regions distributed throughout the conserved 16S sequence (Figure 6). For sequencing of the gene, Illumina amplicon sequencing (~ 300 bp) is often preferred over Sanger sequencing for its higher throughput, coupled with lower cost and effort. For this experimental approach, however, Sanger sequencing of the entire 16S rRNA gene was chosen in order to achieve a high taxonomic accuracy. Sequencing the entire gene further solved potential identification issues caused by high sequence similarities in some regions of the 16S rRNA gene (Lau et al. 2015; Rossi-Tamisier et al. 2015). If only parts of the gene are sequenced, this problem can otherwise only be addressed by applying additional sequencing methods, e.g. multi locus sequence analysis (MLSA) of housekeeping genes (Glaeser and Kämpfer 2015; Wendling et al. 2017). Whereas all hypervariable regions were thus covered in this study, the culture-independent characterization approach implemented by Beemelmanns et al. (2019) targeted only the hypervariable region V4 of the 16S rRNA gene, resulting in a number of unidentified strains. This impedes a direct comparison between the two studies because the datasets contain different taxonomic resolutions that must be considered. Challenges in bacterial species identification also relate to the problem of using the NCBI BLAST database for taxonomic identification. Despite their accessibility, public sequence repositories often lack taxonomic reliability and sufficient annotations, as they are challenged by junk submissions, user-related errors and the absence of proper quality controls (Nilsson et al. 2006). For this explorative research, BLAST was a reasonable choice to describe which bacterial species can be found in pipefish. For future analyses, however, one could consider comparing the results with a broader range of different taxonomies, e.g. SILVA, RDP or Greengenes (Balvočiūte and Huson 2017).

In future studies, I plan to implement microbial manipulation experiments for which it will be necessary to quickly assess and characterize the bacteria found in the pipefish. This suggests that I potentially have to switch to culture-independent next-generation sequencing, however, it could be considered to apply primers which span a larger region of 16S rRNA gene. Although sequencing larger regions implies that the sequence data has to be re-assembled afterwards, it could lead to an overall higher security in taxonomic assignment.

5 Conclusion

Using a cultivation-based sequencing approach, I captured approximately 3% of the sex-specific microbiota associated with the broad-nosed pipefish *Syngnathus typhle.* Coherent with the results of a culture-independent sequencing approach, my data suggests that the microbial community in *S. typhle* is concurrently transferred to the offspring by both, the mother and the father. Furthermore, my data provides evidence that the microbiota changes over the course of pregnancy and that pipefish juveniles inside the male brood pouch develop their own distinct microbiota. Future research intends to shed light on the function of the sex-specific microbiota and potentially reveal a connection with trans-generational immune priming. This will be addressed by implementing a series of microbial manipulation and exchange experiments.

6 Future Perspectives

With this explorative research, I have created a pool of cultivable bacterial strains that can be used in further studies. In the next phase of the project, I want to examine the role of sex-specific microbiota transfer during pregnancy and their effects on the immune system. This could provide evidence for a link between offspring-specific microbiota and trans-generational immune priming. The planned roadmap to explore the function of the microbiota includes three stages to be implemented in the upcoming three years:

(1) Antibiotic resistance testing to find suitable antimicrobial drugs against the natural pipefish microbiota,

(2) Rearing germ-poor pipefish by application of antibiotics to deplete the natural microbiota and

(3) Microbiota manipulation in which I want to examine the role of sex-specific microbiota transfer.

6.1 Antibiotic resistance testing

In medical history, antibiotics are considered the most successful treatment, contributing significantly to the containment of infectious diseases (Fernandes 2006; Aminov 2010). The modern antibiotic age was founded in 1910 by Paul Ehrlich with the discovery of an antimicrobial compound to treat syphilis (Ehrlich and Hata 1910), followed by the isolation of penicillin from a mold by Alexander Fleming (1929). Antibiotics are classified into five groups based on their mode of action: (1) Inhibition of cell wall synthesis, (2) inhibition of protein biosynthesis, (3) inhibition of nucleic acid synthesis, (4) alteration of cell membranes or (5) inhibition of enzymes involved in bacterial growth and replication (Kapoor et al. 2017). I plan a controlled depletion and

manipulation of the sex-specific pipefish microbiota in future studies. The diverse modes of action therefore underline the importance of targeted antibiotic resistance tests.

During this master's thesis, I found four indicator species for maternal contribution to microbiota transfer and four indicator genera for paternal contribution (total: 14 bacterial species, Table 2). I identified an additional 15 species based on their specificity, e.g. isolation from all sexes/developmental stages or presence/absence in a certain sex/developmental stage (Figure 10, Supplemental Table 2). A total of 29 bacterial strains will thus be used for *in vitro* antibiotic resistance testing. Using ETEST (Epsilometer test)-strips (bioMérieux), plastic strips containing a predefined gradient of antibiotic concentrations, I aim at determining the Minimum Inhibitory Concentration (MIC) which is needed to inhibit the growth of the selected strains. I plan to test the resistance against three antibiotics of different families: Chloramphenicol (family phenicols) and tetracycline (family tetracyclines) inhibit protein biosynthesis of the bacterial cells (Brock 1961; Chopra and Roberts 2001). They both belong to the group of broad-spectrum antibiotics that are effective against a wide range of bacteria, often applied in medical fields when the infection-causing bacteria are unknown (Ory and Yow 1963). In contrast, nalidixic acid (family fluoroquinolones) is a narrow-spectrum antibiotic, effective against gram-negative bacteria and commonly used in the treatment of urinary tract infections (Fàbrega et al. 2009). Chloramphenicol and fluoroquinolones were also commonly used in aquaculture, however, their usage has been banned in food production due to health concerns (Chanda et al. 2011). In replicates of three, an overnight culture from the bacterial glycerol stock will be diluted to an optical density (OD) of 0.1. The culture will be spread on a Marine Broth agar plate and the ETEST-strips will be applied at maximum distance. After incubation, an inhibitory zone ellipse appears around the ETEST-strip, indicating the minimum concentration (in µg/ml) at which the antibiotic becomes effective (Figure 17). Two different approaches can be considered for antibiotic resistance tests: Targeting of selected species using narrow-spectrum antibiotics or depletion using broad-spectrum antibiotics. The use of narrow-spectrum antibiotics would lead to a more controlled experimental design through which I could

