

ERC Advanced Grant 2021

Part B2

Section a. State-of-the-art and objectives

Phenotypic differences among species are the outcome of species-specific genetic programs that unfold in the context of environmental stimuli. At the very interface between multicellular hosts and the environment lay complex microbial communities consisting of viruses, bacteria, archaea, fungi and other eukaryotes, which take active part in dynamic host-environment interactions. Nitrogen-fixing microbes associated with roots across several plant species represent a paradigmatic case of highly specialized microbial communities that, by enabling access to environmental nitrogen, directly contribute to host-fitness¹. Similarly, by breaking down long-chain carbohydrates into short-chain fatty acids, complex intestinal microbial communities provide several vertebrates with accessible nutrients and immune-modulatory signals². Host-associated microbes not only process molecules from the external environment, but also actively transform host-derived biomolecules, participating with the host in complex biochemical reactions, such as those involved in the synthesis of secondary bile acids³. Largely exceeding the host's own biochemical repertoire⁴, host-associated microbial consortia generate a staggering diversity of bioactive molecular intermediates that modulate host physiology, actively contributing to the host's homeostatic balance⁵. Microbial consortia participate in virtually all aspects of host biology, including nutrient absorption, toxin degradation, defense against pathogens⁶, maturation and tuning of the immune system⁷, synthesis of essential vitamins⁸, inter-individual communication⁹, escape from predators through bioluminescence¹⁰, and many more. From coral reefs to mammalian guts, whenever the host-microbiota balance is compromised, microbial communities become dysbiotic^{11,12}, pathobionts and opportunistic pathogens take over commensals, ultimately leading to dysfunction, and in extreme cases to disease and death of the host^{13,14}. Given their key role in maintaining homeostasis, microbiota have been also recognized as important modulators of the aging process¹⁵⁻¹⁹.

Different hosts species harbor specific microbial communities, whose community structure and taxonomic diversity in part map to host-species phylogenetic distance, two concepts known as phylosymbiosis^{20,21} and cophylogeny²², respectively. Importantly, host-specific microbial consortia are also shaped by diet^{23,24} and by the host's ecological and social niche^{25,26}. Host-associated microbes are highly dynamic communities and together help their hosts better adapt to the environment, significantly contributing to species-specific phenotypes²⁷. Given the importance that microbial communities play in modulating host biological functions, organisms evolved sophisticated strategies to select, acquire and transmit subsets of microbes from those in the environment. Even though in wild thale cress (*Arabidopsis thaliana*) the overall root microbiome composition is only marginally dictated by the genetic diversity across host plants²⁸, plant roots have been shown to be able to select preferential microbial consortia from the available soil biome in several plant species²⁹. The preferential acquisition of microbes, for instance specialized symbionts, is also prevalent in insects, such as in leaf beetle (*Cassida rubiginosa*), which acquire specific pectinolytic Proteobacteria via vertical transmission upon hatching³⁰. Burying beetle (*Nicrophorus vespilloides*) lay their eggs in the carcasses of birds and small rodents, which generally would be subjected to microbial successions typical of carcass decay. However, by smearing the carcasses with oral and anal secretions rich in antimicrobials, burying beetles control the microbial milieu of the decomposing carcasses to optimize development for their buried larvae³¹. In humans, birth mode by Caesarean or vaginal delivery impacts the microbial species colonizing first the infant's gastrointestinal tract. Moreover, the dynamics of microbial succession in the first months of life depend on feeding on breast milk, solid food, geographical location and, to a lesser extent, on the presence of siblings, maternal BMI and probiotics^{32,33}. While we start understanding how the ecological niche provides a primary source of available microbes, we still know little about the active role of the host in the acquisition and maintenance of complex microbiota in vertebrates, and how the acquisition of these microbial communities, in turn, contributes to species-specific phenotypes (red arrows, **Figure 1**). The analysis of the genetic and mechanistic basis underlying host-microbiota partnerships will contribute to

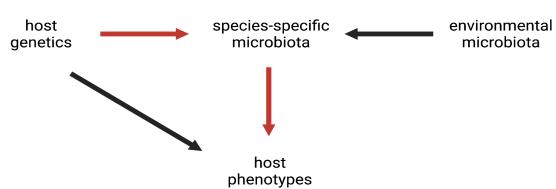


Figure 1. Graphical representation of how genetics and microbiota can affect host phenotypes.

understanding how species actively build their own ecological (external) and metabolic (internal) niche and how microbiota impact phenotype variation among species.

To study how the establishment of microbiota contributes to species-specific phenotypes, I will focus on **African killifish** (oviparous Cyprinodontiformes), a group of vertebrates suitable for studies in nature and in

the laboratory^{34,35}, with the most recent common ancestor dating back to ~ 52 million years ago³⁶. African killifish live in ephemeral water bodies that form during brief rainy seasons, as well as in permanent streams. In the past two decades, killifish have been extensively developed as laboratory model organisms³⁷; our group has sequenced the genomes of over 45 killifish species and we have assembled and annotated five genomes³⁸⁻⁴¹. Using comparative genomics and population genetics in African killifish, we investigated the evolutionary forces that shape genome architecture and life history in this group of teleosts^{38,41-43}. Killifish are now routinely raised and bred in captivity^{44,45} and their rapid life cycle has facilitated the development of efficient transgenesis and genome editing⁴⁶⁻⁴⁸. My research group has significantly contributed to several aspects of killifish development as an accessible model organism, from QTL mapping and population genetics in wild population isolates^{41,42,49}, to the establishment of transgenesis, genome editing^{47,48}, genomics^{38,39,49}, as well as setting the standards for killifish husbandry^{44,47}.

Our research took an unexpected direction when we monitored the life-dependent changes in the composition of the intestinal microbiota of captive turquoise killifish (*Nothobranchius furzeri*). We found that, similar to mice and humans, turquoise killifish undergo dramatic changes in gut microbial community during life, with decreased microbial richness in older individuals compared to adults and increased between-individual microbial diversity. The intestinal microbial composition of aging killifish undergoes a significant decrease in Firmicutes and Bacteroidetes and an increase of Proteobacteria¹⁶. To investigate whether microbial communities associated with adult killifish have a causal influence on homeostatic processes, we performed microbiota transfer experiments, replacing the gut microbiota of middle-age killifish with microbes from young donors, after antibiotic treatment. Microbial transfers resulted in dramatic changes in the gut microbial composition of recipient fish, and, remarkably, we found that transfer of young-derived intestinal contents led to significant lifespan extension and delayed age-dependent decline in locomotor activity¹⁶. Furthermore, microbiota transfer from young individuals led to several transcriptional changes in the intestine that are related to immune regulation. These include upregulation of markers of intestinal B cell proliferation and upregulation of interleukin 10 signaling, an anti-inflammatory cytokine, whose expression is associated with improved health and decreased age-associated inflammation⁵⁰. We found that the genera *Exiguobacterium*, *Propionigenium*, *Planococcus* and *Psychrobacter* are enriched in young-adult killifish intestines and in intestines from fish that received microbiota from young donors. Microbial species from these genera are beneficial to mammalian hosts; they metabolize cellulose and ferment other carbohydrates to produce short chain fatty acids – which are anti-inflammatory mediators with immune-modulatory functions. They additionally hydrolyze gelatin to produce essential amino acids, which can be taken up by the host, and synthesize omega-fatty acids. Together, we found that microbiota associated with young individuals can benefit middle-age subjects. The beneficial effect of microbes transferred to middle-age individuals was dependent on the age of the donors, as microbiota from same-age donors did not lead to lifespan extension in middle-age individuals. We asked if microbiota from old subjects would shorten

lifespan in young killifish and found no effect, suggesting the intriguing possibility that while young-adult hosts may be actively shaping their gut microbiota, systemic aging may lead to microbiota “escaping” host surveillance due to weakened host active control. We then asked whether gut microbiota in laboratory killifish recapitulate those of wild populations and found that although laboratory killifish have lower inter-individual variability compared to wild-caught killifish, the overall taxonomic composition of laboratory killifish recapitulates that of wild killifish populations¹⁶. To note, gut microbiota from different turquoise killifish wild populations largely cluster by locality, possibly reflecting shared micro-habitat characteristics unique to each seasonal pond. Our findings

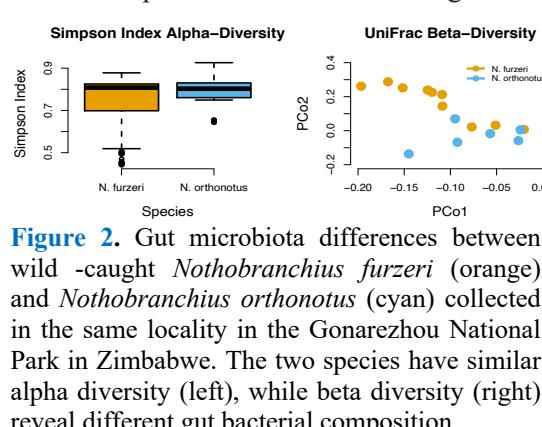


Figure 2. Gut microbiota differences between wild-caught *Nothobranchius furzeri* (orange) and *Nothobranchius orthonotus* (cyan) collected in the same locality in the Gonarezhou National Park in Zimbabwe. The two species have similar alpha diversity (left), while beta diversity (right) reveal different gut bacterial composition.

led us to ask whether different killifish species living in the same locality share the same microbiota, due to shared ecology. We analyzed killifish intestines collected during fieldwork that we conducted in the Gonarezhou National Park in Zimbabwe, which hosts the natural habitat of two species, the spotted and turquoise killifish (*Nothobranchius orthonotus* and *furzeri*, respectively). We found that despite living in the same freshwater ponds and feeding on the same prey⁵¹, age-matched individuals from these two species have distinct and species-specific intestinal microbiota composition (Figure 2), raising the important question of what is the ecological and genetic basis underlying the acquisition and maintenance of distinct microbial communities in host species sharing the same environment.

Thus, leveraging the availability of an accessible model system that can be studied in nature and in the laboratory, the development of state-of-the-art molecular and genomic tools for experimental interventions, a diversity of ecologies and life history traits, a complex and species-distinct microbiota, killifish represent an ideal system to study host-microbiota interactions in the context of species-specific phenotypes.

This proposal aims at investigating the mechanisms responsible for the establishment and maintenance of microbial communities across related killifish species, shedding light on how microbiomes contribute to phenotypic variation across species.

My research plan consists of five main aims (**Figure 3**):

In my first aim, we will exploit i) our established collection of **captive African killifish** species raised in the laboratory and ii) our access and logistic support at **field localities** that host natural killifishes, to extensively chart microbial diversity across phylogeny and ecology. In the second aim, we will study the specificity and efficacy of **antimicrobials** encoded in killifish genomes by screening microbial growth in vitro; while in the third aim we will explore more broadly the **genetic determinants of microbial selection** by killifish hosts. The fourth aim will ask how microbial inputs affect killifish phenotypic responses, including **growth, reproduction and lifespan** across different killifish species, and the fifth aim will be aimed at modulating microbial community assembly by employing a **vaccination-based approach** to harness adaptive immunity to achieve selective microbial exclusion. Together, the last two aims will explore how dialing microbial inputs impacts a range of host short-term, as well as long term, phenotypic responses.

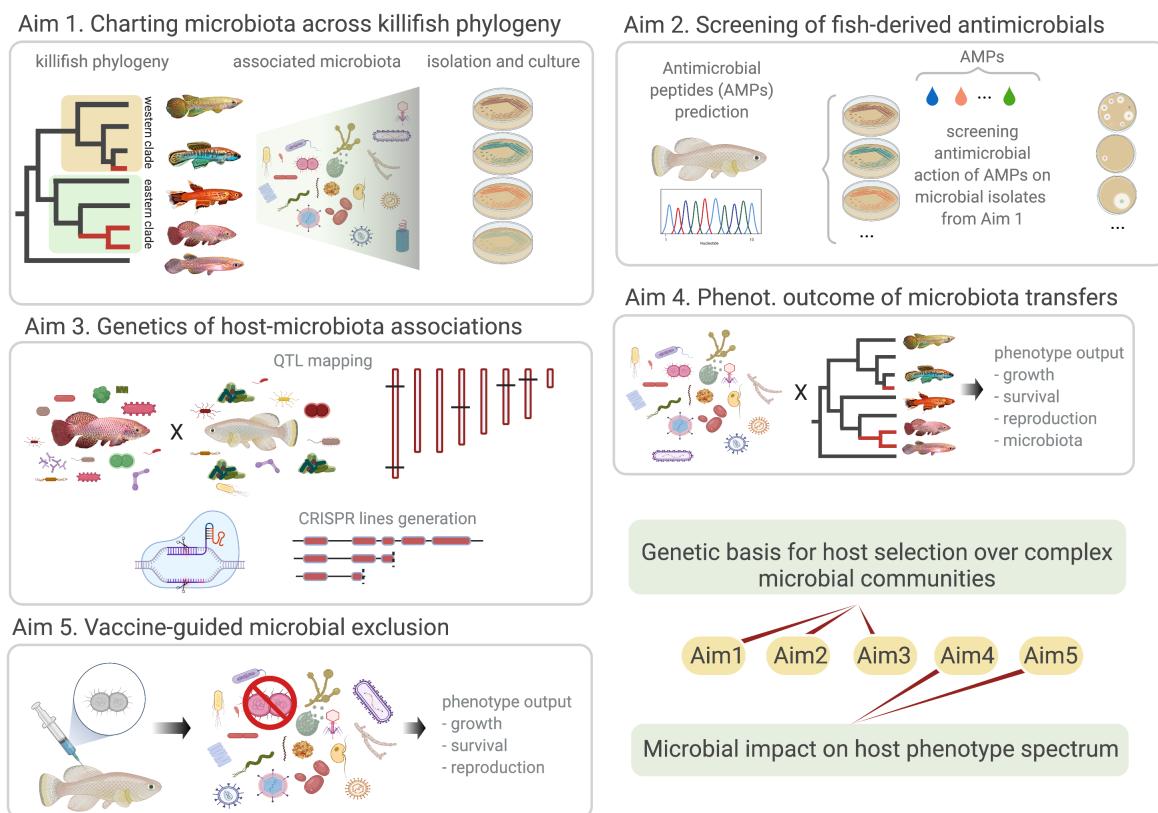


Figure 3. Overview of the research plan for MICROPHENO.

Significance and outlook: Host-microbial partnerships are essential to life on earth and occur throughout the whole tree of life⁵². Microbial communities participate in all aspects of host biology. While commensals help maintain homeostatic balance, enabling nutrient absorption and defense against pathogens, pathobionts, pathogens and parasites represent one of the most serious challenges to homeostasis. Microbiota and hosts have been suggested to be co-evolving units⁵³⁻⁵⁵ and specific microbes (e.g. bacteria of the genus *Wolbachia*) have been shown to be key players even for speciation events⁵⁶. In humans, there are several examples of genomic signatures of past adaptations that protect hosts against microbial parasites^{57,58}. Several eukaryotes show extensive genetic introgression from microbial genomes, which possibly occurred after long periods of pathogen- or commensal-host interactions⁵⁹. However, while the genetics and ecology underlying host-

pathogen and host-parasite interactions have been studied for several decades, we still know little about how different organisms actively assemble their species-specific complex commensal microbial communities and how these communities, in turn, shape species-specific phenotypes. I am extremely excited about this proposal because it tackles one of the most important questions in modern biology. In fact, balanced host-microbiota interactions are key to the health of individual organisms, communities of individuals, as well as ecosystems. The implications of our work will be relevant to evolutionary biology, ecology, conservation, as well as medicine. By leveraging the experimental accessibility of our unique model system – African killifishes – we have an opportunity to mechanistically dissect the evolutionary and ecological basis underlying host-microbiota interactions. My integrated research plan, which includes genomics, screening of antimicrobials, genome editing and immunizations, will provide proof-of-concept for work beyond killifish biology and will set the basis for an integrated approach that bridges ecology and evolution through the microbiome, providing a new perspective to explain phenotype variation. This ambitious proposal represents a phenomenal opportunity for my research team, as capitalizing on the various tools we developed up to date in killifish, we are now equipped to address an urgent and unanswered biological question that has far reaching implications.

Section b: Methodology

Aim 1: Charting microbial diversity across African killifishes

Species phenotype variation is understood to be a function of host genetics (V_G), environmental variability (V_E), and of the interaction between genetics and environment ($V_{G \times E}$). Microbiota associated with hosts can co-vary in composition with both host genetics^{20,60} and with the ecological niche (also in fish²¹), hence de facto co-varying with the key terms that contribute to phenotypic variance. To assess the microbial contribution to host phenotype variation in nature, we seek to chart microbial diversity across host phylogeny and ecology and generate a unique collection of microbial isolates associated with killifish. Killifish repeatedly adapted to annual and non-annual habitats⁶¹ and our past work shows how killifish phenotype diversity is associated with their genomic relatedness^{38,43} and with their ecology. Here, we ask how killifish microbial communities vary as a function of host phylogeny, anatomy, as well as habitat variation. This aim will be foundational to dissect the mechanisms by which specific hosts may actively contribute to shaping their own microbial community assemblies.

1.1 Metagenomics survey of killifish phylogeny

Rationale: African killifish have adapted to a range of habitats: from permanent streams to seasonal savanna ponds. Adaptations to savanna ponds happened multiple times throughout killifish evolution (red branches, **Figure 3**), leading to independent evolution of an annual life cycle^{62,63}, characterized by embryonic diapause and explosive rapid maturation^{34,64}. Repeated adaptations to different habitats in killifish offer a unique opportunity to disentangle the relative contribution of genetics and ecology to the composition of species-specific microbiota. Furthermore, ephemeral ponds at the center of the *Nothobranchius* genus species radiation (coastal Tanzania) contain up to 6 distinct species in the same locality⁶⁵, providing opportunities to test how shared ecology and different genetics shape microbiota in different species.

We will perform amplicon-based (16S) and shotgun metagenomics sequencing in gut, stool and skin samples from a collection of annual and non-annual killifish species collected in the wild. Wild specimens will be accessed in part by our team in Zimbabwe and Seychelles – where we have extensive field work experience and logistical support (see attached support letter in Annex 3) – and in part via our collaborators in Mozambique, Tanzania, Guinea, Sierra Leone, Liberia and Democratic Republic of the Congo. We will employ the same network of collaborators from a previous study we conducted on killifish comparative genomics³⁸. Wild-caught samples will be measured, euthanized and their gut and skin fixed in ethanol. Using shotgun metagenomics in stool samples, we will identify host-specific microbiomes beyond standard amplicon-based approaches that focus exclusively on Bacteria of Fungi. Unpublished work done in my group in captive turquoise killifish shows that killifish stool microbiota is characterized by the presence of Bacteria (~75%), Archaea (~0.05%), Viruses

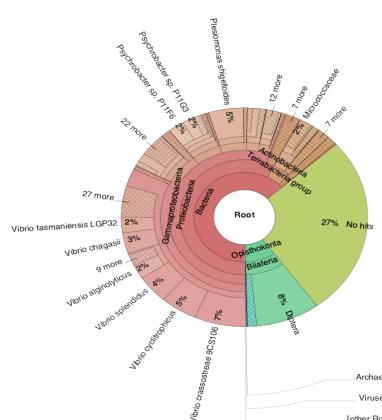


Figure 4. Representative metagenomic sequence of captive turquoise killifish stool microbiota (Davila Aleman et al. *in preparation*). This pie chart shows the presence of Bacteria, Archaea, Viruses, Fungi (other), as well as the sequences from the food source (Diptera).

(~0.05%), Fungi (~0.1 %), host (~ 1%), as well as additional eukaryotic DNA (**Figure 4**). To assess the extent to which organ-specific microbiomes reflect the microbial composition in the habitat, we will conduct metagenomic analyses from independent water sample from each locality. We will analyze the metagenomes using phylogenetic relatedness, body size, stomach content, habitat, and climate as covariates. We will adopt and compare phylogenetic regressions under different models that make different assumptions about the processes leading to phylogenetic divergence, such as Pagel's lambda Brownian motion approach, as well as Ornstein-Uhlenbeck (OU) process⁶⁶. This systematic survey of metagenomes from killifish samples across phylogeny, habitats and locality will allow assessing how tissue-specific microbiome assemblies are constrained by host's evolutionary relatedness and ecology and will help reveal the presence of specific associations between microorganisms and host species. Metagenomics will further enable the identification of host-specific, organ-specific and habitat-specific enrichments for sets of microbial metabolic pathways and genes. To this end, we will use available tools, such as MetaPoap⁶⁷ and metaGEM⁶⁸. To gain insights into whether fish host actively select microbes from the environment, we will compute the Sloan's ecological neutrality index⁶⁹, which compares microbial relative occurrence over mean abundance. For sequencing, we will use a fragmentation-based linked-read technology⁷⁰, combined with Illumina HiSeq and Nanopore sequencing. This approach preserves contiguity over long DNA fragments from metagenomic sequences, facilitating read mapping on reference databases, as well as providing a key resource to catalog and assemble novel uncatalogued microbial genome fragments from natural habitats and killifish epithelia.

Problems/solutions: If access to field samples will be unpractical due to factors outside of our control, such as the travel restrictions caused by the SARS-CoV-2 pandemic, we will focus either on field sites that are accessible, or we will direct our sequencing efforts on six killifish species that we already raise in our laboratory at the FLI under a common garden (see Aim 4.2). This approach will enable us to refocus on how different microbial communities assemble across different species under the same environmental conditions.

1.2 Extensive sampling of killifish-associated microbial isolates

Rationale: While sequencing 16S rRNA amplicons and shotgun metagenomes will give us a deep description of the microbial communities associated with killifish species and their environment, establishing a collection of microbial isolates that occur most frequently in association with killifish will be key for

functional studies of the metabolic crosstalk between hosts and their microbiota, enabling experimental interventions.

We will sample a representative collection of microbial isolates associated with killifish both from field locations, as well as from laboratory-raised fish strains. Microbial isolates will be preserved in agar slants (from wild samples) or directly cultured and cryopreserved (from laboratory-sampled microbes). Following a similar strategy, we have already started a collection of killifish-associated laboratory microbes, which include *Pseudomonas lundensis*, *Schewanella baltica*, *Vagococcus lutrae*, *Aeromonas veronii*, *Carnobacterium maltaromaticum*, *Vibrio metschnikovii*, *Bacillus thuringensis* and two uncharacterized species: *Leucobacter sp.* and *Psuedoclavibacter sp.* Isolation, culturing and stocking of microbial isolates will greatly benefit from the extensive expertise of the **Jena Microbial Research Collection (JMRC)**, a joint institute at the Hans Knoell Institute for Natural Product Research and Infection Biology, which is the neighboring institute to the Leibniz

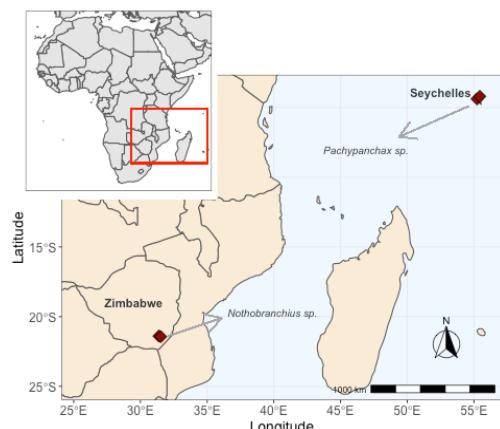


Figure 5. Location (red diamonds) of the main field research areas for the collection of microbial isolates associated with annual (*Nothobranchius* sp. in Zimbabwe) and non-annual (*Pachypanchax* sp. in Seychelles) killifish.