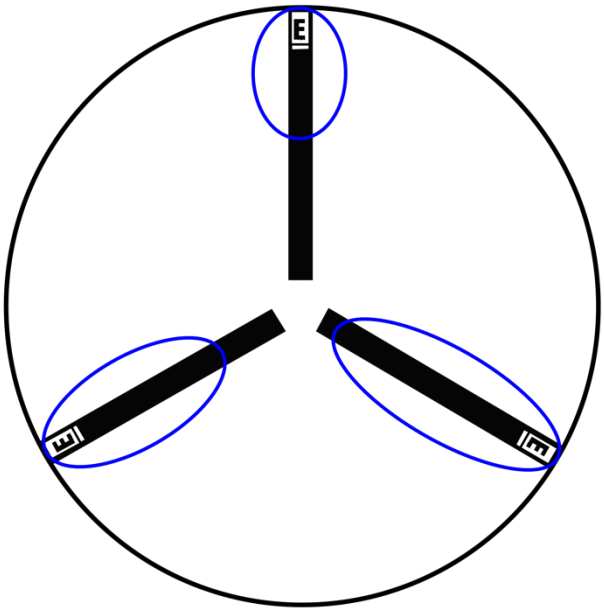


Figure 18: Application scheme for three ETEST-strips on an agar plate and exemplary representation of inhibitory zone ellipses. Graphic modified from biomerieux.de

further assess the role of a specific strain. Yet, it can be challenging to target only selected species: the bacterial strains used in the experiments were extracted from wild fish, thus they may all show strong susceptibility to any of the antibiotics. Furthermore, the strains were selected by a culture-based approach, however, this selection may not represent the most abundant or important strains. In contrast, using only broad-spectrum antibiotics would possibly deplete the majority of the bacteria, therefore reduce the controllability of the experiment.

6.2 Rearing germ-poor pipefish

In this part of the study, I plan to deplete the natural microbiota to have germ-poor pipefish on which I can then perform microbiota manipulation experiments (cf. 6.3 Microbiota Manipulation). Whereas rodents have been established as germ-free *in vivo* models, rearing gnotobiotic teleosts remains a challenge (Martín et al. 2016). Rearing germ-free larvae was first tested on zebrafish (*Danio rerio*) (Pham et al. 2008), followed by a number of other fish species, including salmon (*Salmo salar*) and cod (*Gadus morhua*) (De Swaef et al. 2016). Comparable protocols for *Syngnathus typhle* do not exist yet, therefore I will use wild-caught adult *Syngnathus typhle* and treat them with antibiotics in order to make them germ-poor. Treatment using antibiotics can be performed in three different ways (Yanong 2013): Injection is the most effective and direct way for the antibiotic to reach the blood stream. Oral delivery involves mixing the antibiotic into the fish food with fish oil as a binding agent. Bath treatments require significantly higher amounts of the antibiotic in the water; however, it remains difficult to conclude whether the fish has taken up a sufficient dose. Furthermore, adding antibiotics into the water imposes the risk of environmental bacteria to develop resistances. Based on this, injection appears to be the most efficient and promising strategy. After each treatment, assessing the bacterial load using swabs and culture-independent sequencing will be used to determine the baseline of “germ-poor”, following the protocol of Beemelmanns et al. (2019).

6.3 Microbiota manipulation

By actively manipulating the microbiota of Syngnathus typhle, I aim at revealing the role of the sex-specific microbiota in enhancing offspring development, survival and immune defense. Conversely, depleting the microbiota of mother, father or both would imply that development and immune defense of the embryos is negatively impacted. Bacterial indicator species for either maternal or paternal contribution will be cultivated from glycerol stocks and labeled with Green Fluorescent Protein (GFP). GFP has originally been isolated from the Pacific Northwest jellyfish *Aequorea Victoria* and is commonly applied in experiments involving live-cell imaging (Tsien 1998). Prior to mating, the labeled bacteria will then be added directly to the female gonads, the male brood pouch or to the aquaria system. Using fluorescence microscopy, the GFP signal will be detected to track and visualize the microbiota transfer from parents to offspring.

Furthermore, it is planned to conduct microbial exchange experiments in which: (a) the microbes of either mother or father are depleted, hence only one sex is capable of transferring the microbiota to the offspring, (b) paternal contribution is switched by exchanging the sex-specific microbiota between mother and father and (c) GFP-labeled microbes are added to the water to illuminate microbial colonization through the environment. Finally, it is planned to examine changes over the course of pregnancy, including shifts in microbiota composition and larval development. Following the birth of the offspring generation, I will then assess both, microbiota composition and immunological parameters. Based on this, I hope to unravel maternal and paternal influences of the microbiota on pregnancy and embryonic development.

7 References

Aagaard, K., J. Ma, K. M. Antony, R. Ganu, J. Petrosino, and J. Versalovic. 2014. The placenta harbors a unique microbiome. Sci. Transl. Med. 6.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Aminov, R. I. 2010. A brief history of the antibiotic era: Lessons learned and challenges for the future. Front. Microbiol. 1:1–7.

Azzarello, M. 1991. Some questions concerning the Syngnathidae brood pouch. Bull. Mar. Sci. 49:741–747.

Balvočiūte, M., and D. H. Huson. 2017. SILVA, RDP, Greengenes, NCBI and OTT - how do these taxonomies compare? BMC Genomics 18:1–8.

Beemelmanns, A., M. Poirier, T. Bayer, S. Kuenzel, and O. Roth. 2019. Microbial embryonal colonization during pipefish male pregnancy. Sci. Rep. 9:1–14. Springer US.

Beemelmanns, A., and O. Roth. 2016. Bacteria-type-specific biparental immune priming in the pipefish Syngnathus typhle. Ecol. Evol. 6:6735–6757.

Beemelmanns, A., and O. Roth. 2017. Grandparental immune priming in the pipefish Syngnathus typhle Grandparental immune priming in the pipefish Syngnathus typhle. BMC Evol. Biol., doi: 10.1186/s12862-017-0885-3. BMC Evolutionary Biology.

Bell, G. 1998. Model metaorganism. Science (80-.). 282:248.

Biagi, E., M. Candela, S. Fairweather-Tait, C. Franceschi, and P. Brigidi. 2012. Ageing of the human metaorganism: The microbial counterpart. Age (Omaha). 34:247–267.

Blackburn, D. G. 2015. Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. J. Morphol. 276:961–990.

Blackburn, D. G. 1999. Viviparity and oviparity: Evolution and reproductive strategies. Encycl. Reprod. 4:994–1003.

Bosch, T. C. G., and M. J. McFall-Ngai. 2011. Metaorganisms as the new frontier. Zoology 114:185–190. Elsevier GmbH.

Braga, R. M., M. N. Dourado, and W. L. Araújo. 2016. Microbial interactions: ecology in a molecular perspective. Brazilian J. Microbiol. 47:86–98. Sociedade Brasileira de Microbiologia.

Brock, T. D. 1961. Chloramphenicol. Bacteriol. Rev.

Chanda, M., M. Paul, J. Maity, G. Dash, and S. S. Gupta. 2011. The use of antibiotics and disinfectants in ornamental fish farms of West Bengal, India. J. Nat. Sci. Biol. Med. 139–140.

Chopra, I., and M. Roberts. 2001. Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance. Microbiol. Mol. Biol. Rev. 65:232–260.

Claesson, M. J., S. Cusack, O. O'Sullivan, R. Greene-Diniz, H. De Weerd, E. Flannery, J. R. Marchesi, D. Falush, T. Dinan, G. Fitzgerald, C. Stanton, D. Van Sinderen, M. O'Connor, N.

Harnedy, K. O'Connor, C. Henry, D. O'Mahony, A. P. Fitzgerald, F. Shanahan, C. Twomey, C. Hill, R. P. Ross, and P. W. O'Toole. 2011. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc. Natl. Acad. Sci. U. S. A. 108:4586–4591.

Clements, K. D., E. R. Angert, W. L. Montgomery, and J. H. Choat. 2014. Intestinal microbiota in fishes: what's known and what's not. Mol. Ecol. 1891–1898.

Clutton-Brock, T. H. 1991. The Evolution of Parental Care. Princeton University Press, Princeton.

Crespi, B., and C. Semeniuk. 2004. Parent-offspring conflict in the evolution of vertebrate reproductive mode. Am. Nat. 163:635–653.

Damiano, L., and P. L. Luisi. 2010. Towards an Autopoietic Redefinition of Life. Orig. Life Evol. Biosph. 40:145–149.

De Swaef, E., W. Van den Broeck, K. Dierckens, and A. Decostere. 2016. Disinfection of teleost eggs: a review. Rev. Aquac. 8:321–341.

DiGiulio, D. B., B. J. Callahan, P. J. McMurdie, E. K. Costello, D. J. Lyell, A. Robaczewska, C. L. Sun, D. S. A. Goltsman, R. J. Wong, G. Shawa, D. K. Stevenson, S. P. Holmes, and D. A. Relman. 2015. Temporal and spatial variation of the human microbiota during pregnancy. Proc. Natl. Acad. Sci. U. S. A. 112:11060–11065.

Dunn, A. B., S. Jordan, B. J. Baker, and N. S. Carlson. 2017. The Maternal Infant Microbiome: Considerations for Labor and Birth. MCN Am J Matern Child Nurs. 42:1–7.

Edwards, S. M., S. A. Cunningham, A. L. Dunlop, and E. J. Corwin. 2017. The Maternal Gut Microbiome during Pregnancy. MCN Am. J. Matern. Nurs. 42:310–316.

Egerton, S., S. Culloty, J. Whooley, C. Stanton, R. P. Ross, and R. P. Ross. 2018. The Gut Microbiota of Marine Fish. Front. Microbiol. 9:1–17.

Ehrlich, P., and S. Hata. 1910. Die Experimentelle Chemotherapie der Spirilosen. Julius Springer, Berlin.

Ekkers, D. M., M. S. Cretoiu, A. M. Kielak, and J. D. Van Elsas. 2012. The great screen anomaly-a new frontier in product discovery through functional metagenomics. Appl. Microbiol. Biotechnol. 93:1005–1020.

Epstein, S. S. 2013. The phenomenon of microbial uncultivability. Curr. Opin. Microbiol. 16:636–642. Elsevier Ltd.

Ercolini, D. 2013. High-throughput sequencing and metagenomics: Moving forward in the culture-independent analysis of food microbial ecology. Appl. Environ. Microbiol. 79:3148–3155.

Esser, D., J. Lange, G. Marinos, M. Sieber, L. Best, D. Prasse, J. Bathia, M. C. Rühlemann, K. Boersch, C. Jaspers, and F. Sommer. 2018. Functions of the Microbiota for the Physiology of Animal Metaorganisms. J. Innate Immun. 11:393–404.

Fàbrega, A., S. Madurga, E. Giralt, and J. Vila. 2009. Mechanism of action of and resistance to quinolones. Microb. Biotechnol. 2:40–61.

Fernandes, P. 2006. Antibacterial discovery and development - The failure of success? Nat. Biotechnol. 24:1497–1503.

Fishelson, L., W. L. Montgomery, and A. A. Myrberg. 1985. A unique symbiosis in the gut of tropical herbivorous surgeonfish (acanthuridae: teleostei) from the red sea. Science (80-.). 229:49–51.

Fjellheim, A. J., K. J. Playfoot, J. Skjermo, and O. Vadstein. 2012. Inter-individual variation in the dominant intestinal microbiota of reared Atlantic cod (Gadus morhua L.) larvae. Aquac. Res. 43:1499–1508.

Fleming, A. 1929. On antibacterial action of culture of Penicillium, with special reference to their use in isolation of B. influenzae. Br. J. Exp. Pathol. 10:226–236.

Foster, K. R., J. Schluter, K. Z. Coyte, and S. Rakoff-Nahoum. 2017. The evolution of the host microbiome as an ecosystem on a leash. Nature 548:43–51. Nature Publishing Group.

Gervais, H. 1877. Les Poissons : Synonymie-Description-Moeurs-Frai-Pêche-Iconographie des espèces composant plus particulièrement la Faune francaise par H. Gervais et R. Boulart. Avec une introduction par Paul Gervais. J. Rothschild, Paris.

Ghanbari, M., W. Kneifel, and K. J. Domig. 2015. A new view of the fish gut microbiome: Advances from next-generation sequencing. Aquaculture 448:464–475. Elsevier B.V.

Gill, S. R., M. Pop, R. T. DeBoy, P. B. Eckburg, P. J. Turnbaugh, B. S. Samuel, J. I. Gordon, D. A. Relman, C. M. Fraser-Liggett, and K. E. Nelson. 2006. Metagenomic Analysis of the Human Distal Gut Microbiome. Science (80-.). 312.

Glaeser, S. P., and P. Kämpfer. 2015. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Syst. Appl. Microbiol. 38:237–245. Elsevier GmbH.

Glöckner, F. O., P. Yilmaz, C. Quast, J. Gerken, A. Beccati, A. Ciuprina, G. Bruns, P. Yarza, J. Peplies, R. Westram, and W. Ludwig. 2017. 25 years of serving the community with ribosomal RNA gene reference databases and tools. J. Biotechnol. 261:169–176. Elsevier.

Gomez, D., J. O. Sunyer, and I. Salinas. 2013. The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. Fish Shellfish Immunol. 35:1729–1739. Elsevier Ltd.

Harwani, D. 2012. The Great Plate Count Anomaly and the Unculturable Bacteria. Int. J. Sci. Res. 2:350–351.

Hasselquist, D., and J. Å. Nilsson. 2009. Maternal transfer of antibodies in vertebrates: Trans-generational effects on offspring immunity. Philos. Trans. R. Soc. B Biol. Sci. 364:51–60.

Hau, H. H., and J. A. Gralnick. 2007. Ecology and Biotechnology of the Genus Shewanella. Annu. Rev. Microbiol. 61:237–258.