Institute of Aging, my primary affiliation. To collect microbial isolates from field localities in the Gonarezhou National Park in Zimbabwe and in the Biodiversity Conservation Centre in Seychelles, our team will collaborate with colleagues at local universities, such as our long-term collaborator Prof. Tamuka Nhlawatiwa at the University of Zimbabwe and Dr. Elvina Henriette at the University of Seychelles – as well as with local rangers and field assistants. Each vial will be labeled based on locality, fish ID, source of collection (e.g. gut, stool, skin), date of collection, and the wild isolates will be shipped to the Leibniz Institute on Aging for initial 16S rRNA sequencing and stocking. This collection will occur in three localities along the Guluene river drainage system in the Gonarezhou National Park in Zimbabwe⁷¹, which hosts the natural habitat of the annual turquoise and spotted killifish, as well as in the Mahe North and Praislin Islands in Seychelles, which host the natural habitat of the non-annual killifish golden panchax (*Pachypanchax playfairii*)⁴³ (**Figure 5**). From each locality, we will weekly sample fish over a period of 1 month each year.

To gain a more comprehensive catalog of microbial isolates for each locality/fish species throughout the years, we will sample on year 1, 2, and 3 of the proposal. The primary motivation for this work is to generate a comprehensive microbial collection that reflects ecology and anatomy across African killifishes (annual vs. non-annual). This unique resource will be key to functionally ask what are the mechanistic bases for host-microbiome metabolic crosstalk and what are the biological determinants for species-specific microbial assemblies.

Problems/solutions: Since not all microbial isolates will be amenable to isolation and culturing in the field, we will prioritize the culturing efforts to the most abundant or easily culturable microbes.

Aim 2: Screening killifish-specific antimicrobials against microbial isolates

This aim addresses the contribution of species-specific antimicrobials to shaping host-specific microbial communities. Highly specialized host anatomical structures – such as conspicuous fermenting organs in insects, birds and mammals – create optimal physicochemical conditions for the growth of unique microbial communities. Specialized social behaviors, such as lactation, coprophagy or trophallaxis, enable the transmissions of pre-defined microbial communities – often symbionts – that can benefit the host. Furthermore, the synthesis of antimicrobials can shape host-associated microbial communities (also in fish) by limiting proliferation in subsets of microbes, including pathogens^{72,73}. Does genetic diversity in antimicrobials across hosts affect diversity in host-associated microbial communities? Work done in ants⁷⁴ shows degrees of sequence divergence in the same classes of antimicrobial peptides among related ant species. However, we know little of the extent to which antimicrobials (e.g. antimicrobial peptides) differ both in sequence and function among related host species and how these differences help shape species-specific microbial assemblies⁷⁵.

In Aim 2, we predict the sequence, synthesize and screen the function of antimicrobials encoded in killifish genomes. Tapping on the naturally-occurring diversity of antimicrobials across killifish phylogeny, we leverage on evolution's ingenuity in shaping antimicrobial function between and within killifish species to ultimately screen specificity in antimicrobial functions against a set of microbes (bacteria and fungi) associated with killifish in nature and in captivity.

2.1 Comparative antimicrobial diversity across African killifish phylogeny

Rationale: Our research group has previously generated the genome sequences for over 41 killifish species, as well as the full genome assembly and annotation of five killifish genomes^{38,41,43,71}. These resources represent the basis to bioinformatically identify antimicrobial peptides across killifish. We will employ a combination of standard bioinformatic approaches to identify the sequence of predicted **antimicrobial peptides (AMPs)**. Using *ampir*⁷⁶, we could already identify from the 35247 protein sequences available in Uniprot from the short-lived killifish *Nothobranchius furzeri*, 189 predicted AMPs with 80% prediction probability and 114 with 90% prediction probability. Among the predicted peptides, we could identify two lysozymes, as well as uncharacterized proteins with sequence similarity (based on BLAST) to endocytosis-related proteins and short neurotoxin-like proteins. We will combine initial filtering from *ampir* to prioritize AMPs based on the prediction scores obtained from additional tools, such as iAMPpred⁷⁷, AmPEP⁷⁸ and others. After generating an extensive list of predicted AMPs across all the available killifish genomes, we will study phylogenetic diversification of AMPs among all the re-sequenced (N = 41) and fully assembled (N=5) killifish genomes, studying gene family size expansion/contraction⁷⁹, degree of purifying positive and balancing selection, as well as relaxation of selective constraints^{36,38,80}. To investigate gene expression for the detected AMPs, we will combine available RNASeq data from turquoise killifish intestine¹⁶ and skin⁸¹ with newly generate intestinal and skin transcriptomes in three additional killifish species, i.e. *Nothobranchius orthonotus*, *rachovii*, and *Pachypanchax playfairii*. In these four species, for which we have already extensive population-level genomic information, we will further assess intra-species antimicrobial peptide sequence variability, to directly test for branch-specific positive/negative-selection or neutral evolution in any of the predicted AMPs. To this end, we will employ a range of population-genetics methods, which include Tajima's D and the asymptotic Mc-Donald Kreitman test, which will enable us to assess the density distribution of AMP-specific alleles throughout a range of derived allele frequencies (from low to fixed)⁸².

We will further extend this analysis to the sequences of already known and conserved AMPs, which we could already identify and annotate across killifish genomes. These conserved-function genes include RAG2, BPI, LEAP2, as well as several defensins, including BD5 and many more. The study of AMP diversification across killifish will further enable to directly test whether ecological adaptations, e.g. to annual vs. non-annual environments across killifish, corresponds to specific adaptations in repertoires of antimicrobials.

Problems/solutions: This aim is relatively low risk and rather straightforward; however, the identification of killifish-wide antimicrobials is essential for the next step, which consists in testing antimicrobial function.

2.2 Screening antimicrobial function across killifish-associated microbial isolates

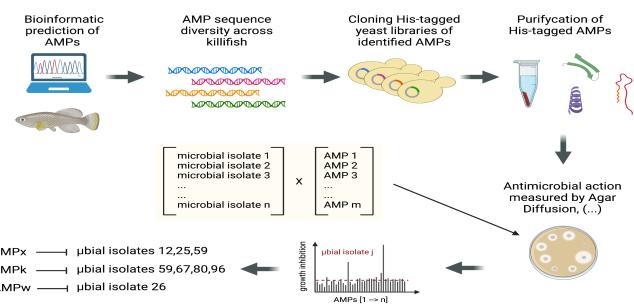
Rationale: Inhibiting growth in targeted microbes can directly favor the host, for instance by excluding pathogens. However, selective microbial inhibition could also result in large-scale remodeling of the microbial consortia. For instance, in the thale cress (*Arabidopsis thaliana*), the microbial community of the leaf is shaped by fungi and oomycetes, which act as **hub species**, establishing complex networks across microbial species, favoring some and inhibiting others⁸³. Hence, targeting a few key microbial taxa (e.g. the hub species) with targeted antimicrobials, can have large-scale impacts on the microbial community associated with the host. To investigate the extent to which genetic variation across hosts could impact the composition of host-specific microbial consortia, we seek to investigate how killifish-encoded antimicrobials modulate growth and survival in microbial isolates from field and laboratory killifish, as well as in a selection of representative microbes from unrelated host species (e.g. plants, mice, humans). We will ask both what is the specificity of different antimicrobials against specific microbial isolates, as well as whether the action of the same antimicrobial changes as a function of single amino acid variation across closely related killifish species. To test the antimicrobial action of the set of antimicrobials obtained in Aim 2.1, we will follow a procedure that involves cloning the full length of each predicted AMP into a His-tagged *Saccharomyces cerevisiae* expression system, allowing for the synthesis of the recombinant peptide, which is then purified using a His-tag purification system employing magnetic beads. The use of yeast instead of *E. coli* enables the inclusion of PTMs (post-translational modifications) in AMP synthesis. The synthesis of all the AMPs (library size of ~10^2 sequences, hence doable by a technician with the help of a student over a 6 months period) will then be followed by screening assays, including classical Agar Diffusion methods⁸⁴, which allow direct testing of microbial growth in the presence of varying concentrations of the AMP (Figure 6). For each AMP-microbe test, we will additionally measure growth with standard OD₆₀₀ measures from liquid cultures using a microplate reader. Together, these tests will help assess the specificity of action of different antimicrobials to inhibit the growth in all the cultured microbial isolates (from Aim 1.2). Our premises at the Leibniz Institute on Aging will enable us to screen the antimicrobial action both in aerobic, as well as anaerobic culturing conditions. This pipeline will lead to the identification of AMP-to-isolate specificity for growth inhibition. Additionally, this same assay will answer the question of whether amino acid variation in the same AMP across related killifish species results in differential growth inhibition across microbial isolates. To ask whether killifish-encoded AMPs are specific towards microbial isolates spanning killifish phylogeny/ecology, we will extend our assay to a limited subset of “control” microbial isolates from distant species, such as plants (e.g. thale cress), insects (e.g. *Drosophila sp.*, in collaboration with the Max Planck Institute for Chemical Ecology), mice (isolated from the FLI mouse facility) and humans (from BIO-ML⁸⁵). This innovative in vitro screening platform will provide a system to translate AMP genetic variation across killifish taxonomy and ecology into a phenotypic output represented by microbial growth inhibition.

Problems/solutions: We have established the cloning and purification procedure for a subset of fish proteases (e.g. for the low choriolytic enzyme, LCE), which pave the way to scaling this protocol up for a larger set of AMPs. A potential challenging aspect of this experiment comes from the fact that the synthesis of a subset of AMPs in *S. cerevisiae* might negatively impact yeast’s growth/survival, especially for anti-fungal AMPs. To address this potential limitation, we will then outsource the synthesis of a subset of AMPs to commercial partners (e.g. ProteoGenics ®).

Aim 3: Functional dissection of host genes that help shape microbial consortia

What host genes affect the acquisition and maintenance of species-specific microbiota? To evaluate the contribution of host genetics in shaping microbiota, we will combine QTL mapping with genome editing via CRISPR/Cas9, leveraging the accessibility of turquoise killifish to rapid and efficient genome editing⁴⁷. We will extensively investigate the in vivo role of genes involved in mucosal innate and adaptive immune function into microbiota acquisition and maintenance in killifish. Then, we will use a co-culture system to assay in vitro how host genes expressed in immune cells affect microbial proliferation.

Figure 6. Screening the antimicrobial activity of antimicrobial peptides identified across African killifish genomes against killifish-associated microbial isolates.