Holmström, C., and S. Kjelleberg. 1999. Marine Pseudoalteromonas species are associated with higher organisms and produce biologically active extracellular agents. FEMS Microbiol. Ecol. 30:285–293.

Hooper, L. V., and A. J. Macpherson. 2012. Interactions Between the Microbiota and the Immune System. Science (80-.)., doi: 10.1126/science.1223490.

Høvik Hansen, G., and R. Sørheim. 1991. Improved method for phenotypical characterization of marine bacteria. J. Microbiol. Methods 13:231–241.

Huber, I., B. Spanggaard, K. F. Appel, L. Rossen, T. Nielsen, and L. Gram. 2004. Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout

(Oncorhynchus mykiss, Walbaum). J. Appl. Microbiol. 117–132.

Kapoor, G., S. Saigal, and A. Elongavan. 2017. Action and resistance mechanisms of antibiotics: A guide for clinicians. J. Anaesthesiol. Clin. Pharmacol. 300–305.

Kassambara, A. 2019. ggpubr: "ggplot2" Based Publication Ready Plots. R package version 0.2.3.

Keller, I. S., T. Bayer, W. Salzburger, and O. Roth. 2018. Effects of parental care on resource allocation into immune defense and buccal microbiota in mouthbrooding cichlid fishes. Evolution (N. Y). 72:1109–1123.

Kissoyan, K. A. B., M. Drechsler, E. L. Stange, J. Zimmermann, C. Kaleta, H. B. Bode, and K. Dierking. 2019. Natural C. elegans Microbiota Protects against Infection via Production of a Cyclic Lipopeptide of the Viscosin Group. Curr. Biol. 29:1030-1037.e5. Elsevier Ltd.

Klein, G. L., E. Soum-Soutéra, Z. Guede, A. Bazire, C. Compère, and A. Dufour. 2011. The anti-biofilm activity secreted by a marine Pseudoalteromonas strain. Biofouling 27:931–940.

Koren, O., J. K. Goodrich, T. C. Cullender, A. Spor, K. Laitinen, H. Kling Bäckhed, A. Gonzalez, J. J. Werner, L. T. Angenent, R. Knight, F. Bäckhed, E. Isolauri, S. Salminen, and R. E. Ley. 2012. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell 150:470–480.

Kvarnemo, C., K. B. Mobley, C. Partridge, A. G. Jones, and I. Ahnesjö. 2011. Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish Syngnathus typhle. J. Fish Biol. 78:1725–1737.

Lagkouvardos, I., J. Overmann, and T. Clavel. 2017. Cultured microbes represent a substantial fraction of the human and mouse gut microbiota. Gut Microbes 0976:1–11.

Lambert, S. M., and J. J. Wiens. 2013. Evolution of Viviparity: A phylogenetic test of the cold-climate hypothesis in phrynosomatid lizards. Evolution (N. Y). 67.

Lane, D. J. 1991. 16S/23S rRNA sequencing. Pp. 115–175 *in* E. Stackebrandt and M. Goodfellow, eds. Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York.

Lau, S. K. P., J. L. L. Teng, C. C. Ho, and P. C. Y. Woo. 2015. Gene amplification and sequencing for bacterial identification. 1st ed. Elsevier Ltd.

Lê, S., J. Josse, and F. Husson. 2008. FactoMineR: An R package for multivariate analysis. J. Stat. Softw. 25:1–18.

Lee, Y. K., and S. K. Mazmanian. 2010. Has the microbiota played a critical role in the evolution of the adaptive immune system? Science (80-.). 330:1768–1773.

Liu, K., C. R. Linder, and T. Warnow. 2011. RAxML and FastTree: Comparing two methods for large-scale maximum likelihood phylogeny estimation. PLoS One 6.

Llewellyn, M. S., S. Boutin, S. H. Hoseinifar, N. Derome, D. G. Biron, J. Romero, and U. De. 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. 5:1–17.

Lokesh, J., and V. Kiron. 2016. Transition from freshwater to seawater reshapes the skin-associated microbiota of Atlantic salmon. Sci. Rep. 6:1–10. Nature Publishing Group.

Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489:220–230.

MacDonald, T. T. 2003. The mucosal immune system. Parasite Immunol., doi: 10.1038/jid.2011.250.

Malavasi, S., A. Franco, F. Riccato, C. Valerio, P. Torricelli, and P. Franzoi. 2007. Habitat selection and spatial segregation in three pipefish species. Estuar. Coast. Shelf Sci. 75:143–150.

Margulis, L., M. Chapman, R. Guerrero, and J. Hall. 2006. The last eukaryotic common ancestor (LECA): Acquisition of cytoskeletal motility from aerotolerant spirochetes in the Proterozoic Eon. Proc. Natl. Acad. Sci. U. S. A. 103:13080–13085.

Martín, R., L. G. Bermúdez-Humarán, and P. Langella. 2016. Gnotobiotic rodents: An in vivo model for the study of microbe-microbe interactions. Front. Microbiol. 7:1–7.

Mazmanian, S. K., and Y. K. Lee. 2014. Interplay between intestinal microbiota and host immune system. J. Bacteriol. Virol. 44:1–9.

McFall-Ngai, M. 2007. Adaptive immunity: Care for the community. Nature 445:153.

McFall-Ngai, M. J., M. G. Hadfield, T. C. G. Bosch, H. V Carey, T. Domazet-Loso, A. E. Douglas, N. Dubilier, G. Eberl, T. Fukami, S. F. Gilbert, U. Hentschel, N. King, S. Kjelleberg, A. H. Knoll, N. Kremer, S. K. Mazmanian, J. L. Metcalf, K. Nealson, N. E. Pierce, J. F. Rawls, A. Reid, E. G. Ruby, M. Rumpho, J. G. Sanders, D. Tautz, and J. J. Wernegreen. 2013. Animals in a bacterial world, a new imperative for the life sciences. PNAS 110:3229–3236.

McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono. 1992. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 10:75–88.

Montalto, M., F. D'Onofrio, A. Gallo, A. Cazzato, and G. Gasbarrini. 2009. Intestinal microbiota and its functions. Dig. Liver Dis. Suppl. 3:30–34. Editrice Gastroenterologica Italiana S.r.l.

Navarrete, P., R. T. Espejo, and J. Romero. 2009. Molecular analysis of microbiota along the digestive tract of juvenile atlantic salmon (Salmo salar L.). Microb. Ecol. 57:550–561.

Nicholls, K. M., J. V. Lee, and T. J. Donovan. 1976. An Evaluation of Commercial Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS). J. Appl. Bacteriol. 41:265–269.

Nilsson, R. H., M. Ryberg, E. Kristiansson, K. Abarenkov, K. H. Larsson, and U. Köljalg. 2006. Taxonomic reliability of DNA sequences in public sequences databases: A fungal perspective. PLoS One 1.

Nuriel-Ohayon, M., H. Neuman, and O. Koren. 2016. Microbial changes during pregnancy, birth, and infancy. Front. Microbiol. 7:1–13.

O'Toole, P. W., and M. J. Claesson. 2010. Gut microbiota: Changes throughout the lifespan from infancy to elderly. Int. Dairy J. 20:281–291. Elsevier Ltd.

Ojetti, V., G. Gigante, M. E. Ainora, F. Fiore, F. Barbaro, and A. Gasbarrini. 2009. Microflora imbalance and gastrointestinal diseases. Dig. Liver Dis. Suppl. 3:35–39. Editrice Gastroenterologica Italiana S.r.l.

Oksanen, J., F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. Mcglinn, P. R. Minchin, R. B. O. Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, E. Szoecs, and H. Wagner. 2019. vegan: Community Ecology Package. R package version 2.5-4.

Oliver, J. D., C. Pruzzo, L. Vezzulli, and J. B. Kaper. 2013. Vibrio species. Pp. 401–439 *in* Food microbiology. American Society of Microbiology.

Ory, E. M., and E. M. Yow. 1963. The Use and Abuse of the Broad Spectrum Antibiotics. JAMA J. Am. Med. Assoc. 185:273–279.

Pannaraj, P. S., F. Li, C. Cerini, J. M. Bender, S. Yang, A. Rollie, H. Adisetiyo, S. Zabih, P. J. Lincez, K. Bittinger, A. Bailey, F. D. Bushman, J. W. Sleasman, and G. M. Aldrovandi. 2017. Association between breast milk bacterial communities and establishment and development of the infant gut microbiome. JAMA Pediatr. 171:647–654.

Perez-Muñoz, M. E., M. C. Arrieta, A. E. Ramer-Tait, and J. Walter. 2017. A critical assessment of the “sterile womb” and “in utero colonization” hypotheses: Implications for research on the pioneer infant microbiome. Microbiome 5:1–19. Microbiome.

Pham, L. N., M. Kanther, I. Semova, and J. F. Rawls. 2008. Methods for generating and colonizing gnotobiotic zebrafish. Nat. Protoc. 3:1862–1875.

Pruesse, E., J. Peplies, and F. O. Glöckner. 2012. SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28:1823–1829.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 41:590–596.

R Core Team. 2019. R: A language and environment for statistical computing.

Rambaut, A. 2018. FigTree, version 1.4.4.

Rappé, M. S., and S. J. Giovannoni. 2003. The Uncultured Microbial Majority. Annu. Rev. Microbiol. 57:369–394.

Rees, T., T. Bosch, and A. E. Douglas. 2018. How the microbiome challenges our concept of self. PLoS Biol. 16:1–7.

Rodrigues, S., C. Paillard, A. Dufour, and A. Bazire. 2014. Antibiofilm Activity of the Marine Bacterium Pseudoalteromonas sp. 3J6 Against Vibrio tapetis, the Causative Agent of Brown Ring Disease. Probiotics Antimicrob. Proteins 7:45–51.

Rohwer, F., V. Seguritan, F. Azam, and N. Knowlton. 2002. Diversity and distribution of coral-associated bacteria. Mar. Ecol. Prog. Ser. 243:1–10.

Romalde, J. L., A. L. Diéguez, A. Lasa, and S. Balboa. 2014. New Vibrio species associated to molluscan microbiota: A review. Front. Microbiol. 4:1–11.

Romero, J., E. Ringø, and D. L. Merrifield. 2014. The Gut Microbiota of Fish. Aquac. Nutr. 75–100.

Romero, R., J. Espinoza, L. F. Gonçalves, J. P. Kusanovic, L. Friel, and S. Hassan. 2007. The role of inflammation and infection in preterm birth. Semin. Reprod. Med. 25:21–39.

Rosenberg, E., and I. Zilber-Rosenberg. 2016. Microbes Drive Evolution of Animals and Plants: the Hologenome Concept. MBio 7:1–8.

Rossi-Tamisier, M., S. Benamar, D. Raoult, and P. E. Fournier. 2015. Cautionary tale of using 16s rRNA gene sequence similarity values in identification of human-associated bacterial species. Int. J. Syst. Evol. Microbiol. 65:1929–1934.

Roth, O., G. Joop, H. Eggert, J. Hilbert, J. Daniel, P. Schmid-Hempel, and J. Kurtz. 2010. Paternally derived immune priming for offspring in the red flour beetle, Tribolium castaneum. J. Anim. Ecol. 79:403–413.

Roth, O., I. Keller, S. H. Landis, W. Salzburger, and T. B. H. Reusch. 2012a. Hosts are ahead in a marine host-parasite coevolutionary arms race: Innate immune system adaptation in pipefish syngnathus typhle against vibrio phylotypes. Evolution (N. Y). 66:2528–2539.

Roth, O., V. Klein, A. Beemelmanns, J. P. Scharsack, and T. B. H. Reusch. 2012b. Male pregnancy and biparental immune priming. Am. Nat. 180:802–814.

Roth, O., J. P. Scharsack, I. Keller, and T. Reusch. 2011. Bateman's principle and immunity in a sex-role reversed pipefish. J. Evol. Biol. 24:1410–1420.

Sakata, T., H. Sugita, T. Mitsuoka, D. Kakimoto, and H. Kadota. 1981. Characteristics of Obligate Anaerobic Bacteria in the Intestines of Freshwater Fish. Bull. Japanese Soc. Sci. Fish. 47:421–427.

Schaedler, R. W., R. Dubus, and R. Costello. 1965. The Development of the Bacterial Flora in the Gastrointestinal Tract of Mice. J. Exp. Med. 122:59–66.

Schloss, P. D., K. D. Iverson, J. F. Petrosino, and S. J. Schloss. 2014. The dynamics of a family's gut microbiota reveal variations on a theme. Microbiome 2:1–13.

Schoenmakers, S., R. Steegers-Theunissen, and M. Faas. 2019. The matter of the reproductive microbiome. Obstet. Med. 12:107–115.

Sommer, F., and F. Bäckhed. 2013. The gut microbiota-masters of host development and physiology. Nat. Rev. Microbiol. 11:227–238. Nature Publishing Group.

Spanggaard, B., I. Huber, J. Nielsen, T. Nielsen, K. F. Appel, and L. Gram. 2000. The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. Aquaculture.

Staley, J. T., and A. Konopka. 1985. Measurements of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. 39:321–346.

Stamatakis, A. 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313.

Stewart, E. J. 2012. Growing unculturable bacteria. J. Bacteriol. 194:4151–4160.

Stölting, K. N., and A. B. Wilson. 2007. Male pregnancy in seahorses and pipefish: Beyond the mammalian model. BioEssays 29:884–896.