3.1 QTL mapping of genes associated with host-specific microbial communities

Rationale: That microbiota composition is an individual-specific and species-specific mappable trait, has been shown in microbiota mapping efforts pursued in particular in mice, which have revealed a complex genetic architecture, often related to host metabolic functions⁸⁶⁻⁸⁸, as well as antimicrobial pathways⁸⁹. Mapping microbiota composition across killifish species will give us a direct tool for rapidly testing how individual host genes affect microbiota composition (Aim 3.4). In coral fish, the community structure of fish skin microbiota has been shown to correlate in part with host phylogenetic relatedness²¹. We recently found that different killifish species (turquoise and spotted killifish) living in the same environment and with largely overlapping diets show distinct intestinal microbiota (**Figure 2**). To investigate the genetic substrate underlying gut microbiota differences among these two species, we generated a pilot laboratory cross. The F1 generation is largely incompatible to intercrossing, as they generate embryos unable to hatch or fry that die soon after hatching. However, backcrosses between F1 fish and individuals from either species is successful, enabling mapping of the loci segregating with individual microbiota composition in the F2 backcrossed generation. We will sample gut and skin from the F2 backcross generation at 2 months of age, i.e. when individuals from either species are reproductively active. We will raise over 400 F2 backcross fish to map the QTLs that explain the largest amount of phenotypic variance in microbial diversity, using all diversity indexes in the Hill's diversity spectrum for microbial diversity⁹⁰. From each individual/tissue, we will generate 16S rRNA amplicons for the V4 segment, uniquely tag each individual DNA library, multiplex and sequence them using the HiSeq Illumina platform. For the same individuals, we will extract genomic DNA and perform multiplex shotgun genotyping (MSG)⁹¹, which enables genotyping a large number of individuals for QTL mapping. We will adopt the R/qtl and pLink softwares to perform mapping, as well as custom python scripts, following a similar approach to the one followed by our group in the past^{41,49}. Importantly, we have already generated genome assemblies and annotations for both these species^{38,41}, which will help better identify the genes and genomic regions underlying each QTL (Aim 3.4). This work will be instrumental to map species-specific loci associated to differences in microbiota composition.

Genes involved in immune or metabolic function can be likely underlying genomic loci associated with species-specific microbiota differences. It is also possible that genes that control body size, intestine length and other morphological/anatomical traits may be associated with differences in microbiota between species. For instance, longer/shorter intestines or differences in compartmentalization of the intestine may be affecting microbiota composition, e.g. due to differences in oxygen concentration or pH. Hence, at the time of sample collection, we will also measure body length, morphology, total fish weigh, intestine size and shape. This information will enable us to associate microbiota composition with morphology, quantified using geometric morphometrics⁹².

Problems/solutions: *If mapping microbiota composition will result prohibitive due to very large trait complexity, hence a large number of QTL explain a small portion of the phenotypic variance, we will focus on mapping the genomic regions associated with specific bacterial taxa that are largely dissimilar between the two species. If the cross yields a limited number of F2 backcross individuals, insufficient for mapping, we will generate a cross between two closer-related species Nothobranchius furzeri and Nothobranchius kadleci, which have previously shown to be compatible for the generation of viable F2 offspring⁹³.*

The development of genome editing technologies in killifish^{46,47} provides us with a tool to directly test the phenotypic importance of a set of candidate genes in shaping the commensal microbial community in killifish. However, we will be able to test the *in vivo* effect of the candidate genes identified in Aim 3.1 only after the genetic mapping is done (Aim 3.4).

3.2 Genome editing of innate immune genes

Rationale: Innate immune function sets the first line of defense against pathogens as it is able to deploy rapid and efficient responses (e.g. inflammation) against external insults. Through the activation of special classes of extra- and intra-cellular receptors (e.g. TLRs, NLRs, etc.), it generates automatic responses to a known set of external stimuli, such as nucleic acids, bacterial proteins, endotoxin, etc. For hosts to entertain beneficial interactions with their microbial partners, it is necessary to dial down pro-inflammatory responses and establish a more permissive immune environment to microbial communities. The expression of immune genes importantly covaries with gut microbiome composition in sticklebacks⁹⁴. Whether innate immunity contributes to establishing commensal, species-specific microbiota is an important open question⁹⁵. Using a technology routinely used in our lab (see **Figure 7**), we will generate killifish lines that lack major innate immune effectors, such as MyD88 and IRF8, both of which are present and annotated in the turquoise killifish genome. MyD88 is downstream to several TLRs, integrating signals from different microbial epitopes, and its dysfunctions in humans are associated with susceptibility to bacterial infections⁹⁶. IRF8 is a

transcription factor with an important role in myeloid cell differentiation into monocytes⁹⁷. Defects in the human IRF8 lead to immunodeficiencies, including increased susceptibility to mycobacterial infections⁹⁸. We will test whether the lack of either of these two innate immune effectors leads to imbalanced microbiota in both gut and skin, compared to wild-type fish. Whether any of the microbiota-QTL identified in Aim 3.1 maps in proximity to the genomic regions containing MyD88, IRF8 or any of the direct interaction partners, and if the mapping resolution is sufficient, we will use BAC transgenesis (our lab has acquired the turquoise killifish BAC library) to integrate the locus of interest in the turquoise killifish genome^{48,99}. We will then assess whether fish carrying this construct have a different microbiota from wild-type fish.

Killifish bacterial microbiome is dominated by bacteria of the genus *Vibrio* (Figure 4), flagellated Proteobacteria that can be toxicogenic or non-toxicogenic and that are generally highly abundant among aquatic species. Since TLR5 (Toll-Like Receptor 5) specifically senses the flagellar protein flagellin, to test whether lack of TLR5 leads to altered microbiota composition, we will generate knock-out killifish lines using CRISPR/Cas9.

Innate immune responses play also important modulatory roles for the adaptive immune system. PIGR (Polymeric Immuno-Globulin Receptor) is a transmembrane Fc receptor that binds to antigen-bound polymeric secreted antibodies (IgAs in mammals) on the surface of mucosal epithelia, facilitating effector responses^{100,101}. To test the potential role of innate-immune modulation of adaptive immune function in shaping microbiota composition, we will generate homozygous knock-out PIGR turquoise killifish and analyze changes in their gut and skin microbiota.

In all transgenic and edited killifish lines, we will employ shotgun metagenomics in gut and skin to identify differences in microbiota between knock-out/transgenic and wild-type killifish. Additionally, we will measure growth rate, body size at sexual maturity, as well as survivorship and serum metabolomics. Transgenesis and genome editing in killifish for candidate innate immune genes will help us answer what is the role of this ancient and powerful line of defense in shaping host microbiota.

3.3 Genome editing in genes involved in adaptive immune function

Rationale: Lymphocyte-based adaptive immune function enables vertebrates to mount targeted immune responses against rapidly evolving and vastly heterogeneous pathogens. The adaptive immune system is also capable of establishing immune tolerance (e.g. against the self). However, its role in shaping commensal microbial consortia is less well understood. Antibodies and T cell receptors recognize a large number of antigens/epitopes, triggering downstream responses that range from targeted defense to tolerance. Both these mechanisms can be the key to establishing and maintaining a commensal microbiota, while protecting oneself from pathogens¹⁰¹.

Hence, in genes that are key to adaptive immune function there may lay part of the mechanisms through which hosts actively modulate their microbiota. The innovation of adaptive immune function has been suggested to have been a key transition that allowed vertebrates maintain a complex commensal gut microbial community, contributing to vertebrate phenotypic/species radiation¹⁰². To directly investigate the connection between adaptive immune function and commensal microbiota selection by the host, we will study microbiome composition in a series of killifish lines that have genetically altered adaptive immune function. First, we will sequence by 16S rRNA amplicon sequencing the gut and skin microbiome at different life stages in fish lacking Rag1 function. Rag1 is involved in VDJ recombination in B and T cells, enabling lymphocyte functional maturation. Using CRISPR/Cas9, we already generated a homozygous *Rag1*^{-/-} turquoise killifish line, which shows a lack of mature lymphocytes and remarkably displays normal lifespan under laboratory conditions (Figure 7). Recent work from our group, where we characterized the evolution of the IgH locus in teleost fish, led us to discover that throughout the killifish lineage, the fish-specific mucosal antibody class IgZ has undergone rapid evolution and has been lost in both annual and non-annual species of the eastern African killifish clade¹⁰³.

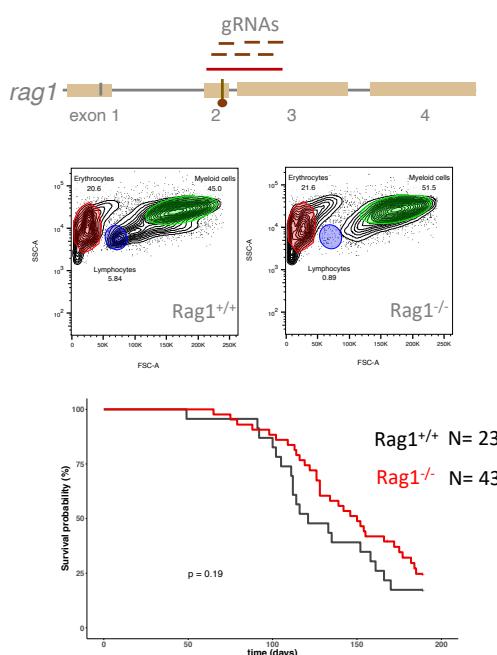


Figure 7. Rag1 knock-out killifish line. Using CRISPR/Cas9, we generated a killifish line lacking a functional *Rag1* gene. Mature lymphocytes are significantly depleted (middle plots). *Rag1*^{-/-} fish show similar lifespan to wild-type killifish (bottom plot).

(**Figure 3**). We ignore the phenotypic consequences of the loss of this mucosal antibody class. We will explore whether the loss of this mucosal antibody isoform leads to a change in microbiota composition. This will be done by knocking out the constant region of the IgZ locus in the annual killifish *Callopanchax toddi* from the western African killifish clade, which has intact IgZ, and for which we have generated an assembled genome and can readily raise in the lab³⁸. Unlike IgZ, IgD is a highly conserved antibody class, maintained from fish to mammals. However, its physiological function still remains elusive. IgD has been recently suggested to enhance mucosal homeostasis and to contribute to aspects of microbiota-host interaction¹⁰⁴. We will explore the impact of this highly conserved class of antibody in shaping the microbiota, by knocking it out via CRISPR/Cas9 in turquoise killifish. Once we have established a homozygous IgD^{-/-} killifish line, we will sequence their microbiome composition via 16S rRNA amplicon sequencing in intestine content and skin. Similarly, we will target via CRISPR/Cas9 also IgM, the main class of teleost mucosal antibody¹⁰³. These experiments will help us answer whether different branches of adaptive immune function lead to enrichment/depletion of specific components of the microbiota. However, if the lack of specific classes of mucosal antibodies does not affect the microbiota composition, we will study the temporal dynamics of microbiota composition during killifish life in wild-type and knock-out lines. If any of these knock-out lines will have a significantly altered microbiota, we will then generate reporter lines to image timing and localization of expression of each candidate gene. To generate reporter lines, we will knock-in a fluorescent marker (GFP) via CRISPR/Cas9, following a method already established in killifish^{46,47}.

We expect knock-out lines for immune genes to have systemic effects that may go beyond microbes-host dynamics. Hence, changes in microbiome composition in any knock-out fish line may directly or indirectly be a consequence of the individual gene function. To connect gene function with changes in microbiome, we will use a broad set of phenotypic measures for each knock-out line showing an altered microbiome. These include serum metabolomics, growth rate, fertility, body size and survivorship. If any of the knock-out lines will show dramatically altered microbiome composition, i.e. loss of microbial richness and prevalence of specific microbial taxa (e.g. pathogenic *Vibrio*), we will perform single-cell RNA Sequencing (scRNA Seq) in gut or skin, depending on the organ displaying most microbiome changes in knock-out lines, to investigate overall changes in host cell composition and function (**Figure 9**). To perform scRNA Seq, we will employ a scalable, low-cost, high-throughput combinatorial indexing platform that we have been developing in the past year in our laboratory, inspired by the work of Cole Trapnell and Jay Shendure¹⁰⁵.

Problems/solutions: If knock-out killifish lines for *Rag1*, IgZ and IgD and IgM will not show changes in the microbiome, compared to wild-type killifish, we will study the responses of the gut microbiome to pathogen challenges in wild-type and knock-out killifish lines. To this end, we will expose killifish to inactivated *Vibrio anguillarum* and to extracts from the fish parasitic nematode *Anisakis simplex*, following a protocol already used in zebrafish¹⁰⁶. To track microbiome responses to infection, we will sequence fish stool before and after pathogen exposure. These experiments will tell us whether maintenance (rather than establishment) of a constant microbial community in response to pathogenic insult depends on IgM/Z/D function.

3.4 Genome editing of genes identified during the antimicrobial assay and the QTL study

Rationale: The antimicrobial assay (Aim 2.2) and the QTL study (Aim 3.1) will provide a list of candidate genes and genomic regions that will help functionally connect microbiota composition with host genomics. We will focus on a subset of genes based on novelty and conservation, as well as relevance with immune function. For each of these candidate genes, we will compare gene sequence among the three annual (*Nothobranchius furzeri*, *Nothobranchius orthonotus* and *Callopanchax toddi*) and the two non-annual (*Aphyosemion australe* and *Pachypanchax playfairii*) killifish species to identify coding and non-coding variants that differ among them. Genes that are associated with differential microbial responses (Aim 2.2) and that have unique sequence variation among killifish, will then be targets for genome editing via CRISPR/Cas9. Following an approach similar to Aim 3.2-3, we will study whether altering the candidate gene function results in changes in the species-specific microbiome and in other phenotypes, including serum metabolites, growth rate, body size and survivorship. These experiments will help assess the phenotypic relevance of genes implicated in establishing a species-specific microbiome.

3.5 An in vitro assay for cell-microbe interaction

Rationale: To test direct interactions between host cells and host-associated microbial consortia, our lab recently developed an in vitro assay that assesses bacterial growth when co-cultured with immune cells from different sources. We engineered the killifish gut resident microbe *Pseudomonas lundensis* to constitutively express GFP, which we knocked in within the essential *glmS* gene, taking advantage of the *Tn7* transposition machinery¹⁰⁷, in collaboration with Prof. Karen Guillemin's group at University of Oregon, whose group

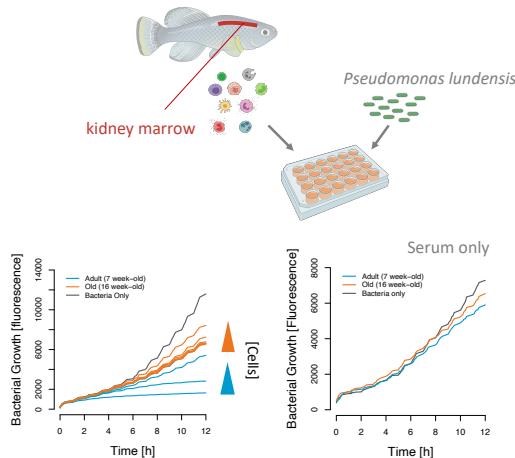


Figure 8. Assaying the influence of immune cell on bacterial growth. Immune cells from the kidney marrow (the fish main hematopoietic organ) are co-cultured with labelled bacteria from the stool microbiota. Immune cells from young donors inhibit bacterial growth more than cells from old donors.

consider them as new candidates for transgenesis/genome editing in the whole fish (Aim 3.2-3-4). Beyond *Pseudomonas lundensis*, we will engineer additional microbial species, prioritizing two classes of microbes: those that are equally abundant across killifish species, and those that are associated with individual killifish species, based on the results of Aim 1.2.

Problems/solutions: Individual microbes do not represent the complexity of the microbiota associated with the intestinal or the skin epithelia. We shall therefore develop further the host cell-microbiota co-culture system, increasing complexity in the cultured microbiota by taking advantage of the technological advancements achieved in culturomics¹⁰⁸.

Aim 4: Microbial influence on host phenotype variation

Our ultimate goal is to understand how microbiota affect phenotype variation across hosts (red arrow, **Figure 1**). To tackle this challenging task, we will expose different killifish species to the same microbiota input and assess a range of phenotypic responses.

4.1 Species-wide phenotypic response to the same microbial input

Rationale: Disentangling how microbiota affect host phenotypes can be confounded by host genetics and environmental microbiota (**Figure 1**). We will control both variables as follows. We will expose four different killifish species (two annuals and two non-annuals) to two different microbiota sources, one from seasonal water pools (annual environments) and one from permanent water habitats (non-annual environment). To prime their gut microbiota, we will hatch fish in a microbe-rich solution containing substrate collected from either annual or non-annual environments, mimicking natural hatching conditions (**Figure 10**). As control, we will also hatch fish in sterile autoclaved tank water, following killifish husbandry standards⁴⁴. To note, killifish fry hatch with an open mouth (unlike zebrafish), therefore they start acquiring their gut microbiota immediately at birth, similar to mammals⁴⁴. We will maintain fry in fresh, microbe-rich substrate, for the first week of life, before transferring them to a centralized water recirculation system, where they will be individually housed in 2.8L tanks and fed sterile commercial pellet food. For each of the four killifish species, we will analyze a set of phenotypes, including growth rate, size at sexual maturity (indicated by the appearance of male sexual coloration), fecundity (measured as eggs produced per female) and lifespan. We will collect stool at regular intervals throughout individual fish life, starting at week four, then every other week, in order to trace microbiota composition in individual fish over time. We will then generate a reference environmental microbiome by sequencing the sediment microbiome using

developed this method. We assayed bacterial growth based on fluorescence using a plate reader (**Figure 8**). We will assay whether immune cells extracted from different fish species, e.g. the short-lived annual turquoise killifish vs. the long-lived non-annual killifish *Pachypanchax playfairii*, have similar impact on bacterial growth. We will further test the impact on microbial growth in immune cells extracted from young, adult and old fish donor. Whenever bacterial growth changes as a function of the immune cell source, we will run a battery of assays, including single-cell RNA Seq (**Figure 9**), metabolomics, proteomics, as well as in vivo imaging via holotomography (e.g. using Nanolive® to visualize engulfment or killing) in the cultured cells. If any candidate host immune gene, pathway or cellular mechanism will emerge as significantly associated with altered bacterial proliferation in vitro, we will transfet immune cells to achieve functional rescue and target candidate pathways via CRISPR/Cas9 technology, a method that we have developed in cultured killifish skin cells using the X-tremeGENE™ 360 transfection system. Whenever individual host genes or pathways are validated to affect bacterial growth in our in vitro system, we will

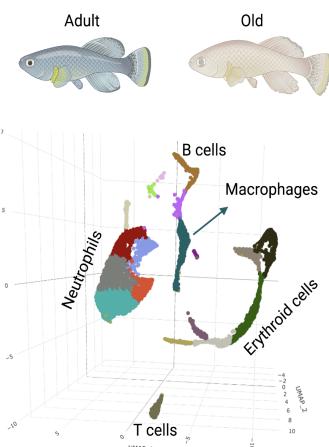


Figure 9. Single-cell RNA Seq in kidney marrow cells from young and old turquoise killifish reveals the main killifish immune cell types.

shotgun metagenomics and 16S rRNA amplicon sequencing. These datasets will enable us to answer several important questions. First, we will know whether each killifish species establishes its own microbiota even though they are exposed to a common source. Second, if species do not show significantly diverging gut microbiota – i.e. their between-species microbiome diversity falls within the within-species microbiota diversity – we will ask whether the scored phenotypes vary between control fish raised in normal laboratory conditions, and those exposed to a microbial-rich hatching environment. Since host genetics is kept constant, phenotype differences between control and microbial-rich conditions will be a direct measure of microbiota influence on host phenotypes. If microbiota interventions will lead to phenotypic changes in a species-specific manner, we will explore further the mechanistic connection between microbiota composition and host phenotypes by performing analysis of the fish metabolome, proteome, as well transcriptome in the intestine and in the primary immune organs (kidney marrow) between the control fish raised under normal laboratory conditions, and those exposed to the microbial-rich hatching environment. Deep phenotyping will provide us with candidate host genes that connect microbiota composition and function with host phenotypes. Strong candidate genes involved in immune/metabolic pathways, or in proximity with the microbiome QTLs, will be tested by editing the killifish genome using CRISPR/CAS9 (Aim 3).

Problems/solutions: *If exposure to a microbial-rich hatching solution will result in harm to fry development, we will dilute it until fish reach sexual maturation at the same time as control laboratory-raised fish. If, on the contrary, microbial-rich hatching medium will not affect microbiota composition compared to normal laboratory conditions, we will develop germ-free killifish culturing conditions, following a strategy developed in zebrafish^{109,110}. Germ-free raised killifish will enable us to study how individual members of the microbiota contribute to fry health and growth capacity. However, we do acknowledge that germ-free culturing conditions in fish is still a technology under development and that there may be challenges in raising gnotobiotic killifish to adulthood. In this case, we will focus on manipulating the microbiota in the early stages of killifish development.*

4.2 Killifish-specific microbial assemblies in a pseudo-natural killifish biotope

Rationale: To address how different killifish species assemble their own microbial communities from shared available microbial sources in an environment that mimics natural conditions, we will take advantage of the unique **killifish natural biotope** that our group is setting up at the FLI in Jena (see enclosed support document, Annex 2). This biotope consists of a series of artificial pools of ~2200 liters that recapitulate the killifish natural habitat, including changes in lighting and daily temperature. In each pool, we will house killifish multi-species communities (e.g. the annual *Nothobranchius furzeri* and the non-annual *Pachypanchax playfairii* in common garden), spiked with unique starting microbial communities, obtained from soil sediment and water samples from natural killifish habitats that we have in part already collected from the Gonarezhou National Park in Zimbabwe (**Figure 10**). Each artificial pond, seeded with soil containing microbes from annual and non-annual killifish environments, respectively, will allow studying individual species time series in gut and stool microbiome throughout life. We will mark-recapture individual fish throughout their life using elastomere tags¹¹¹, measure fish growth and survivorship, and collect stool upon fish isolation in individual tanks. We will periodically sample the water microbiome. This unique setup will be key to assessing individual fish phenotype responses (including microbiome composition) to defined ecological conditions and will help clarify whether same microbial input communities impact phenotype variation across different hosts in a species-specific manner.

Aim 5: Physiologic response to immunizations against members of the microbiota

Rationale: Artificially selecting the microbial composition of complex microbiota associated with a given host would allow testing the phenotypic response of individual members of the microbiota. While we cannot yet culture individually all microbes present in a complex microbiota and then piecewise “assemble” artificial microbial communities, we will leverage the intrinsic capacity of the host immune system to target specific microbes, to actively shape microbiota.

As recently shown by our group, killifish, similarly to other teleosts, are fully equipped with an adaptive immune system capable of responding to a highly diverse set of antigenic challenges¹⁰³, and their **B cell receptor repertoire diversity** undergoes major changes during aging¹¹². We recently confirmed that turquoise killifish undergo canonical VDJ recombination at the **T cell receptors locus**, and express



Figure 10. Sediment of annual killifish ponds from the Gonarezhou National Park in Zimbabwe as appears at the end of the wet season, when water evaporates and killifish survive as dormant embryos in the substrate.

recombined and functional T cell receptors, which are expected to play key roles in adaptive immune responses. Harnessing the extensive technical knowledge acquired in the field of aquaculture¹¹³⁻¹¹⁵, we will immunize fish against specific microbes as a means to modulate their microbiota composition. Our ultimate goal is to inject fish with inactivated cultured bacteria and a proper adjuvant, largely following protocols used in the fish industry¹¹⁴. We will carry out this aim in collaboration with GSK Vaccine, which is a world-leader in the development of commercial vaccines and adjuvants and which will provide us with guidance and technical assistance (see support letter in Annex 1). For this aim, we will focus specifically on bacteria, because they are the most abundant members of killifish microbiota – based on our metagenomics results (**Figure 4**) – and because we have already gained extensive experience in isolating and culturing them from fish intestine and stool. Previous work from our group has shown that the genera *Propionibacterium*, *Delftia* and *Citrobacter* are stably more abundant in old vs. young killifish intestines¹⁶. Based on these results and on the results from Aim 1.2, we will vaccinate turquoise killifish (*Nothobranchius furzeri*) – i.e. our most used laboratory killifish model – against bacteria that consistently appear to become more abundant at late age. We will vaccinate fish at 4-6 weeks of age, using heat inactivated bacteria injected intramuscularly. Vaccinating killifish earlier than 4 weeks of age may result not effective as B cell maturation in killifish occurs around 3-4 weeks of age. Vaccine dosage will depend on fish responses in terms of survival after vaccination (e.g. > 95% survival within a week from vaccination) and will start from 10 µg/g of fish body weight following standard protocols in the field¹¹⁶. After vaccination, we will test whether vaccinated fish present different abundance of the target bacterium compared to sham-vaccinated fish. We will collect gut and stool samples every 3 weeks post vaccination. We will use Illumina MiSeq to cover the V3-V4 regions of the 16S rRNA locus to sequence microbiota from gut samples; while we will employ shotgun metagenomics using the Illumina HiSeq technology in stool samples, which are enriched for microbial DNA over host DNA (**Figure 4**). This approach will help answer whether preventing the age-dependent increased titer of a subset of aging-associated bacteria has systemic effects, such as changes in body size, fecundity and population lifespan. To link microbial exclusion to host-specific phenotypes, we will extend this **vaccination-guided commensal microbial exclusion** approach to the non-annual species golden panchax. If this vaccination approach turns out to be effective, i.e. vaccinated fish develop a long-term microbial-antigen-specific antibody response, we will extend it to a selection of annual and non-annual killifish species, generating fish cohorts where subsets of host-associated microbial isolates are specifically used to achieve immunizations. This approach will be key to addressing the fundamental question of what is the impact of individual or collective microbial consortia in modulating host phenotypes. Overall, our approach will open novel strategies to actively manipulate host microbiota and ultimately affect host phenotypes. In future work, we will extend our strategy to train the host immune system against a broader range of microbes, which also includes archaea, viruses and fungi, and engineer individual microbes to ultimately modulate the host phenotypic spectrum.

Problems/solutions: *If we won't be successful in isolating and culturing target bacteria (Aim 1.2), such as *Propionibacterium*, *Delftia* and *Citrobacter*, we will obtain these bacteria from third parties and heat inactivate them in our laboratory. If heat-inactivated bacteria won't achieve robust immune responses, we will strategize with our partner GSK-vaccines to develop an mRNA-vaccine strategy¹¹⁴.*

Risk assessment and outlook:

MICROPHENO goes beyond the current state-of-the-art in research in host-microbiota interactions. This ambitious project lays out a comprehensive program that generates resources and develops novel technologies to study how the acquisition and maintenance of host-specific microbial communities affect species-specific phenotypes. This proposal taps on my group's expertise in developing novel tools in killifish and combines relatively lower risk Aims (Aims 1, 3 and 4) with high-risk Aims (Aim 2, and 5). Meeting the high-risk/high gain profile supported by ERC, Aim 2 could lead to the development of a disruptive and versatile screening system to test the role of host-specific antimicrobials in affecting growth in host-associated microbes. To mitigate the chance that the yeast-based cloning approach may be intrinsically limited in the capacity to screen for AMPs, we will collaborate with commercial partners for the synthesis of custom peptides, based on our novel AMP list. Collection of naturally-occurring microbial isolates, as well as testing of host-derived antimicrobials, will greatly benefit from our partners and neighbors at the Hans Knoell Institute for Natural Product Research and Infection Biology (HKI) in Jena, which host the **Jena Microbial Research Collection (JMRC)**.

Generating transgenic killifish lines and testing their phenotypic spectrum (Aim 3) will be possible thanks to the unique infrastructures at the Leibniz Institute on Aging in Jena (FLI), which – to my knowledge – has the largest killifish facility in the world. My group has access to an extensive fish facility with several thousand tanks with a centralized water filtration/recirculating system. Additional to this infrastructure that will be

crucial for Aims 1, 3 and 4, the recent approval to build a **natural killifish biotope** at the FLI (see enclosed statement in Annex 2), which reproduces culturing conditions similar to the natural killifish habitat, will enable common garden experiments to assess the impact of a shared environmental microbiota on the phenotype spectrum of different host species. The development of an *in vitro* immune-microbiome co-culture system is innovative and potentially high-risk. However, we have already established labelled microbial cultures that will help screen how genetic manipulations in host immune cells affect microbial proliferation. To mitigate the limited numbers of immune cells extracted from each fish, we will pool immune cells from different killifish donors and additionally use *Nothobranchius ocellatus* as a source of immune cells, i.e. the largest-known annual killifish species, which can reach a size of ~18 cm, as opposed to ~5-6 cm of turquoise killifish. We expect that *N. ocellatus* will lead to 3X as many immune cells per age-group than other smaller-sized killifish.

I am very excited about the most ambitious and potentially groundbreaking part of this proposal: Aim 5. If successful, this aim has the potential to help establish a new strategy for selective microbial exclusion via the involvement of adaptive immunity and could have important implications for actively engineering hosts microbiota. Additionally, the results of Aim 5 could generate knowledge and technology useful to devise strategies to help patients or livestock to protect themselves from the health-related risks of age-dependent dysbiosis.

This project will generate a large amount of data as all the proposed phenotyping strategies involve extensive use of “omics” technologies. The computational and storage requirements will be mitigated by the presence of two core facilities at the FLI in Jena, the IT department, which will provide support for data storage and computational hardware infrastructures, as well as the Life Science Computing, which will work in close proximity with us to adopt and develop state-of-the-art computational strategies to meet our analytical demands. The FLI in Jena has a sequencing core facility, with state-of-the-art sequencing platform (Illumina NovaSeq6000, Illumina MiSeq, Oxford Nanopore MinION, etc.), which we will actively involve in all our sequencing projects.

Timeplan:

Table 1: Time plan for experiments described

| Experiment | Year 1 | Year 2 | Year 3 | Year 4 | Year 5 |
|---|--------|--------|--------|--------|--------|
| Aim 1 | | | | | |
| 1) metagenomics across killifish phylogeny | | | | | |
| 2) isolation of natural microbial isolates | | | | | |
| Aim 2 | | | | | |
| 1) Bioinformatic AMP prediction | | | | | |
| 2) Screening AMPs <i>in vitro</i> | | | | | |
| Aim 3 | | | | | |
| 1) QTL mapping | | | | | |
| 2) genome editing of innate immunity genes | | | | | |
| 3) genome editing of adaptive immunity genes | | | | | |
| 4) genome editing of genes from QTL | | | | | |
| 5) assay for host cells-microbiota interactions | | | | | |
| Aim 4 | | | | | |
| 1) microbiota transfers - phenotyping | | | | | |
| 2) common garden experiment - phenotyping | | | | | |
| Aim 5 | | | | | |
| 1) microbial vaccines | | | | | |

Reference

- 1 Garrido-Oter, R. *et al.* Modular Traits of the Rhizobiales Root Microbiota and Their Evolutionary Relationship with Symbiotic Rhizobia. *Cell Host Microbe* **24**, 155-167 e155, doi:10.1016/j.chom.2018.06.006 (2018).
- 2 Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P. & Forano, E. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* **3**, 289-306, doi:10.4161/gmic.19897 (2012).
- 3 Ridlon, J. M., Harris, S. C., Bhowmik, S., Kang, D. J. & Hylemon, P. B. Consequences of bile salt biotransformations by intestinal bacteria. *Gut Microbes* **7**, 22-39, doi:10.1080/19490976.2015.1127483 (2016).
- 4 Koppel, N. & Balskus, E. P. Exploring and Understanding the Biochemical Diversity of the Human Microbiota. *Cell Chem Biol* **23**, 18-30, doi:10.1016/j.chembiol.2015.12.008 (2016).
- 5 Lin, L. & Zhang, J. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunol* **18**, 2, doi:10.1186/s12865-016-0187-3 (2017).
- 6 Yoon, M. Y., Lee, K. & Yoon, S. S. Protective role of gut commensal microbes against intestinal infections. *J Microbiol* **52**, 983-989, doi:10.1007/s12275-014-4655-2 (2014).
- 7 Tibbs, T. N., Lopez, L. R. & Arthur, J. C. The influence of the microbiota on immune development, chronic inflammation, and cancer in the context of aging. *Microb Cell* **6**, 324-334, doi:10.15698/mic2019.08.685 (2019).
- 8 Hill, M. J. Intestinal flora and endogenous vitamin synthesis. *Eur J Cancer Prev* **6 Suppl 1**, S43-45, doi:10.1097/00008469-199703001-00009 (1997).
- 9 Theis, K. R. *et al.* Symbiotic bacteria appear to mediate hyena social odors. *Proc Natl Acad Sci U S A* **110**, 19832-19837, doi:10.1073/pnas.1306477110 (2013).
- 10 Freed, L. L. *et al.* Characterization of the microbiome and bioluminescent symbionts across life stages of Ceratioid Anglerfishes of the Gulf of Mexico. *FEMS Microbiol Ecol* **95**, doi:10.1093/femsec/fiz146 (2019).
- 11 Wilkins, L. J., Monga, M. & Miller, A. W. Defining Dysbiosis for a Cluster of Chronic Diseases. *Sci Rep* **9**, 12918, doi:10.1038/s41598-019-49452-y (2019).
- 12 Ricci, F. *et al.* Beneath the surface: community assembly and functions of the coral skeleton microbiome. *Microbiome* **7**, 159, doi:10.1186/s40168-019-0762-y (2019).
- 13 Kamada, N., Chen, G. Y., Inohara, N. & Nunez, G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol* **14**, 685-690, doi:10.1038/ni.2608 (2013).
- 14 Bourne, D. G., Morrow, K. M. & Webster, N. S. Insights into the Coral Microbiome: Underpinning the Health and Resilience of Reef Ecosystems. *Annu Rev Microbiol* **70**, 317-340, doi:10.1146/annurev-micro-102215-095440 (2016).
- 15 Han, B. *et al.* Microbial Genetic Composition Tunes Host Longevity. *Cell* **173**, 1058, doi:10.1016/j.cell.2018.04.026 (2018).
- 16 Smith, P. *et al.* Regulation of life span by the gut microbiota in the short-lived African turquoise killifish. *eLife* **6**, doi:10.7554/eLife.27014 (2017).
- 17 Kundu, P., Blacher, E., Elinav, E. & Pettersson, S. Our Gut Microbiome: The Evolving Inner Self. *Cell* **171**, 1481-1493, doi:10.1016/j.cell.2017.11.024 (2017).
- 18 Seidel, J. & Valenzano, D. R. The role of the gut microbiome during host ageing. *F1000Res* **7**, doi:10.12688/f1000research.15121.1 (2018).
- 19 Boehme, M. *et al.* Microbiota from young mice counteracts selective age-associated behavioral deficits. *Nature Aging*, doi:10.1038/s43587-021-00093-9 (2021).
- 20 Lim, S. J. & Bordenstein, S. R. An introduction to phylosymbiosis. *Proc Biol Sci* **287**, 20192900, doi:10.1098/rspb.2019.2900 (2020).
- 21 Chiarello, M. *et al.* Skin microbiome of coral reef fish is highly variable and driven by host phylogeny and diet. *Microbiome* **6**, 147, doi:10.1186/s40168-018-0530-4 (2018).
- 22 Blasco-Costa, I., Hayward, A., Poulin, R. & Balbuena, J. A. Next-generation cophylogeny: unravelling eco-evolutionary processes. *Trends in Ecology & Evolution*, doi:10.1016/j.tree.2021.06.006.
- 23 Baldo, L. *et al.* Convergence of gut microbiotas in the adaptive radiations of African cichlid fishes. *ISME J* **11**, 1975-1987, doi:10.1038/ismej.2017.62 (2017).
- 24 Muegge, B. D. *et al.* Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* **332**, 970-974, doi:10.1126/science.1198719 (2011).
- 25 Spor, A., Koren, O. & Ley, R. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat Rev Microbiol* **9**, 279-290, doi:10.1038/nrmicro2540 (2011).
- 26 Tung, J. *et al.* Social networks predict gut microbiome composition in wild baboons. *eLife* **4**, doi:10.7554/eLife.05224 (2015).
- 27 Lynch, J. B. & Hsiao, E. Y. Microbiomes as sources of emergent host phenotypes. *Science* **365**, 1405-1409, doi:10.1126/science.aay0240 (2019).
- 28 Thiergart, T. *et al.* Root microbiota assembly and adaptive differentiation among European Arabidopsis populations. *Nat Ecol Evol* **4**, 122-131, doi:10.1038/s41559-019-1063-3 (2020).
- 29 Wippel, K. *et al.* Host preference and invasiveness of commensal bacteria in the Lotus and Arabidopsis root microbiota. *Nat Microbiol*, doi:10.1038/s41564-021-00941-9 (2021).
- 30 Salem, H. *et al.* Drastic Genome Reduction in an Herbivore's Pectinolytic Symbiont. *Cell* **171**, 1520-1531 e1513, doi:10.1016/j.cell.2017.10.029 (2017).

- 31 Shukla, S. P. *et al.* Microbiome-assisted carrion preservation aids larval development in a burying beetle. *Proc Natl Acad Sci U S A* **115**, 11274-11279, doi:10.1073/pnas.1812808115 (2018).
- 32 Moore, R. E. & Townsend, S. D. Temporal development of the infant gut microbiome. *Open Biol* **9**, 190128, doi:10.1098/rsob.190128 (2019).
- 33 Stewart, C. J. *et al.* Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* **562**, 583-588, doi:10.1038/s41586-018-0617-x (2018).
- 34 Cellerino, A., Valenzano, D. R. & Reichard, M. From the bush to the bench: the annual *Nothobranchius* fishes as a new model system in biology. *Biol Rev Camb Philos Soc* **91**, 511-533, doi:10.1111/brv.12183 (2016).
- 35 Poeschla, M. & Valenzano, D. R. The turquoise killifish: a genetically tractable model for the study of aging. *J Exp Biol* **223**, doi:10.1242/jeb.209296 (2020).
- 36 Cui, R. *et al.* Genomics of a killifish from the Seychelles islands supports transoceanic island colonization and reveals relaxed selection of developmental genes. *bioRxiv*, 2020.2008.232421, doi:10.1101/2020.08.03.232421 (2020).
- 37 Valenzano, D. R., Aboobaker, A., Seluanov, A. & Gorbunova, V. Non-canonical aging model systems and why we need them. *EMBO J* **36**, 959-963, doi:10.15252/embj.201796837 (2017).
- 38 Cui, R. *et al.* Relaxed Selection Limits Lifespan by Increasing Mutation Load. *Cell* **178**, 385-399 e320, doi:10.1016/j.cell.2019.06.004 (2019).
- 39 Cui, R., Willemsen, D. & Valenzano, D. R. *Nothobranchius furzeri* (African Turquoise Killifish). *Trends Genet* **36**, 540-541, doi:10.1016/j.tig.2020.01.012 (2020).
- 40 Reichwald, K. *et al.* Insights into Sex Chromosome Evolution and Aging from the Genome of a Short-Lived Fish. *Cell* **163**, 1527-1538, doi:10.1016/j.cell.2015.10.071 (2015).
- 41 Valenzano, D. R. *et al.* The African Turquoise Killifish Genome Provides Insights into Evolution and Genetic Architecture of Lifespan. *Cell* **163**, 1539-1554, doi:10.1016/j.cell.2015.11.008 (2015).
- 42 Willemsen, D., Cui, R., Reichard, M. & Valenzano, D. R. Intra-species differences in population size shape life history and genome evolution. *eLife In Press*, doi: <https://doi.org/10.7554/eLife.55794> (2020).
- 43 Cui, R. *et al.* Ancestral transoceanic colonization and recent population reduction in a nonannual killifish from the Seychelles archipelago. *Mol Ecol* **30**, 3610-3623, doi:10.1111/mec.15982 (2021).
- 44 Dodzian, J., Kean, S., Seidel, J. & Valenzano, D. R. A Protocol for Laboratory Housing of Turquoise Killifish (*Nothobranchius furzeri*). *J Vis Exp*, doi:10.3791/57073 (2018).
- 45 Polacik, M., Blazek, R. & Reichard, M. Laboratory breeding of the short-lived annual killifish *Nothobranchius furzeri*. *Nat Protoc* **11**, 1396-1413, doi:10.1038/nprot.2016.080 (2016).
- 46 Harel, I. *et al.* A platform for rapid exploration of aging and diseases in a naturally short-lived vertebrate. *Cell* **160**, 1013-1026, doi:10.1016/j.cell.2015.01.038 (2015).
- 47 Harel, I., Valenzano, D. R. & Brunet, A. Efficient genome engineering approaches for the short-lived African turquoise killifish. *Nat Protoc* **11**, 2010-2028, doi:10.1038/nprot.2016.103 (2016).
- 48 Valenzano, D. R., Sharp, S. & Brunet, A. Transposon-Mediated Transgenesis in the Short-Lived African Killifish *Nothobranchius furzeri*, a Vertebrate Model for Aging. *G3 (Bethesda)* **1**, 531-538, doi:10.1534/g3.111.001271 (2011).
- 49 Valenzano, D. R. *et al.* Mapping loci associated with tail color and sex determination in the short-lived fish *Nothobranchius furzeri*. *Genetics* **183**, 1385-1395, doi:10.1534/genetics.109.108670 (2009).
- 50 Dagdeviren, S. *et al.* IL-10 prevents aging-associated inflammation and insulin resistance in skeletal muscle. *FASEB J* **31**, 701-710, doi:10.1096/fj.201600832R (2017).
- 51 Polacik, M. & Reichard, M. Diet overlap among three sympatric African annual killifish species *Nothobranchius* spp. from Mozambique. *J Fish Biol* **77**, 754-768, doi:10.1111/j.1095-8649.2010.02717.x (2010).
- 52 McFall-Ngai, M. *et al.* Animals in a bacterial world, a new imperative for the life sciences. *Proc Natl Acad Sci U S A* **110**, 3229-3236, doi:10.1073/pnas.1218525110 (2013).
- 53 Theis, K. R. *et al.* Getting the Hologenome Concept Right: an Eco-Evolutionary Framework for Hosts and Their Microbiomes. *mSystems* **1**, doi:10.1128/mSystems.00028-16 (2016).
- 54 Moran, N. A. & Sloan, D. B. The Hologenome Concept: Helpful or Hollow? *PLoS Biol* **13**, e1002311, doi:10.1371/journal.pbio.1002311 (2015).
- 55 Douglas, A. E. & Werren, J. H. Holes in the Hologenome: Why Host-Microbe Symbioses Are Not Holobionts. *mBio* **7**, e02099, doi:10.1128/mBio.02099-15 (2016).
- 56 Brucker, R. M. & Bordenstein, S. R. The hologenomic basis of speciation: gut bacteria cause hybrid lethality in the genus *Nasonia*. *Science* **341**, 667-669, doi:10.1126/science.1240659 (2013).
- 57 Enard, D. & Petrov, D. A. Evidence that RNA Viruses Drove Adaptive Introgression between Neanderthals and Modern Humans. *Cell* **175**, 360-371 e313, doi:10.1016/j.cell.2018.08.034 (2018).
- 58 Pauling, L., Itano, H. A. & et al. Sickle cell anemia a molecular disease. *Science* **110**, 543-548, doi:10.1126/science.110.2865.543 (1949).
- 59 Gasser, M. T., Chung, M., Bromley, R. E., Nadendla, S. & Dunning Hotopp, J. C. Complete Genome Sequence of wAna, the Wolbachia Endosymbiont of *Drosophila ananassae*. *Microbiol Resour Announc* **8**, doi:10.1128/MRA.01136-19 (2019).
- 60 Youngblut, N. D. *et al.* Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. *Nat Commun* **10**, 2200, doi:10.1038/s41467-019-10191-3 (2019).

- 61 Furness, A. I., Lee, K. & Reznick, D. N. Adaptation in a variable environment: Phenotypic plasticity and bet-hedging during egg diapause and hatching in an annual killifish. *Evolution* **69**, 1461-1475, doi:10.1111/evo.12669 (2015).
- 62 Cui, R. *et al.* Relaxed Selection Limits Lifespan by Increasing Mutation Load. *Cell* **180**, 1272-1279, doi:10.1016/j.cell.2020.02.038 (2020).
- 63 Furness, A. I. The evolution of an annual life cycle in killifish: adaptation to ephemeral aquatic environments through embryonic diapause. *Biol Rev Camb Philos Soc* **91**, 796-812, doi:10.1111/brv.12194 (2016).
- 64 Hu, C. K. *et al.* Vertebrate diapause preserves organisms long term through Polycomb complex members. *Science* **367**, 870-874, doi:10.1126/science.aaw2601 (2020).
- 65 B.R., W., Nagy, B., van der Merwe, P. D. W., Cotterill, F. P. D. & Bellstedt, D. U. Redescription of the seasonal killifish species *Nothobranchius ocellatus* and description of a related new species *Nothobranchius matanduensis*, from eastern Tanzania (Teleostei: Nothobranchiidae). *Ichthyological Exploration of Freshwaters*, doi:10.23788/IEF-1149 (2020).
- 66 McElreath, R. *Statistical Rethinking. A Bayesian Course with Examples in R and STAN*. 2nd edn, 594 (Chapman and Hall/CRC, 2020).
- 67 Ward, L. M., Shih, P. M. & Fischer, W. W. MetaPOAP: presence or absence of metabolic pathways in metagenome-assembled genomes. *Bioinformatics* **34**, 4284-4286, doi:10.1093/bioinformatics/bty510 (2018).
- 68 Zorrilla, F., Patil, K. R. & Zelezniak, A. metaGEM: reconstruction of genome scale metabolic models directly from metagenomes. *bioRxiv*, 2020.2012.2031.424982, doi:10.1101/2020.12.31.424982 (2021).
- 69 Sloan, W. T. *et al.* Quantifying the roles of immigration and chance in shaping prokaryote community structure. *Environ Microbiol* **8**, 732-740, doi:10.1111/j.1462-2920.2005.00956.x (2006).
- 70 Chen, Z. *et al.* Ultralow-input single-tube linked-read library method enables short-read second-generation sequencing systems to routinely generate highly accurate and economical long-range sequencing information. *Genome Res*, doi:10.1101/gr.260380.119 (2020).
- 71 Willemsen, D., Cui, R., Reichard, M. & Valenzano, D. R. Intra-species differences in population size shape life history and genome evolution. *Elife*, doi:<https://doi.org/10.1101/852368> (2020).
- 72 Chen, C. *et al.* The protective effect of fish-derived cathelicidins on bacterial infections in zebrafish, *Danio rerio*. *Fish Shellfish Immunol* **92**, 519-527, doi:10.1016/j.fsi.2019.06.029 (2019).
- 73 Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-395, doi:10.1038/415389a (2002).
- 74 Zhang, Z. & Zhu, S. Comparative genomics analysis of five families of antimicrobial peptide-like genes in seven ant species. *Dev Comp Immunol* **38**, 262-274, doi:10.1016/j.dci.2012.05.003 (2012).
- 75 Lazzaro, B. P., Zasloff, M. & Rolff, J. Antimicrobial peptides: Application informed by evolution. *Science* **368**, doi:10.1126/science.aau5480 (2020).
- 76 Fingerhut, L., Miller, D. J., Strugnell, J. M., Daly, N. L. & Cooke, I. R. ampir: an R package for fast genome-wide prediction of antimicrobial peptides. *Bioinformatics* **36**, 5262-5263, doi:10.1093/bioinformatics/btaa653 (2021).
- 77 Meher, P. K., Sahu, T. K., Saini, V. & Rao, A. R. Predicting antimicrobial peptides with improved accuracy by incorporating the compositional, physico-chemical and structural features into Chou's general PseAAC. *Sci Rep* **7**, 42362, doi:10.1038/srep42362 (2017).
- 78 Bhadra, P., Yan, J., Li, J., Fong, S. & Siu, S. W. I. AmPEP: Sequence-based prediction of antimicrobial peptides using distribution patterns of amino acid properties and random forest. *Sci Rep* **8**, 1697, doi:10.1038/s41598-018-19752-w (2018).
- 79 De Bie, T., Cristianini, N., Demuth, J. P. & Hahn, M. W. CAFE: a computational tool for the study of gene family evolution. *Bioinformatics* **22**, 1269-1271, doi:10.1093/bioinformatics/btl097 (2006).
- 80 Wertheim, J. O., Murrell, B., Smith, M. D., Kosakovsky Pond, S. L. & Scheffler, K. RELAX: detecting relaxed selection in a phylogenetic framework. *Mol Biol Evol* **32**, 820-832, doi:10.1093/molbev/msu400 (2015).
- 81 Petzold, A. *et al.* The transcript catalogue of the short-lived fish *Nothobranchius furzeri* provides insights into age-dependent changes of mRNA levels. *BMC Genomics* **14**, 185, doi:10.1186/1471-2164-14-185 (2013).
- 82 Messer, P. W. & Petrov, D. A. Frequent adaptation and the McDonald-Kreitman test. *Proc Natl Acad Sci U S A* **110**, 8615-8620, doi:10.1073/pnas.1220835110 (2013).
- 83 Agler, M. T. *et al.* Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. *PLoS Biol* **14**, e1002352, doi:10.1371/journal.pbio.1002352 (2016).
- 84 Balouiri, M., Sadiki, M. & Ibnsouda, S. K. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal* **6**, 71-79, doi:10.1016/j.jpha.2015.11.005 (2016).
- 85 Poyet, M. *et al.* A library of human gut bacterial isolates paired with longitudinal multiomics data enables mechanistic microbiome research. *Nat Med* **25**, 1442-1452, doi:10.1038/s41591-019-0559-3 (2019).
- 86 Benson, A. K. *et al.* Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci U S A* **107**, 18933-18938, doi:10.1073/pnas.1007028107 (2010).
- 87 Belheouane, M., Gupta, Y., Kunzel, S., Ibrahim, S. & Baines, J. F. Improved detection of gene-microbe interactions in the mouse skin microbiota using high-resolution QTL mapping of 16S rRNA transcripts. *Microbiome* **5**, 59, doi:10.1186/s40168-017-0275-5 (2017).

- 88 Wang, J. *et al.* Analysis of intestinal microbiota in hybrid house mice reveals evolutionary divergence in a vertebrate hologenome. *Nat Commun* **6**, 6440, doi:10.1038/ncomms7440 (2015).
- 89 Schlamp, F. *et al.* High-resolution QTL mapping with Diversity Outbred mice identifies genetic variants that impact gut microbiome composition. *bioRxiv*, 722744, doi:10.1101/722744 (2021).
- 90 Ohlmann, M. *et al.* Diversity indices for ecological networks: a unifying framework using Hill numbers. *Ecol Lett* **22**, 737-747, doi:10.1111/ele.13221 (2019).
- 91 Andolfatto, P. *et al.* Multiplexed shotgun genotyping for rapid and efficient genetic mapping. *Genome Res* **21**, 610-617, doi:10.1101/gr.115402.110 (2011).
- 92 Cooke, S. B. & Terhune, C. E. Form, function, and geometric morphometrics. *Anat Rec (Hoboken)* **298**, 5-28, doi:10.1002/ar.23065 (2015).
- 93 Ng'oma, E. *et al.* The age related markers lipofuscin and apoptosis show different genetic architecture by QTL mapping in short-lived Nothobranchius fish. *Aging (Albany NY)* **6**, 468-480, doi:10.18632/aging.100660 (2014).
- 94 Fuess, L. E. *et al.* Immune Gene Expression Covaries with Gut Microbiome Composition in Stickleback. *mBio* **12**, doi:10.1128/mBio.00145-21 (2021).
- 95 Thaiss, C. A., Zmora, N., Levy, M. & Elinav, E. The microbiome and innate immunity. *Nature* **535**, 65-74, doi:10.1038/nature18847 (2016).
- 96 Maglione, P. J. *et al.* IRAK-4 and MyD88 deficiencies impair IgM responses against T-independent bacterial antigens. *Blood* **124**, 3561-3571, doi:10.1182/blood-2014-07-587824 (2014).
- 97 Salem, S., Salem, D. & Gros, P. Role of IRF8 in immune cells functions, protection against infections, and susceptibility to inflammatory diseases. *Hum Genet* **139**, 707-721, doi:10.1007/s00439-020-02154-2 (2020).
- 98 Hambleton, S. *et al.* IRF8 mutations and human dendritic-cell immunodeficiency. *N Engl J Med* **365**, 127-138, doi:10.1056/NEJMoa1100066 (2011).
- 99 Suster, M. L., Abe, G., Schouw, A. & Kawakami, K. Transposon-mediated BAC transgenesis in zebrafish. *Nat Protoc* **6**, 1998-2021, doi:10.1038/nprot.2011.416 (2011).
- 100 Kaetzel, C. S. Cooperativity among secretory IgA, the polymeric immunoglobulin receptor, and the gut microbiota promotes host-microbial mutualism. *Immunol Lett* **162**, 10-21, doi:10.1016/j.imlet.2014.05.008 (2014).
- 101 Pabst, O. & Slack, E. IgA and the intestinal microbiota: the importance of being specific. *Mucosal Immunol* **13**, 12-21, doi:10.1038/s41385-019-0227-4 (2020).
- 102 Popkes, M. & Valenzano, D. R. Microbiota-host interactions shape ageing dynamics. *Philos Trans R Soc Lond B Biol Sci* **375**, 20190596, doi:10.1098/rstb.2019.0596 (2020).
- 103 Bradshaw, W. J. & Valenzano, D. R. Extreme genomic volatility characterizes the evolution of the immunoglobulin heavy chain locus in cyprinodontiform fishes. *Proc Biol Sci* **287**, 20200489, doi:10.1098/rspb.2020.0489 (2020).
- 104 Gutzeit, C., Chen, K. & Cerutti, A. The enigmatic function of IgD: some answers at last. *Eur J Immunol* **48**, 1101-1113, doi:10.1002/eji.201646547 (2018).
- 105 Cao, J. *et al.* Comprehensive single-cell transcriptional profiling of a multicellular organism. *Science* **357**, 661-667, doi:10.1126/science.aam8940 (2017).
- 106 Hernandez, P. P. *et al.* Single-cell transcriptional analysis reveals ILC-like cells in zebrafish. *Sci Immunol* **3**, doi:10.1126/sciimmunol.aau5265 (2018).
- 107 Wiles, T. J. *et al.* Modernized Tools for Streamlined Genetic Manipulation and Comparative Study of Wild and Diverse Proteobacterial Lineages. *mBio* **9**, doi:10.1128/mBio.01877-18 (2018).
- 108 Lagier, J. C. *et al.* Culturing the human microbiota and culturomics. *Nat Rev Microbiol* **16**, 540-550, doi:10.1038/s41579-018-0041-0 (2018).
- 109 Melancon, E. *et al.* Best practices for germ-free derivation and gnotobiotic zebrafish husbandry. *Methods Cell Biol* **138**, 61-100, doi:10.1016/bs.mcb.2016.11.005 (2017).
- 110 Rawls, J. F., Samuel, B. S. & Gordon, J. I. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proc Natl Acad Sci U S A* **101**, 4596-4601, doi:10.1073/pnas.0400706101 (2004).
- 111 Racz, A. *et al.* Identification of Individual Zebrafish (*Danio rerio*): A Refined Protocol for VIE Tagging Whilst Considering Animal Welfare and the Principles of the 3Rs. *Animals (Basel)* **11**, doi:10.3390/ani11030616 (2021).
- 112 Bradshaw, W. J., Poeschla, M., Placzek, A. & Valenzano, D. R. Antibody repertoire sequencing reveals systemic and mucosal immunosenescence in the short-lived turquoise killifish. *bioRxiv*, 2020.2008.2021.261248, doi:10.1101/2020.08.21.261248 (2020).
- 113 Gudding, R., Lillehaug, A. & Evensen, O. Recent developments in fish vaccinology. *Vet Immunol Immunopathol* **72**, 203-212, doi:10.1016/s0165-2427(99)00133-6 (1999).
- 114 Ma, J., Bruce, T. J., Jones, E. M. & Cain, K. D. A Review of Fish Vaccine Development Strategies: Conventional Methods and Modern Biotechnological Approaches. *Microorganisms* **7**, doi:10.3390/microorganisms7110569 (2019).
- 115 Bailone, R. L. *et al.* Zebrafish as an alternative animal model in human and animal vaccination research. *Lab Anim Res* **36**, 13, doi:10.1186/s42826-020-00042-4 (2020).
- 116 Dubey, S. *et al.* Aeromonas hydrophila OmpW PLGA Nanoparticle Oral Vaccine Shows a Dose-Dependent Protective Immunity in Rohu (*Labeo rohita*). *Vaccines (Basel)* **4**, doi:10.3390/vaccines4020021 (2016).