Tanaka, T., K. Kawasaki, S. Daimon, W. Kitagawa, K. Yamamoto, H. Tamaki, M. Tanaka, C. H. Nakatsu, and Y. Kamagata. 2014. A hidden pitfall in the preparation of agar media undermines microorganism cultivability. Appl. Environ. Microbiol. 80:7659–7666.

Tarnecki, A. M., N. P. Brennan, R. W. Schloesser, and N. R. Rhody. 2019. Shifts in the Skin-Associated Microbiota of Hatchery-Reared Common Snook Centropomus undecimalis During Acclimation to the Wild. Microb. Ecol. 77:770–781. Microbial Ecology.

Tavaré, S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Pp. 57–86 *in* Lectures on mathematics in the life sciences.

Tetreau, G., J. Dhinaut, B. Gourbal, and Y. Moret. 2019. Trans-generational immune priming in invertebrates: Current knowledge and future prospects. Front. Immunol. 10.

Thaiss, C. A., N. Zmora, M. Levy, and E. Elinav. 2016. The microbiome and innate immunity. Nature 535:65–74.

The Human Microbiome Project Consortium. 2012. Structure, function and diversity of the healthy human microbiome. Nature 486:207–214. Nature Publishing Group.

Tsien, R. Y. 1998. The Green Fluorescent Protein. Annu. Rev. Biochem. 67:509–544.

van den Elsen, L. W. J., J. Garssen, R. Burcelin, and V. Verhasselt. 2019. Shaping the gut microbiota by breastfeeding: The gateway to allergy prevention? Front. Pediatr. 7:1–10.

Verstraelen, H., R. Vilchez-Vargas, F. Desimpel, R. Jauregui, N. Vankeirsbilck, S. Weyers, R. Verhelst, P. De Sutter, D. H. Pieper, and T. Van De Wiele. 2016. Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16S rRNA gene. PeerJ 2016:1–23.

Wang, A. R., C. Ran, E. Ringø, and Z. G. Zhou. 2018. Progress in fish gastrointestinal microbiota research. Rev. Aquac. 626–640.

Wassenaar, T. M., and P. Panigrahi. 2014. Is a foetus developing in a sterile environment? Lett. Appl. Microbiol. 59:572–579.

Weisberg, S., and J. Fox. 2019. An R Companion to Applied Regression. Sage, Thousand Oaks, CA.

Wendling, C. C., A. Piecyk, D. Refardt, C. Chibani, R. Hertel, H. Liesegang, B. Bunk, J. Overmann, and O. Roth. 2017. Tripartite species interaction: eukaryotic hosts suffer more from phage susceptible than from phage resistant bacteria. BMC Evol. Biol. 17:1–12. BMC Evolutionary Biology.

Wickham, H. 2009. ggplot2: Elegant Graphics for Data Analysis. Springer, New York.

Yanong, R. P. E. 2013. Use of Antibiotics in Ornamental Fish Aquaculture.

Yatsunenko, T., F. E. Rey, M. J. Manary, I. Trehan, M. G. Dominguez-Bello, M. Contreras, M. Magris, G. Hidalgo, R. N. Baldassano, A. P. Anokhin, A. C. Heath, B. Warner, J. Reeder, J. Kuczynski, J. G. Caporaso, C. A. Lozupone, C. Lauber, J. C. Clemente, D. Knights, R. Knight, and J. I. Gordon. 2012. Human gut microbiome viewed across age and geography. Nature 486:222–227.

Yilmaz, P., L. W. Parfrey, P. Yarza, J. Gerken, E. Pruesse, C. Quast, T. Schweer, J. Peplies, W. Ludwig, and F. O. Glöckner. 2014. The SILVA and “all-species Living Tree Project (LTP)” taxonomic frameworks. Nucleic Acids Res. 42:643–648.

Younge, N., J. R. McCann, J. Ballard, C. Plunkett, S. Akhtar, F. Araújo-Pérez, A. Murtha, D. Brandon, and P. C. Seed. 2019. Fetal exposure to the maternal microbiota in humans and mice. JCI Insight 4:1–14.

Zhang, S., Z. Wang, and H. Wang. 2013. Maternal immunity in fish. Dev. Comp. Immunol. 39:72–78. Elsevier Ltd.

8 Supplementary material

Supplemental Table 1: Overview of all isolated bacterial species and their presence/absence in the sampled sexes/stages of *S. typhle*: Female gonads (FG), Non-pregnant males (NP), Early pregnancy pouch (EPP), Early pregnancy juveniles (EPJ), Late pregnancy pouch (LPP), Late pregnancy juveniles (LPJ), Seawater (SW) and PBS control (PBS). Numbers denote in how many samples of the respective sex/stage the strain was found

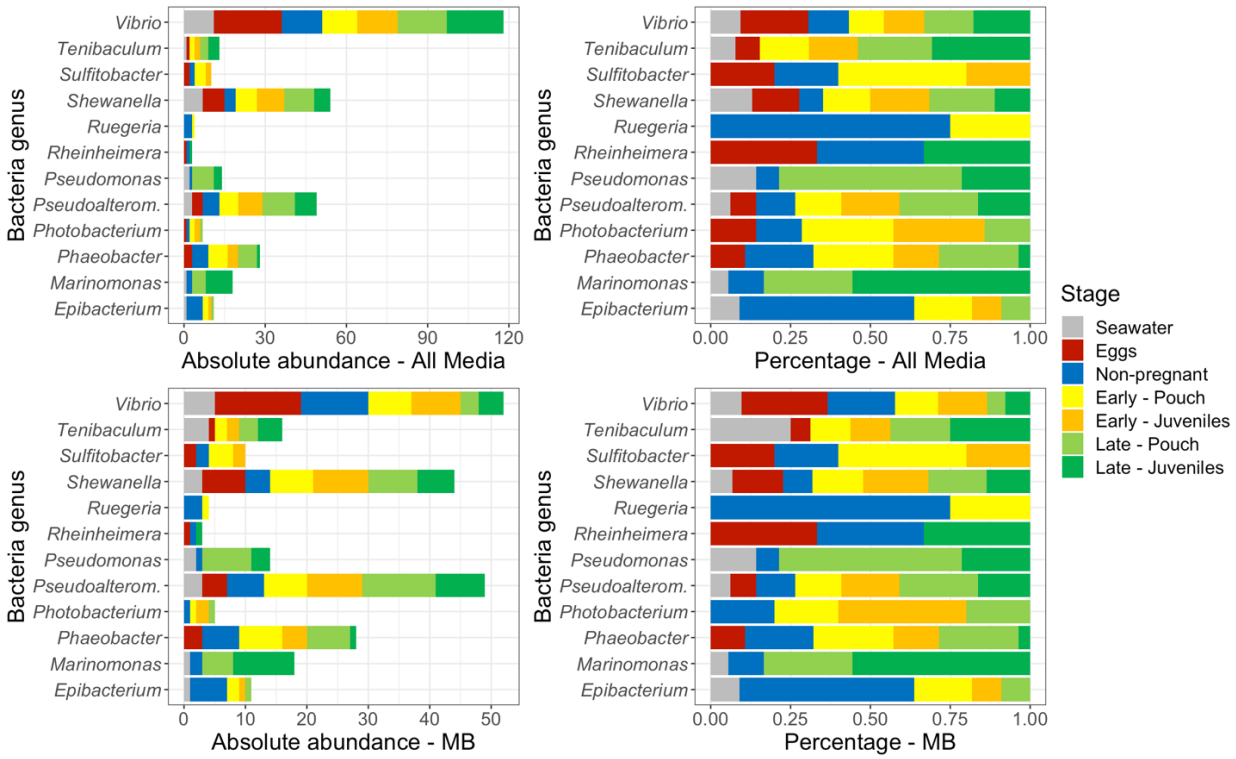
Bacterial species	FG	NP	EPP	EPJ	LPP	LPJ	SW	PBS
Agrobacterium salinitolerans	0	1	1	0	0	0	0	0
Ahrensia kielensis	0	1	0	0	0	0	0	0
Aliivibrio finisterrensis	0	0	0	1	0	0	2	0
Brevibacterium frigoritolerans	0	0	0	0	1	0	0	0
Cellulophaga lytica	0	1	0	0	0	0	0	0
Cobetia amphilecti	0	0	0	0	1	0	0	0
Colwellia aestuarii	2	0	0	0	0	0	1	0
Dokdonia flava	0	1	0	0	0	0	0	0
Enterovibrio calviensis	0	0	1	0	0	0	0	0
Epibacterium mobile	0	5	1	1	1	0	1	0
Epibacterium scottomollicae	0	1	0	0	0	0	0	0
Exiguobacterium indicum	0	1	0	0	0	0	0	0
Exiguobacterium profundum	1	0	0	0	0	0	0	0
Fictibacillus phosphorivorans	0	0	0	0	1	0	0	0
Kiloniella laminariae	0	0	0	0	1	0	0	0
Kordia antarctica	0	0	1	0	0	0	0	0
Leisingera aquimarina	0	1	0	0	0	0	0	0
Litoreibacter albidus	0	0	1	0	0	0	0	0
Luteimonas aestuarii	0	0	0	1	0	0	0	0
Marinobacter dokdonensis	0	0	1	0	0	0	0	0
Marinococcus halotolerans	1	0	0	0	0	0	0	0
Marinomonas communis	0	0	0	0	0	2	1	0
Marinomonas dokdonensis	0	0	0	0	2	1	0	0
Marinomonas gallaica	0	2	0	0	0	0	0	0
Marinomonas rhizomae	0	0	0	0	2	4	0	0
Microbacterium oxydans	0	1	1	0	0	0	2	0
Neptunomonas concharum	0	0	1	0	0	0	0	0
Phaeobacter inhibens	1	0	1	1	1	0	0	0
Phaeobacter piscinae	2	6	6	3	4	1	0	0
Photobacterium ganghwense	0	1	1	2	1	0	0	0
Photobacterium swingsii	1	0	1	0	0	0	0	0
Pseudoalteromonas agarivorans	0	0	0	0	0	0	1	0
Pseudoalteromonas arabiensis	0	0	0	0	2	1	0	0
Pseudoalteromonas carrageenovora	0	0	1	0	0	1	0	0
Pseudoalteromonas distincta	0	0	0	0	1	0	0	0
Pseudoalteromonas elyakovii	0	1	0	0	1	0	0	0
Pseudoalteromonas espejiana	0	0	0	2	0	0	0	0
Pseudoalteromonas gelatinilytica	0	0	0	0	3	1	0	0
Pseudoalteromonas hodoensis	1	0	1	1	0	0	0	0
Pseudoalteromonas issachenkonii	0	0	0	1	0	0	0	0
Pseudoalteromonas lipolytica	0	0	0	0	0	2	0	0
Pseudoalteromonas neustonica	0	0	0	0	1	1	0	0
Pseudoalteromonas phenolica	0	0	0	0	0	1	1	0
Pseudoalteromonas piratica	0	2	1	0	0	0	0	0

Bacterial species	FG	NP	EPP	EPJ	LPP	LPJ	SW	PBS
Pseudoalteromonas piscicida	0	0	0	1	0	0	0	0
Pseudoalteromonas prydzensis	0	0	1	0	0	0	0	0
Pseudoalteromonas shioyasakiensis	1	0	0	0	0	1	0	0
Pseudoalteromonas spiralis	0	0	1	0	0	0	0	0
Pseudoalteromonas tetraodonis	0	2	1	1	0	0	0	0
Pseudoalteromonas ulvae	1	0	1	3	0	0	1	0
Pseudoalteromonas undina	1	1	0	0	0	0	0	0
Pseudomonas chengduensis	0	0	0	0	0	0	1	0
Pseudomonas marincola	0	0	0	0	2	0	0	0
Pseudomonas pachastrellae	0	0	0	0	0	0	1	0
Pseudomonas segetis	0	1	0	0	1	2	0	0
Pseudophaeobacter arcticus	0	1	0	0	0	0	0	0
Psychrobacter faecalis	0	1	0	0	0	0	0	0
Rheinheimera pacifica	1	1	0	0	0	1	0	0
Rhodococcus erythropolis	0	0	0	0	0	3	0	0
Rhodococcus qingshengii	0	0	0	0	0	0	0	1
Ruegeria conchae	0	2	1	0	0	0	0	0
Ruegeria profundi	0	1	0	0	0	0	0	0
Salinibacterium amurskyense	0	1	0	0	0	0	0	0
Shewanella aestuarii	2	1	1	1	2	3	0	0
Shewanella baltica	0	0	0	2	1	1	0	0
Shewanella colwelliana	2	0	1	0	2	0	1	0
Shewanella hafniensis	0	0	1	1	1	2	0	0
Shewanella marinintestina	4	2	4	5	3	0	3	0
Sulfitobacter brevis	0	1	0	0	0	0	0	0
Sulfitobacter geojensis	0	0	1	0	0	0	0	0
Sulfitobacter pacificus	1	0	1	1	0	0	0	0
Sulfitobacter pontiacus	1	1	1	1	0	0	0	0
Tenibaculum litopenaei	0	0	1	0	0	0	0	0
Tenibaculum mesophilum	1	0	1	2	3	4	1	1
Thalassospira mesophila	0	0	0	0	0	1	0	0
Thalassotalea ganghwensis	1	0	0	0	0	0	1	0
Thalassotalea piscium	0	0	0	0	0	1	0	0
Vibrio aestuarianus	0	1	0	0	0	0	0	0
Vibrio aestuarianus, subsp. Francensis	0	0	0	0	1	1	0	0
Vibrio alginolyticus	3	2	0	2	0	1	3	0
Vibrio anguillarum	2	2	4	3	4	5	0	0
Vibrio atlanticus	0	2	0	1	0	1	1	0
Vibrio atypicus	0	0	2	0	0	0	0	0
Vibrio cortagadensis	0	0	1	0	0	0	0	0
Vibrio cyclitrophicus	2	2	1	0	3	0	2	0
Vibrio fortis	0	0	0	0	0	0	1	0
Vibrio gallaecicus	1	0	0	0	0	1	0	0
Vibrio gigantis	5	0	0	0	1	2	0	0
Vibrio ichthyoenteri	1	0	0	0	0	0	0	0
Vibrio kanaloae	2	1	0	0	1	1	1	0
Vibrio lentus	0	1	1	2	1	3	0	0
Vibrio pacinii	1	0	1	0	0	0	0	0
Vibrio pectenicida	0	0	0	0	0	0	1	0

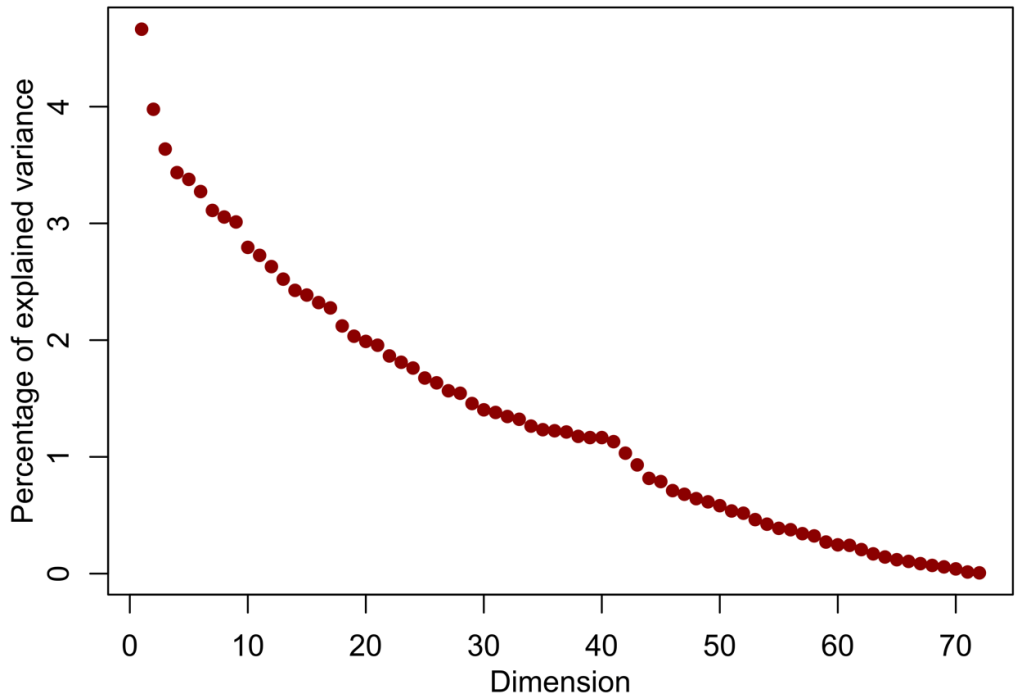
Bacterial species	FG	NP	EPP	EPJ	LPP	LPJ	SW	PBS
Vibrio rotiferianus	1	0	0	0	0	2	1	0
Vibrio sinaloensis	2	0	0	2	0	0	0	0
Vibrio splendidus	0	0	0	0	1	0	0	0
Vibrio tapetis	0	0	1	0	0	0	0	0
Vibrio vulnificus	2	3	1	2	1	0	1	0

Supplemental Table 2: Overview of 29 bacterial species isolated from reproductively relevant tissue of *S. typhle* that showed certain specificities such as (a) universal presence across all stages, (b) stage-specifically grouping or (c) stage, respectively sex specificity presence and absence based on the presence-absence matrix (Supplemental Table 1). Shown are the species name and the specificity criterion after which the strain was selected

Bacterial strain	Specificity
Colwellia aestuarii	Only in FG and SW (c)
Epibacterium mobile	Indicator paternal contribution (c)
Epibacterium scottomollicae	Indicator paternal contribution (c)
Marinomonas communis	Indicator paternal contribution (c)
Marinomonas dokdonensis	Indicator paternal contribution (c)
Marinomonas gallaica	Indicator paternal contribution (c)
Marinomonas rhizomae	Indicator paternal contribution (c)
Phaeobacter piscinae	Present in all groups (a)
Photobacterium ganghwense	Only in males (c)
Pseudoalteromonas arabiensis	Only in late pregnancy (LPP, LPJ) (b)
Pseudoalteromonas gelatinilytica	Only in late pregnancy (LPP, LPJ) (b)
Pseudoalteromonas neustonica	Only in late pregnancy (LPP, LPJ) (b)
Pseudoalteromonas shioyasakiensis	Indicator maternal contribution (c)
Pseudomonas marincola	Indicator paternal contribution (c)
Pseudomonas segetis	Indicator paternal contribution (c)
Ruegeria conchae	Indicator paternal contribution (c)
Ruegeria profundi	Indicator paternal contribution (c)
Shewanella aestuarii	Present in all groups (a)
Shewanella marinintestina	Present in all groups except LPJ (a)
Sulfitobacter pontiacus	Not present in late pregnancy (b)
Tenacibaculum mesophilum	Indicator maternal contribution (c)
Vibrio aestuarianus subsp. Francensis	Only in late pregnancy (LPP, LPJ) (b)
Vibrio alginolyticus	Not present in pouch during pregnancy (c)
Vibrio anguillarum	Present in all groups (a)
Vibrio cyclitrophicus	Not in present in juveniles (c)
Vibrio gallaecicus	Indicator maternal contribution (c)
Vibrio lentus	Only in males (c)
Vibrio pacinii	Indicator maternal contribution (c)
Vibrio vulnificus	Present in all groups except LPJ (a)



Supplemental Figure 1: Comparison of cultivation media (general: Marine Broth and selective: TCBS) in terms of absolute and relative abundances. Shown are results from both media (top) and from general cultivation media only (bottom).



Supplemental Figure 2: Multiple correspondence analysis (MCA) eigenvalues visualize the percentage of explained variance for all 72 dimensions.

Declaration of Authorship

I, Kim-Sara Wagner, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Title of thesis:

Cultivation and characterization of sex-specific microbiota in the broad-nosed pipefish *Syngnathus typhle*

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission.

Date:

Signed: