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Effect of early rearing conditions on the behaviour and microbiome of fish with low genetic diversity

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SUMMARY

Fish performance is influenced by their genotype and environment. For populations with low genetic diversity, adaptation to environmental change can be compromised, but it has been suggested that the microbiome can act as an additional source of variability. Early rearing conditions can be particularly important for fish development and behaviour, due to their dependence on the environmental conditions. This thesis explored the interactions between fish genotype and rearing environment (diet and enrichment) on behaviour, metabolic rate and microbiome (gut, skin) using captive and wild populations of naturally inbred mangrove killifishes (*Kryptolebias* sp.), where the exploratory behaviour and closed respirometry were used to test the captive killifish and microbiome diversity analyses were performed in both captive and wild killifishes.

Behavioural trials in self-fertilizing killifish (*Kryptolebias marmoratus*) indicated a significant effect of both genetic strain and rearing environment (environmental enrichment and diet) on fish activity measurements. Incubation time also had a substantial role on both fish behaviour and microbiome diversity. Gut-microbiome alpha diversity was shaped by strain, diet, and hatching time in *K. marmoratus* with interactions between diet and physical enrichment.

An intergenerational influence of rearing environment on fish behaviour was detected in *K. marmoratus*, where parental activity was found to influence offspring activity. Gut-microbial comparisons between parents and offspring identified *Vibrionaceae* as the dominant colonizers in laboratory reared *K. marmoratus*. A dominant effect of the rearing environment over strain on both microbiome composition and distribution was observed. The influence of the interaction between parental and own environments on microbiome alpha diversity in *K. marmoratus* offspring suggests long-term effects of the rearing environment on the fish (gut) microbiome.

In the wild, results from the outcrossing *K. ocelatus* and the self-fertilising *K. hermaphroditus* identified that the diversity and community composition of the skin microbiome were strongly shaped by their environment but also by the species and host genetic diversity at different levels. This study also found first-time evidence of a relationship between microbiome and epigenetic diversity in these wild populations, suggesting that both mechanisms could be potential sources of additional variability for fish species with low genetic diversity.

The global findings of this thesis on mangrove killifishes from different origins (laboratory and natural conditions) highlighted the importance of the interactions between genotype and environment in shaping fish microbiome composition and diversity.

DECLARATIONS

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed.....[redacted].....

Date.....28/03/2024.....

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references.
A bibliography is appended.

Signed.....[redacted].....

Date.....28/03/2024.....

I hereby give consent for my thesis, if accepted, to be available for electronic sharing

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Date.....28/03/2024.....

The University's ethical procedures have been followed and, where appropriate, that ethical approval has been granted.

Signed.....[redacted].....

Date.....28/03/2024.....

AUTHORSHIP DECLARATION

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DISCLAIMER

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Ethics

Experimental methods and protocols required for animal model studies in this thesis were approved by Swansea University Ethics Review Committee and reference numbers are stated in relevant chapters.

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Chapter 1

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Chapter 2

IA, SC, and CGL generated the research plan, IA conducted the experiment for behaviour and respirometry. SM helped to derive the behavioural data from recorded videos of both parental and offspring behaviours using BORIS software. IA analysed both the data from behavioural and respirometry experiments and wrote the chapter with the contribution of the supervisors.

Chapter 3

IA, SC, and CGL generated the research plan. IA conducted the experiment. IA performed gut sampling of fish from both parental and offspring generation. IA have done the DNA extractions, PCR amplifications, and library preparations of the gut samples. MH helped to sequence the samples for the gut microbiome. IA worked for the molecular analysis, and bioinformatics of the microbiome with the contribution of TUW and SC. IA analysed and wrote the chapter with the contribution of the supervisors.

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I-V: GENERAL INTRODUCTION

I. Influence of the rearing environment on fish individual fitness

Environmental factors can influence the fitness of fish (Aarestrup et al., 2018), although different fish species have different phenotypic responses to environmental changes due to their unique metabolic budget and developmental process (Raventos et al., 2021). As fish are ectotherms, they depend on the external environment for temperature regulation and metabolism, which makes early life conditions particularly crucial. An unfavourable environment can result in high mortalities at early stages, particularly compared to mammals and birds, and can impact a wide range of behavioural responses, for example, the swimming behaviour during their dispersal stages (Faillettaz et al., 2018). Environmental factors (i.e., from water quality to stocking density) have also been found to influence the fitness traits of fish under aquaculture conditions (e.g., growth, survival, and behaviour are all influenced by feed and tank culture systems in Tilapia, *Oreochromis niloticus*) (Abd El-Hack et al., 2022). Rearing conditions become even more important under extreme environmental conditions, and already a wide range of aquaculture practices and diverse fish species have been affected by extreme temperatures (hot and cold) due to climate change (Islam et al., 2022). In addition, environmental stressors can affect the growth and fitness of farmed fish depending on their metabolic scope (e.g., positive correlation between ammonia and standard metabolic rate was observed in traditional pond culture in *Carassius auratus gibelio*) (Yao et al., 2020). This relationship between rearing environment and fish fitness is particularly important at the very early life stages of fish due to its influence on fish behaviour and later-life performance.

I.I. Early rearing conditions and environmental enrichment

Environmental enrichment (e.g., physical or structural) has been used in aquaculture to provide fish a better welfare and yield by introducing some positive aspects to their environment and to

mimic more natural conditions (Brydges & Braithwaite, 2009). Structural enrichment is already a well-known approach for fish in both farmed and laboratory settings (Jones et al., 2021) because of both the positive (e.g. improved/natural behaviour) and negative effects (e.g. increased aggression) of enrichments. But there are significant differences between the early rearing conditions of fish from farmed and natural environment.

Environmental factors in the natural habitat of wild fish (Roni, 2019), and different rearing conditions for fish in the captivity including tank enrichment (Lee et al., 2019), diet and nutrition (Gisbert et al., 2022), and temperature, play an essential role in fish performance (i.e., hatching and survival (Sunde et al., 2019)), and stress response (e.g., cold temperature affecting mucus functioning in gilthead sea bream (Sanahuja et al., 2019)). Environmental enrichment at juvenile stage can have positive effects (i.e., increased survival) on hatchery-reared Atlantic salmon parr during the post-release phase in the river (Mes et al., 2019). An enriched environment can also play an important role in lowering stress (e.g., in *Sebastes schlegelii*) (Zhang et al., 2021), developing more stable fish behaviour and higher survival, both in captive (Braithwaite & Ahlbeck Bergendahl, 2020) and wild fish (Mes et al., 2018), however no direct influence of environmental enrichment on enhanced microbiome of fish environment has been reported yet, and it was stated as a possible outcome of enrichment in fish as previously enhanced microbiome richness in rearing tank and water biofilm (in Atlantic Salmon) was observed (Minich et al., 2020) in the hatchery environment. In captivity, environmental enrichment (physical structures) reduces aggression and increases growth, for example in rainbow trout (*Oncorhynchus mykiss*) (Brunet et al., 2022), as a barren environment can induce increased activity or other restless behaviours in fish (Fureix & Meagher, 2015). Environmental enrichment in hatcheries can also have positive impacts on fish

(produce larger fish) with higher morphological variability (i.e., differentiated fins) in piracanjuba (*Brycon orbignyanus* (Saraiva & Pompeu, 2019)), for example, which might influence fish behaviour later on. However, the behavioural response to environmental enrichment varies among organisms and across different settings. For instance, it has been reported that the influence of environmental enrichment on behaviour and physiology depends on the interaction between the genotype and the environmental setting in five isogenic *Drosophila melanogaster* lines (Akhund-Zade et al., 2019). In contrast, no influence of enrichment was observed on brain size or behaviour in three-spined sticklebacks (Toli et al., 2017) while spatial enrichment had an effect on brain size in mosquitofish (Turschwell & White, 2016). Moreover, enrichment can result in different gradients of aggression, for example, increased aggression caused by tank enhancement (structural) was observed in zebrafish (*Danio rerio*) (Woodward et al., 2019). So, it seems that the impact on fish behaviour of even microenvironmental changes is highly variable and depends both on the species and on the type of environmental modification.

I.II. Role of diet and probiotics in fish fitness

Diet is essential for fish growth, reproduction (Fowler et al., 2019; Butts et al., 2020), and health (e.g., through dietary supplementation) (von Danwitz & Schulz, 2020). However, nutrient absorption in fish (captive and wild) depends on individual preferences and condition (Naznin, 2021). Formulated, or supplemented diets (e.g., with pre or probiotics, prebiotics are non-digestible fibre such as B-glucan that can enhance the growth of beneficial bacteria or probiotics such as *Lactobacillus* in gut (Guerreiro et al., 2016, Das, et al., 2017)) are designed to improve welfare and performance in farmed fish. Diet enrichment like probiotics addition (Sayes et al., 2018) has been reported to reduce gut inflammation and preserve gut integrity in fish (Merrifield

et al., 2010). *Bacillus* probiotics can also improve water quality, growth, and immunity in a wide range of fish species including Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) (Mohammadi et al., 2020; Mohammadi et al., 2021; Putra et al., 2021; Van Doan et al., 2021). Probiotics can be administered to the fish either through direct application to water or through bio-encapsulation using live feed like *Artemia* (Cruz et al., 2012; Vázquez-Silva et al., 2017). Combining multiple probiotic strains has been found to improve the digestibility, for example in Siberian sturgeon (*Acipenser baerii*) (Ghodrati et al., 2021), and to lower stress, such as in zebrafish (*Danio rerio*) (Gioacchini et al., 2014). Moreover, probiotics have been observed to influence the reproduction and metabolism of zebrafish through an effect on its endocrine system (Carnevali et al., 2017). Probiotic diets have also been found to improve the activity of *Cyprinus carpio* (Sharma & Thakur, 2020; Sharma, 2021). Although probiotic diets seem mostly advantageous, there is still a lack of research on the effect of probiotic diet on the gut microbiome in fish (Xia et al., 2018; Borges et al., 2021), both at individual and inter- or trans-generational level, especially under different rearing environmental conditions.

II. Interaction between the genetic background and the rearing environment on fish performance

Genetic diversity can generate phenotypic plasticity in an organism sometimes through a genotype by environment interactions (G x E) (Saltz et al., 2018). Thus, fish phenotypic responses (e.g., behaviour) can be influenced partly by the genetic background and the environment of fish, although it is usually very difficult to disentangle their relative roles, particularly in natural populations. Fish from different environmental origins vary widely in terms of genetic diversity,

for example, marine fishes tend to have higher genetic diversity compared to freshwater fish species. Therefore, fish genetic diversity can also be driven by their habitat (Martinez et al., 2018).

It has become evident that G x E interactions have an important effect on fish performance, particularly when fish are farmed under varied environmental conditions. For example, the best performing genotypes of Nile Tilapia reared in one environment (pond) were not growing the same in a different rearing environment (river-cages) (Thodesen et al., 2011; Trọng et al., 2013). Fish productivity in different aquaculture practices is also highly dependent on both G x E and the sensitivity to its micro-environmental variations, as has been observed in Genetically Improved Farmed Tilapia (*Oreochromis niloticus*) (Agha et al., 2018), which display a strong influence of rearing environments on the genetic gain (life-long development).

II.I. Adaptation of fish with low genetic diversity

The matting of closely related individuals (inbreeding) represents an extreme example of low genetic diversity and can result in inbreeding depression, with negative consequences for individual and population survival (Syukri et al., 2020; Kardos et al., 2023). This is because genetic diversity influences the species' ability to survive and deal with environmental change, and thus contributes to their resilience against multiple environmental instabilities (Gandra et al., 2021; McKenzie et al., 2021). Yet, there are species which are able to survive, or even thrive, with low genetic diversity, for example, Hawaiian Crow (*Corvus hawaiiensis*) survived through a prolonged (~100 years) population bottleneck (Flanagan et al., 2021), and some species of cheetah survived through population decline instead of reduced genetic diversity (Tommasi et al., 2021). The mangrove killifishes (*Kryptolebias marmoratus* and *K. hermaphroditus*), for example, are unique

self-fertilising species, naturally inbred, which have been found to survive and disperse despite of low levels of genetic diversity (Tatarenkov et al., 2017a). Their living environment (mangrove ecosystems) is quite dynamic and it has been suggested that epigenetic processes can play a role in the adaptation of their wild fish populations (Cayuela et al., 2021).

Epigenetic mechanisms can produce phenotypic differences in populations upon exposure to diverse environmental challenges, through the regulation of gene expression without the involvement of mutations (Berger et al., 2009), and can be important for fish during their early developmental stages (e.g., influence of speciation and diet on methylome divergence in African cichlids *Astatotilapia calliptera*) (Vernaz et al., 2022), in combination with the underlying genetic variation (Fargeot et al., 2021). Some of the epigenetic marks induced by the environment can persist the whole life of the individual, and in some cases can have transgenerational effects in fish (Bhandari, 2016).

III. Fish physiology and behavioural responses to environmental conditions

Environmental factors affect fish physiology (i.e., basal metabolism) and behaviour (i.e., activity or exploration). For example, elevated rearing temperature (e.g., 27 ℃) and low nutrient diet can cause higher basal metabolic rate (BMR) and deformity (notochord) in Japanese eel (*Anguilla japonica*) (Okamura et al., 2018). The effects of environmental enrichment on fish welfare, physiology and behaviour have been reported already under different laboratory rearing conditions (i.e., in zebrafish, three-spined stickleback, in guppies, and in goldfish) (Williams et al., 2009; Stevens et al., 2021), but their genetic basis is still very poorly explored.

Exposure to environmental challenges can have both positive and negative influence on the offspring through a wide range of phenotypes. For instance, a higher survival was observed in zebrafish after parental exposure to oil through ambient water (Bautista & Burggren, 2019). In contrast, cardiac teratogenesis was caused by polycyclic aromatic hydrocarbons, PAHs was observed in the offspring of exposed Atlantic killifish (*Fundulus heteroclitus*) (Clark et al., 2014). The transmission of severe maladaptive phenotype (i.e., skeletal deformities) from parents to offspring was also observed in a multigenerational comparative study using Benzo[a]pyrene (BaP) exposure in zebrafish (Corrales et al., 2014). Behavioural impairment (i.e., boldness) in adults and altered foraging behaviour in larvae was also observed in a multigenerational study of mixed chemicals exposure in fathead minnows (*Pimephales promelas*) (Swank et al., 2021)). Anxiety like fish behaviour can also be influenced by its environment (e.g., persistent organic pollutants) in both parental and offspring generation (Alfonso et al., 2019). However, research focusing the intergenerational influence of rearing conditions on fish with low genetic diversity is still limited.

IV. The fish microbiome

Fish microbiome consists of a diverse communities of protists, fungi, virus, bacteria, and archaea colonising the mucosal surfaces which play a key role in overall fish fitness, survival, and immunity. The microbiome of fish varies (diversity and composition) depending on the organ colonised (i.e., skin, gill, gut), beneficial (e.g., probiotic bacteria- *Bacillus*) or pathogenic (e.g., many *Vibrio* species) nature in fish, and based on the rearing conditions experienced by the fish. For example, environmental influence (i.e., tank rearing systems) was observed on the gill and skin microbiome (structure) of yellowtail kingfish, *Seriola lalandi*, along with a lower influence of the diet on shaping the microbiome (Minich et al., 2021). In the same study, it was found that microbiome alpha diversity from gill and skin was higher than that in the gut (more or less stable),

and a higher diversity was reported for the gut microbiome with growing age, reflecting the general influence of developmental stages on gut microbiome in fish over time (Niu et al., 2020). Early rearing conditions, usually more dynamic, also influence the microbiome in wild fish population (i.e., coastal pelagic *Seriola spp.*) (Ben-Aderet, 2017).

A host's microbiome can be determined and interact with intrinsic (host) and extrinsic (environment) factors. Thus, the fish microbiome can greatly vary even within a species, influenced by diet and environmental conditions (Star et al., 2013), for example the gut microbiome of Atlantic salmon *Salmo salar* L. has seasonal fluctuations and changes throughout different developmental stages of fish, for example, the predominant *Vibrionaceae* were detected in midsummer and early earing stages of salmon development, but then declined as fish were reaching their harvesting size (Zarkasi et al., 2014), suggesting that to test the effect of the variable factors on microbiome on different fish stages and over time (Gallo et al., 2020) may be necessary. The comparison between the microbiome of fish gut and its environment tends to display a specific pattern of microbiome composition for specific fish species, referred to as the core microbiome of fish (Wong et al., 2013). However, the effect of environment and host on microbiome has also been observed in Freshwater smelt (*Hypomesus nipponensis*) (skin and gut) (Park & Kim, 2021).

V. Using model species to understand the relative role of genetics and environment on fish behaviour and microbiome composition

Zebrafish (*Danio rerio*) is a commonly used model species to study fish physiology (i.e., metabolism), behaviour and stress response (Zago et al., 2018). Different fish models (i.e., zebrafish and three spined stickleback) have also been used for microbiome related research

(Soares et al., 2019). Mangrove killifish species (*Kryptolebias hermaphroditus* and *K. marmoratus*), because of their unique reproductive mode and the capacity to produce genetically homogeneous individuals naturally, allows us control over the genetic variability of individual fish, to explain the relative contribution of both genotype and environment on phenotypic responses. *K. hermaphroditus* and *K. marmoratus* are the only known self-fertilizing vertebrates (Tatarenkov et al., 2017a), while closely related species such as *K. ocellatus* reproduce through outcrossing (Waldir M Berbel-Filho et al., 2020). *K. marmoratus* and *K. hermaphroditus* are mainly selfing, although a very low level of outcrossing has also been reported (mixed-mating) to produce heterozygous individuals (Mackiewicz et al., 2006; Avise & Tatarenkov, 2015). This unique feature has made these mangrove killifish species very useful to investigate the response effects caused by their genetic diversity and the environment.

V.I. *Kryptolebias marmoratus* as biological model species

K. marmoratus have been found in natural tropical estuarine habitats (Costa, 2011; Lira et al., 2015) covering a wide range of geographic area (Figure V.I.1). Several selfing lines have been identified (Tatarenkov et al., 2010) in the laboratory which made this species useful for emerging research covering feeding behaviour (Pandey et al., 2008), developmental biology (Kanamori et al., 2006; Mourabit et al., 2011), growth (i.e., using PAN-RS and DAN lines and hybrids) (Nakamura et al., 2008), and environmental (i.e., salinity, ammonia) influence on fish physiology and stress (Frick & Wright, 2002), some advancement in the key research areas also includes the Genome project (Rhee et al., 2014), behavioural study of strain HON9 (Edenbrow & Croft, 2012), developmental genetic study using mutagen N-ethyl-N-nitrosourea (ENU) (Moore et al., 2012), and mutants identification (Saud et al., 2021).

K. marmoratus (strains DAN and HON9, DAN was originated from Belize and HON9 was originated from Honduras (Tatarenkov et al., 2010)). The whole genome (2, 106, 131 SNPs) of variable *K. marmoratus* lineages were reported in (Lins et al., 2017), where the genetic structuring analysis revealed the population clustering of lineages from Belize and Honduras except some exceptions with some other lineages.

Epigenomic study of DAN line tested the line R and reported the greater influence of genotypes over DNA methylation (an epigenetic marker) due to environmental enrichment (Berbel-Filho et al., 2019). RNAseq analysis of HON9 (with 2 other lines) was used to identify mutant alleles followed by phenotypes (e.g., aggression, morphological structures), this can be very useful to detect individual variations among different clonal lines (Kudoh et al., 2024).

Behavioural plasticity was tested in *K. marmoratus* (20 genotypes including DAN and HON9), where it was found that the fish were more exploratory during their early developmental stage compared to their age at sexual maturity (Edenbrow & Croft, 2011). The colour pattern of DAN and HON9 were showing the distinct greyish colour for the hermaphrodites while slight to dark orangish colour for the male individuals. has been used as biological model in this thesis for assessing genetic and environmental factors related to how low genetically diverse fish respond to environmental rearing conditions (Figure V.I.2).

Figure V.I.1 Map generated from https://obis.org/ using dataset covering time range 1900-2023, green dots showing the natural distribution locations of mangrove killifish species, *K. marmoratus*.

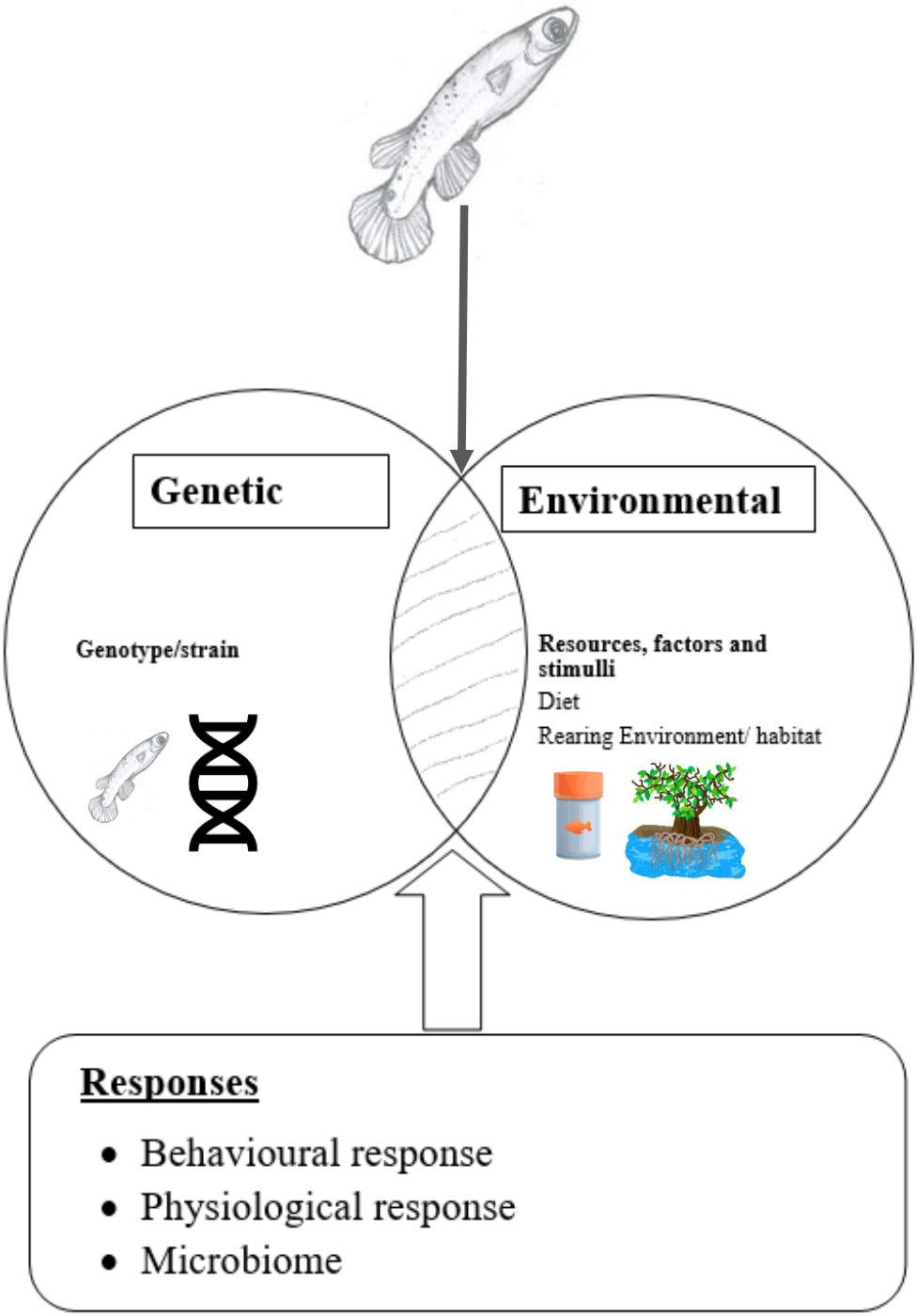


Figure V.I.2 Genetic and Environmental factors, and responses in mangrove killifish, *K. marmoratus*.

VI. THESIS OBJECTIVES

VI. Thesis objectives

This thesis consists of 4 Chapters (1, 2, 3 and 4) where the following research questions and objectives were addressed-

Q 1. How do the relative roles of genotype and environment influence physiology, behaviour, and microbiome in fish?

In **Chapter1**, **Objective 1** was set to investigate the effects of early rearing conditions (diet and environmental enrichment) on the phenotypic responses (behaviour and physiology) and gut microbiome of mangrove killifish from two different self-fertilising strains. Considering the influence of environmental enrichment on killifish (different strain from this study) in a previous study, the main **hypothesis** for **Chapter 1** was to observe differences in behavioural response between two different lines of killifish due to early rearing environmental conditions (physical enrichment and probiotic diet).

Q 2. Are the behavioural effects of early rearing environment transmitted between generations?

Chapter 2 investigated **Objective 2,** to detect potential intergenerational influences of rearing environment (standard poor vs enriched) on the behaviour and basal metabolism of two different strains of *Kryptolebias marmoratus*. The **hypothesis** for **Chapter 2** was to have positive influence (more activity) of environmental enrichment on exploratory behaviour (inspection of novel object, active crosses) and basal metabolism across generations with a difference between two killifish genotypes tested.

Q 3. To what extent, early rearing environment and genotype can influence the gut microbiome in fish across generations?

In **Chapter 3**, **Objective 3** was established to explore the contribution of genotype and rearing environment (parental vs offspring's own environment) on the offspring's gut microbiome through a comparison between two generations (parent vs offspring) of mangrove killifish, *K. marmoratus*,

from two different strains. **Hypothesis** of **Chapter 3** was to have differences in microbiome diversity and/or composition between lines as an effect of rearing environmental condition of parents.

Q 4. What are the relative roles of the environment and the genetic background (species and individual genetic diversity) in shaping the fish microbiome in the wild?

Objective 4 in **Chapter 4** assessed the influence of species and sampling locations on the skin microbiome of wild fish from two different mangrove killifish species (*K. ocelatus* and *K. hermaphroditus*) with different mating systems (outbreeding and selfing), living in sympatry and allopatry. The **hypothesis** for **Chapter 4** was to observe the relationship among microbiome (skin) diversity and host genetics, epigenetics (DNA methylation patterns) and fluctuating asymmetry by controlling environmental influences (shared and sympatry).

CHAPTER 1: Influence of early rearing environment and genetic background on fish behaviour and microbiome

1.1. Abstract

Early rearing conditions are critical for fish fitness and survival and can be manipulated in captivity to improve welfare. Environmental enrichment can lower stress and promote more natural behaviour in fish, while probiotics can modulate the microbiome-gut-brain axis and also influence behaviour. Research on the relative roles of the genetic background and environmental conditioning on fish performance is still limited. I investigated the role of fish genotype and early rearing environment (physical enrichment and probiotics) on the phenotypic responses and gut-microbiome of naturally inbred mangrove killifish (*Kryptolebias marmoratus*) of two genetically different strains. Behavioural and physiological responses were analysed using the novel object exploration test and basal metabolic rate (BMR), and 16S rRNA amplicon sequencing and analysis of the gut-microbiome. Fish activity was significantly influenced by incubation time, strain, and diet, while BMR was not influenced by any of the variables. *Vibrio* was the most abundant bacteria, followed by *Photobacterium*, *Lactobacillus*, and *Shewanella*. Fish strain, diet, and incubation time significantly influenced the microbiome alpha diversity and evenness, where positive influence of strain was observed in HON9 with a higher Chao1 richness and EDS diet had positive influence in fish from both strains with higher microbiome evenness compared to the probiotic fed fish groups. An interaction of diet-environmental enrichment had influenced microbiome evenness, while beta diversity was only influenced by incubation time. The results indicate an effect of the genotype, diet, environment, and hatching time on fish behaviour and microbiome, with potential implications for farmed fish husbandry.

1.2. Introduction

Among multiple environmental components that influence fish phenotypic variations, rearing environment has been found to influence cognitive behaviour and interact with individual genetic architecture (Salena et al., 2021; Venney et al., 2021). In captive conditions like hatcheries and aquaculture, the addition of substrates such as artificial plants or logs can support fish in different developmental stages creating a richer (enriched) environment compared to the standard barren (poor) one. Environmental enrichment has a significant effect on stress, physiology and behaviour in fish (e.g., in black rockfish, *Sebastes schlegelii*) (Zhang et al., 2020; Zhang et al., 2021).

In the early rearing stage of fish, enriched hatchery environment seems to be an option to decrease stress, and to increase the chances in fish to behave more naturally (Corcoran, 2015) as well as to improve the foraging performance (i.e., for Atlantic salmon *Salmo salar L.* parr) (Brown et al., 2003). An enriched structural environment at an appropriate early life stage can promote the development of better learning capability for fish to cope with environmental change in its life later on (Makino et al., 2015). It has also been reported that the beneficial effects of the enrichment (i.e. coloured substrate) are related to social exchanges and are considered as the foundation of a less stressful social association in enriched-reared fish groups (Batzina et al., 2014). However, in a recent lab-based study in rare minnows (*Gobiocypris rarus*), the effect of environmental enrichment has been explored regarding its physiological, and anxiety-like behavioural status which referred that environmental enrichment had a limited effect on fish growth and anxiety-like behaviours (Xu et al., 2022). Thus, the effect of environmental enrichment varies depending on fish species as well their different habitat types ranging from wild to captive conditions. In a captive environment, enrichment can also influence other behavioural attributes such as aggression

in fish, while it depends on the specific requirements of the individual species and its life stage, its natural history and preferences (Näslund & Johnsson, 2016), and nutrient availability.

Diet can also influence fish physiology and behaviour. For example, dietary xenobiotics (e.g., persistent organic pollutants (POPs), Polycyclic aromatic hydrocarbons (PAHs)) can cause greater mobility, lower exploratory activity and higher anxiety in fish, with subsequent disrupted behavioural performance (Vignet et al., 2014). Moreover, diet can induce unusual behaviour and increased cortisol (stress indicator) in fish. For example, a recent experiment in zebrafish revealed that high caffeine concentrations (i.e., 200 mg/L) can induce abnormal swimming behaviour and higher cortisol than the baseline level (Rosa et al., 2018). However, as most studies have been carried out under controlled laboratory conditions, the dynamics in the natural environment can be much more complicated.

Dietary supplements (immunostimulants) have been introduced and used in aquaculture with beneficial effects for fish health and wellbeing (Yeganeh et al., 2015; Vallejos-Vidal et al., 2016). Recently, immunostimulants including prebiotics and probiotics have received an increasing attention as an environment-friendly approach for improving fish health, and have been suggested as an alternative to the use of therapeutics and antibiotics in the aquaculture during the past few years (Hoseinifar et al., 2015). Moreover, probiotics (e.g., many *Bacillus* species) and prebiotics (e.g., B-glucan) have been observed to impact the physiology and stress responses of host organisms. Prebiotic supplements (non-digestible food ingredients that selectively work on the host to stimulate the growth and/or activity of a limited number of gut bacteria (Gibson et al., 2004; Akhter et al., 2015)) added to the diet of different fish species trigger promising results in immune

response (Song et al., 2014; Carbone & Faggio, 2016; Guerreiro et al., 2018). An experiment with dietary prebiotics (mannan-oligosaccharide) in zebrafish (*Danio rerio*) showed that prebiotic diet helped the diet deprived fish to behave more similar to the normally fed (not diet deprived) ones (Forsatkar et al., 2017). Some plant based essential oils have also been evaluated to enhance disease resistance and growth by improving gut health and biological activities (i.e., stress response and immunity) in aquatic animals (Sutili et al., 2018). However, inconsistencies have been observed in some studies using prebiotics (Dobšíková et al., 2013; Eshaghzadeh et al., 2015) which then led to another alternative, the use of probiotics.

Probiotics can be incorporated as beneficial live organisms into the diet to enhance fish immunity, to prevent and control of various diseases in aquaculture (Harikrishnan et al., 2011; Yang et al., 2014). Alongside, the impact of probiotic enriched diet has also become an emerging interest for their effects on fish behaviour. In Nile Tilapia fry, probiotic diet (*Bacillus subtilis* and Biogen®) had shown significant improvement on survival and feeding behaviour, with no effect on activity (Soltan & El-L, 2008). Probiotic diet can also play a vital role in host health by increasing the number of beneficial bacteria in the gut (Luan et al., 2023), which ultimately can influence the composition of gut microbiome and host performances (i.e., growth and immune response) (Merrifield & Carnevali, 2014; Allameh et al., 2017). Probiotics have been reported to modulate microbiome-gut-brain axis and shoaling behaviour in zebrafish (Borrelli et al., 2016). Evidence of other behavioural changes such as anxiety related behaviour in zebrafish (Davis et al., 2016; Valcarce et al., 2020) using probiotics has also been observed. But there is limited research in this field in fish compared to mammals, especially regarding the homeostasis in fish behavioural and physiological response to environmental conditions. Moreover, the combined effect of probiotic

diet and rearing environment on fish behaviour has not been explored. The tested probiotic diet by INVE aquaculture in this study have been tested as an effective option for other fish species previously. Therefore, we combined this probiotic diet along with the rearing environments under laboratory conditions in this study to investigate exploratory behaviour of fish.

Exploratory behaviour is a commonly studied trait (Burghardt, 2013). Behavioural differences between wild and domesticated zebrafish were found to vary due to their rearing conditions. However, sex differences and genetic variations (different strains) can also have influence on fish behaviour (Gorissen et al., 2015; van den Bos et al., 2017; Genario et al., 2020), as well as individual differences, reported in zebrafish for example (Demin et al., 2019; Volgin et al., 2019). Fish physiology and behavioural responses under different environmental settings can interplay with the genotype, making it difficult to disentangle then genetic from the environmental effects. Genotype and environmental interactions (G x E) in fish can generate ranges of phenotypic responses (plastic or non-plastic) depending on individual and species level. These interactive effects (G x E) can be measured following the changes between or across environments (Christensen et al., 2021). Some examples of G x E in fish include temperature-induced sex determination (Geffroy et al., 2021), growth (Srimai et al., 2019; Freitas et al., 2021; Gonzalez et al., 2022), heritability of particular traits of importance for production (Mengistu et al., 2020) and selective breeding in fish farming (Gulzari et al., 2022).

Inbred fish, which have a less diverse genetic background due to mating between close relatives, can help to investigate the behavioural and physiological responses to changes in the environment, by reducing the interference of the genetic background. The naturally inbred and self-fertilising

mangrove killifish (*Kryptolebias marmoratus*) are ideal for this kind of research because genotypes can easily be tracked under controlled laboratory conditions, where genetically homogenous lines can be compared under precise environmental conditions. It allows us to have control on its genotype and to explore the impacts of its early-life physiological factor (e.g., hatching) and different early rearing environments (Figure 1.1.). The main aim of this study was to disentangle the effects of the genotype and the rearing environment (diet and environmental enrichment) on phenotypic responses (behaviour and physiology) and gut microbiome, using the mangrove killifish (*K. marmoratus*) as a biological model.

1.3. Materials and methods

1.3.1. Experimental setting

Two different genetic strains (DAN and HON9) of naturally inbred mangrove killifish *K. marmoratus* were used for the experiments. Those have been maintained in the laboratory for > 30 generations of inbreeding. This two genetically different strains of killifish have been maintained in the facilities since 2013 and the two strains were genetically characterized using the Microsatellite (27) genotyping with multiplex 1,2, 3, and 4 prior to set the experiment. Fish were reared under two environments (enriched and poor) and two diet enrichments - Easy Dry Seclo (EDS) and Probiotic (Table S1.1., Table S1.2. and Figure S1.3.). Inclusion rate for probiotic solution followed protocols for Cutthroat Trout larvae and fry (Arndt & Wagner, 2007) (see also supplementary material S1.4.). Fish were reared in rectangular plastic tanks (~1L capacity) 16.5 cm long, 11 cm wide and 10 cm high. All tanks, enrichment substrates, fish handling equipment and water holding tanks were cleaned using laboratory grade sterilizing solutions prior to installation and fish releasing. Water level was maintained at ~500 ml/tank and fish were reared individually in separate tanks from hatching. Each enriched tank was set up with one artificial

plastic log (~2 inch long and ~1.5-inch diameter) and two artificial small plants and poor environment tanks lack these. The experimental plan was approved by the Faculty of Science and Engineering Ethics Committee at Swansea University (AWERB IP Reference: IP-2021-07).

1.3.2. Parental generation

Eggs were collected daily from self-fertilizing adults (~10) *K. marmoratus* (DAN and HON9 each) and located in 50 ml jars. Collected eggs were recorded and registered individually based on their parental strains. Eggs were monitored on a regular basis to check developmental stages (Figure S1.5.A., and Table S1.5.B.) and remove any dead eggs. Eggs were reared under standard conditions (12:12 hours light/darkness, Temperature 24-26°C, ~14 ppt salinity) throughout the incubation period. Eggs were hatched either naturally (for DAN, n=16 and for HON9, n=36) or through manual artificial dechorionizing due to diapause (for DAN n=24 and for HON9 n=4) if they had not naturally hatched for 30 days (Figure S1.5.C.). Individual alevins (N=80) were then allocated to individual tanks corresponding to four experimental groups (10/group and 40/strain) (Figure S1.3.).

1.3.3. Behavioural test tank preparation and measurement

A novel object exploration test (Berbel-Filho et al., 2020) was performed on individual fish ~10 months old. Fish were not fed for 24 hours before the experiment, and then were transferred carefully from the rearing tank to the custom-made behavioural tank set up (Figure S1.6.) and acclimated for ~15 minutes in an isolated area. Tanks were divided into 5 zones, with 0 being the acclimation zone and 5 the furthest from it, the novel object (a Lego piece) was located in the middle of the tank (zone 3). Individual fish behaviour was recorded for 20 minutes using overhead

cameras. All the recorded videos were then stored for further processing until the end of the experiment. The videos were analysed using BORIS v. 7. 12. 2 software (Friard & Gamba, 2016) and the following behaviours (Table 1.1.) were recorded (Table S1.8):

Table 1.1. Behavioural parameters with definition used for the experimental *K. marmoratus*

Behaviour	**Definition**
Latency	The waiting time (seconds) of fish in the acclimation zone (Zone 0) counted as the time duration from the time the gate opens until starting to explore the first exploratory zone
Exploration	Total time spent in all exploratory zones (Zone 0-5) throughout the experiment duration (20 minutes)
Inspection time	Total time spent in the novel object zone (Zone 3) during the overall exploration
Inspections	The frequency of inspecting the novel object zone (Zone 3) by fish
Contacts	The number of touches to the novel object by fish in the total time of exploration
Activity	Total number of crosses among zones (Zone 0-5). A fish was considered entering a new zone once the entire fish has left the existing zone

1.3.4. Basal metabolic rate

One week after the behavioural test, fish were individually tested for respirometry to avoid any stress interference in the results. Basal metabolic rate (BMR) was measured as a proxy to stress as

it was previously observed that there is a positive corelation between BMR and cortisol in killifish. One-week interval was maintained to make sure that the fish were recovered fully from the exploratory behavioural experiments to avoid any stress interventions. So, I could acclimate the fish and confirmed the measurement of the resting metabolic rate of the fish. Basal Metabolic Rate (BMR) was measured though the consumption of oxygen over time to estimate the amount of energy expended at resting period of fish in more stable temperature environment. Fish were fasted overnight (Killen et al., 2007) following the regular husbandry regime prior to acclimation of ~4 hours (Pope et al., 2014), which had been previously established for mangrove killifish (LeBlanc et al., 2010). During acclimation, water in the chamber was flow-through. Fish were transferred carefully with a scoop of small net from the home tank to the respirometer chamber prior to set for acclimation to minimize stress. Fish were weighed to normalize the readings before setting for the acclimation. Two blank trials were carried out to confirm no leakage of oxygen. Oxygen levels were calibrated using saturated oxygenated water (100% dissolved oxygen) and anoxic water (~0% dissolved oxygen by using OXCAL 0% O_2 calibration capsules for optical oxygen sensors by Pyroscience, GmbH).

To measure BMR, we maintained a homogenous oxygen concentration within the chamber following sealing of both inlet and outlet valves, using a magnetic stirrer in a false bottom in the chamber. After acclimation, oxygen consumption was measured once for each fish for ~40 min. We measured the decrease in oxygen concentration using closed respirometry in real time with fibre optic probes, FireStingO2 Optical Oxygen Meter with respective sensor spots (OXSP5) all from Pyroscience (GmbH), which allowed us to ensure the oxygen concentration does not drop below a specified threshold level (typically 75%). A temperature channel was also used to consider

compensation temperature in the measurement process, and four respirometer chambers were run simultaneously (Figure S1.7.). At least 6.3 mg/l O_2 concentration and 70% oxygen saturation at 25℃ were maintained, saturation was detected looking into the normogram (Oprean et al., 2008). Finally, BMR was calculated considering the rate of oxygen decreases in the chamber, mass of the individual (weight), volume of water (60 ml) inside the respirometer chamber (50ml) and time of measurement (~40 minutes) (Table S1.9). Background respiration (average 19.36%) was corrected using blank runs.

1.3.5. Gut microbiome analyses

1.3.5.1. Sampling

After the BMR measurements, individual killifish from each experimental group were euthanized using anaesthetics solution of 2-phenoxyethanol (1ml/L). Fish were then placed on a clean Petri dish, to be beheaded and the caudal peduncle cut off, and the peritoneal cavity being opened to dissect the gut. Individual whole gut sample was then placed in the RNAlater solution (~1ml) and kept at room temperature overnight. Samples were stored at -80 ℃ the following day until further analyses.

1.3.5.2. DNA extraction, library preparation, and sequencing

DNA extraction was performed following the extraction protocol for microbial DNA using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN) for the gut samples (Uren Webster et al., 2018). Amplification of the 16S rRNA-V4 region (Klindworth et al., 2013) was performed using primers, 515F-806R (Caporaso et al., 2012) with updated sequences 515F:GTGCCAGCMGCCGCGGTAA (Parada et al., 2016) and 806R:GGACTACHVGGGTWTCTAAT (Apprill et al., 2015). PCR_1

consisted of a total volume of 22.5 µL incorporating 12.5 µL of Platinum™ II Hot-Start PCR Master Mix (2X) (Thermo Fisher Scientific), 0.5 µL of Forward (FP) and Reverse (RP) primers (10 uM), 9 µL of Ultra-pure water (UPW) and 2.5 µL of DNA. The PCR began with a 3 min denaturation step at 95°C followed by 27 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, then a final elongation step at 72°C for 5 minutes. During PCR_2, indexing with Nextera ® XT Index Kit v2 (Illumina, Inc., San Diego, CA, 92122 United States) was performed. PCR_2 was made with a total volume of 27.5 µL per sample, containing 2.5 µL of PCR_1 product, 1.25 µL of each index, 12.5 µL of Platinum™ taq and 10 µL of UPW. The reaction conditions were as above and with 11 cycles for PCR_2. Final PCR products were pooled from samples (N=46) based on agarose gel band intensity and cleaned using AMPure XP beads (Beckman Coulter Genomics, Brea, CA, United States). Final library quantification was performed using qPCR (NEB Illumina quantification kit), prior to sequencing on a MiSeq Illumina platform (300 bp, paired end). Samples were then further processed for bioinformatics to get the working samples depending on the higher non-chimeric reads yield.

1.3.5.3. Bioinformatics analyses

All Sequence data were then processed in Qiime2 (version: qiime2-2022.2) (Bolyen et al., 2019) for bioinformatics. DADA2 (Callahan et al., 2016) was used to truncate forward (220 bp), and reverse (155 bp) reads based on the quality filtering. After filtering mitochondrial, chloroplast and unclassified reads, and observing the rarefaction curves (to decide a common depth) (Figure S1.11.1) a sub-sampling (as a normalization step for the samples from a widely varied range of sequence reads) was done with a total of 9,906 features (to make an even sampling depth for all samples) where 15.32% amplicon sequence variants (ASVs) were retained from all the samples

(N=46). An extra filtering (sample contingency-based filtering, minimum 2 features' presence in at least 2 samples were retained across all samples) was performed and finally 1524 ASVs were left. The most abundant 30 ASVs were first analysed in fish samples (N=46) (Table S1.10) naturally hatched and artificially dechorionated; and then only in naturally hatched ones (N=25; DAN n=7 and HON9 n=18). Taxonomic classification was performed using the Silva reference taxonomy (v138) (Quast et al., 2012). Microbiome alpha (Chao1 richness and Simpson's evenness) (Table S1.11) and beta (Bray-Curtis and weighted UniFrac distances) diversity were estimated.

1.3.6. Statistical analysis

All analyses were carried out in R v. 4.2.2 (Team, 2013). Generalised linear models were used to analyse the effects of size, genotype (2 strains), environment (enriched/poor), diet (probiotic/standard), and hatching/incubation time (natural or artificial dechorionization) and the best model was chosen using *glmulti* (Calcagno et al., 2020) based on Akaike's Information Corrected Criterion (AICC) and likelihood-Ratio chi-squared test (LR Chisq). Basal metabolic rate (BMR) was calculated considering the decline in dissolved oxygen (DO) (mg O_2) per unit body mass in weight (g) of individual fish and per unit time (h) of the respirometry. A linear model was performed to assess the effect of fish strain, diet, and environment on BMR.

The influence of strain, diet, environment, and hatching (time) on microbiome alpha (Chao1 richness and Simpson's evenness) and beta diversity (Bray-Curtis and weighted UniFrac distances) was analysed. Alpha diversity of samples from (a) both natural and artificially hatched fish and (b) only naturally hatched fish groups was analysed using *glmulti* to choose the best model among best possible three models, considering the best-fit indicator (lowest Corrected Akaike Information

criteria, AICC). Beta diversity was visualised with non-metric multidimensional scaling using *vegan* (Oksanen et al., 2007) followed by further multivariate analysis of variance using *adonis2* (based on PERMANOVA, *vegan* package) with 99,999 permutations. To analyse the differentially abundant ASVs across all the samples (N=46), DEseq2 (M. I. Love et al., 2014) was used to compare between strains (DAN and HON9), diets (EDS and Probiotic) and rearing environments (Enriched and Poor). Differentially abundant ASVs (DAA) were then detected using DeSeq2 (Love et al., 2014) in R based on False Discovery Rate (FDR)<0.05 and among all the sample groups were observed with pheatmap (Kolde & Kolde, 2015) in R.

1.4. Results

1.4.1. Behaviour and BMR analysis

There were no statistically significant differences in length between strains (t-test= 0.08; p=0.93) and between environments (t-test=-0.58; p=0.56), therefore, length was excluded from further analysis. From all the recorded behavioural parameters (Table 1.1.), I excluded the contacts from further analysis because the contacts were on average <1. Total exploration and inspection time were also excluded as no effective model was run with any of the predictors for inspection time spent by individuals, and the best model for total exploration was run only including diet (best model AICC 1180.112, weights 0.247) but with no significant effect (Diet LR Chisq 2.86, Df= 1, p = 0.091). Inspection behaviour was significantly influenced by strains. Inspections were significantly influenced by strain (LR Chisq=106.726, Df= 1, p <0.001), environment (LR Chisq=6.446, Df= 1, p=0.01), and hatching type (LR Chisq=6.891, Df=,1 p=0.01)(Figure 1.2., Table 1.2., model comparisons in Table S1.12.). Among rest of the other behavioural parameters studied, the influence of diet was detected for the activity. Activity was significantly influenced by hatching type (LR Chisq=9.67, Df= 1, p=0.01), strain (LR Chisq=419.97, Df=1, p<0.001), and

diet (LR Chisq=84.27, Df= 1, p<0.001) (Figure 1.3., Table 1.2., model comparisons in Table S1.13.). A strong correlation (using Spearman Correlation Coefficients test) (Myers & Sirois, 2004) was observed for both strains [DAN (R=0.81, p<0.001) and HON9 (R=0.79 , p<0.001)] between active crosses (activity) and inspection time (s) spent by fish in the novel object zone (zone 3) (Figure S1.14.) indicating that more active fish tend to spend more time in the novel object zone. Probiotic diet fed fish from both strains were found as more active compared to EDS diet fed fish (Figure 1.3.). Neither strain, environment, or diet, nor their interactions affected BMR (Df=1, W = 0.989, p-value = 0.7265) (Table S1.15.).

1.4.2. Microbiome analysis

For the samples, the sequence resulted with lowest >1,900 reads and the highest > 78,000. Blank was sequenced alongside the samples and recorded ~46 reads. After denoising, the lowest non-chimeric reads obtained was >1,500 and the highest >49,000. Then the feature count for the samples was found as a total of 10,624. After the removal of mitochondria or chloroplast, total 10,415 features were retained. Then the sub-sampling and sample contingency-based filtering left total 1,524 ASVs . Of the most abundant 30 ASVs, *Vibrio* was the most abundant throughout all samples, followed by *Photobacterium*, *Lactobacillus*, *Shewanella* and *Prevotella*. (Figure 1.4.). Chao1 diversity (microbiome abundance and richness) was influenced by host strain (LR Chisq=10.133, Df=1, p<0.01), hatching type (LR Chisq=13.322, Df=1, p <0.001) and the interaction between diet and hatching type (LR Chisq=11.227, Df=1, p <0.001) (Figure 1.5.a., best model results in Table 3, models' comparison in S1.16.). The evenness of the microbiome distribution was then analysed using Simpson's evenness index, and diet (LR Chisq=8.475, Df=1, p <0.01), hatching type (LR Chisq=10.648, Df=1, p <0.01), and the interaction between diet and

environment (LR Chisq=9.904, Df=1, p <0.01) had a statistically significant effect (Figure 1.5.b., Table 1.3., models' comparison in S1.17.). By contrast, only hatching type influenced beta diversity (Bray-Curtis distance, Df=1, F-value=5.144, p<0.001 and Weighted UniFrac distance, Df=1, F-value=6.671, p=0.01) (Figure 1.6., Table 1.4.). Differential abundance analysis (DAA) identified 17 differentially abundant taxa (families) across all samples. *Vibrionaceae* and *Halieaceae* were found to be the most abundant in both DAN and HON9, whereas taxa such as *Flammeovirgaceae* were only found in HON9 (Figure S1.18.1, Table S.18.2.). Naturally hatched alevins were the one which did not enter diapause and the artificially hatched embryos were the one which entered diapause. So, the difference of the microbiome between these two hatched fish groups may be an influence of diapause.

We also analysed separately the microbiome of the fish which hatched naturally (N=25). *Vibrio*, *Photobacterium* and *Shewanella* were the most abundant ASVs across all naturally hatched fish gut samples. The most abundant 30 ASVs in the naturally hatched fish groups indicated a remarkable difference of distribution and diversity between strains (DAN and HON9) and diets (EDS and Probiotic) (Figure 1.7.). In the DAN-EDS-Enriched group (n=2), *Halieaceae* and *Staphylococcus* were the most prominent ones while in the HON9-Probiotic-Poor group (n=5), *Thermus*, *Rhizobiaceae*, uncultured *Bacteria*, *Mycoplasma* and *Gimesia* were the most abundant (Figure 1.7.).

Then, microbiome alpha and beta diversity of these fish samples were analysed for statistical significance tests, where chao 1 was influenced by strain (LR Chisq=5.82, Df=1, p <0.05) and diet (LR Chisq=6.64, Df=1, p=0,01); and Simpson's evenness was influence only by diet (LR

Chisq=4.72, Df=1, p <0.05) (Table S.1.19. with best model 1 and model comparisons). There was no effect of strain, diet or environment on beta diversity (Bray-Curtis and Weighted UniFrac distance) was also observed (Table S.1.20.). Despite the lower sample size, these results support those from both groups of hatching pooled, with both strain and diet influencing alpha diversity but not beta diversity.

1.5. Discussion

Individual genotype (heterogenicity) can influence behaviour such as social aggression and disease susceptibility (White et al., 2020) depending on the environment. We used the naturally inbred mangrove killifish (*K. marmoratus*) to disentangle the influence of genotype and environment on fish behaviour and microbiome. We found that fish strain (genetic background), diet and hatching type had significant effect on fish activity, while inspections (frequency) were influenced by fish strain, environment, and hatching type. Effect of diet on fish activity has been observed previously, for example, probiotic diet affected stress coping styles in triploid juvenile farmed Chinook Salmon (*Oncorhynchus tshawytscha*) with increased boldness and exploration behaviours under exogenous feeding (regular and probiotic feed) condition (St Louis, 2021). However, increased activity (observed in rainbow trout) may also manifest an amplified stress response to novelty (McGlade et al., 2022), and the performance and welfare conditions can vary between diploid and triploid individuals even if they are from same genetic line (Madaro et al., 2022). Thus, genotype and diet can influence fish behavioural responses, and reveal whether patterns of genetic or neuronal function are generalized or species specific in terms of relationship between genotype and behavioural phenotypes (Gallant & O'Connell, 2020).

Diet also plays an important role to maintain energy supply for the body (at least in mammals), more specifically influencing the changes in bile acids intestinal gut microbes and contributing to glucose tolerance and homeostasis (Sedgeman et al., 2018). However, we found that neither genetic background nor environmental conditions affected fish resting metabolic rate (BMR). Similar findings had also been observed in a study of genotype by temperature interactions in Glanville fritillary butterfly (Niitepõld, 2010). No effect of genotype and age groups interactions on metabolism were observed either in *Tafazzin*-Knockout mice (Tomczewski et al., 2023).

In fish, host diet, genetics, and specific rearing conditions (i.e., aquaculture) can affect the composition and function of the microbiome (Ghanbari et al., 2015). We observed the effect of genotype (strain) and diet on microbiome alpha diversity (chao1 richness and Simpson's evenness) and found a significant role of hatching time on gut microbiome structural composition (Beta diversity metrics, Bray-Curtis and Weighted-Unifrac distance). In a recent study in zebrafish, the developmental stages (larvae to adult) rather than environment (rearing) had significant influence on the gut microbiome (Xiao et al., 2021), which could explain the effect of hatching time, given that artificially dechorionated fish typically have spent longer time before hatching.

The fish microbiome can also be modified by diet (probiotic) which can ultimately influence their immune system. But, the interactions between host and bacteria partly depends on diet and other environmental factors (López Nadal et al., 2020). Here, *Proteobacteria*, *Vibrio* and *Shewanella* sp. were identified as the predominant groups of bacteria. Similar dominant bacteria were also recorded in the gut microbiome of another marine fish, such as *Sardinella longiceps* (Johny et al., 2022). Although we were expecting to see an effect of probiotic diet on the gut microbiome

composition in our study, no taxa of probiotic bacterial origin (combination of *Bacillus* sp.) were observed in the list of most abundant 30 ASVs in the samples. Instead, the gut microbiome composition (top30 ASVs) was dominated by the genus *Vibrio* regardless of variations among different treatment groups. Although *Vibrio* are generally considered as an indicator of disease in fish (Ofek et al., 2022), we did not find evidence of infectious disease in our experimental fish. However, fish can also act as natural reservoirs of pathogenic *Vibrio* species (e.g., *V. cholerae*) on a small scale (Senderovich et al., 2010), but here we could only classify *Vibrio* up to genus level, so its potential pathogenicity is unclear.

The second most abundant genus identified in our study was a member of Gram-negative bacteria *Photobacterium*, that can also have pathogenic potential. *Photobacterium* can be isolated from different marine environments, can pose sometimes negative influence (as pathogen) on the host (Labella et al., 2017). The next most abundant genus in our findings was *Lactobacillus*. Some isolates of *Lactobacillus* have been identified for their ability to inhibit the *in-vitro* growth of various fish pathogens and have been studied in estuarine fish *Mugil cephalus* as a potential probiotics for both freshwater and marine water aquaculture (Hatha et al., 2014). Dietary *Lactobacillus acidophilus* enhanced growth performance, antioxidant profiles and modulated immune-related gene expressions in the common carp, *Cyprinus carpio* (Adeshina et al., 2020). A recent study in zebrafish (*Danio rerio*) demonstrated the potential of certain *Lactobacillus* bacteria (*L. delbrueckii*) to decrease some anxiety and stress-related behaviours in fish through the gut microbiome-brain axis (Olorocisimo et al., 2022). Another prominent bacterium in the killifish microbiome was the genus *Shewanella*. *Shewanella* sp. has been found to restore lipopolysaccharide-induced intestinal microbiota dysbiosis in turbot along with the beneficial role

in upgrading the host gut-health (Zhang et al., 2020). *Shewanella putrefaciens* has also been used as probiotic in fish (increased growth in *Sparus aurata* L juveniles) (De La Banda, 2012).

Globally, our result indicates that the fish genotype (strain) and the addition of probiotics had a strong effect both on fish behaviour (activity was higher in probiotic fed DAN strain) and microbiome diversity (probiotic fed HON9 strain showed higher microbiome richness) (but not community structuring), with a less marked effect of the early rearing environmental enrichment. We also found a strong effect of the incubation time both in microbiome composition and activity, which warrants further research, particularly considering the potential impact of husbandry practices on the fitness of farmed fish. Yet, the effect of genotype and diet on microbiome diversity was supported by separate analyses of the naturally hatched group of fish, supporting the link between microbiome and host genotype as well as the potential of the probiotics to modify microbiome composition and potentially fish behaviour. Overall, fish genotype, diet, and hatching time significantly influenced the microbiome alpha diversity, where positive influence of strain was observed in HON9 with higher diversity and EDS diet had positive influence on both strains with higher evenness.

TABLES

Table 1.2. Results from the novel object exploration analysis, the best fitted linear models were chosen based on the Likelihood-Ratio chi-squared test (LR Chisq), and by using multi-model (generalized linear model) averaged approaches based on the corrected Akaike information criterion (AICC) and the weightings.

Models and variables	**LR Chisq**	**Df**	***P*- value**	**AICC**	**weight**
Inspections					
Model 1				1316.917	0.570
Inspections ~ 1 + Strain + Environment + Hatching type					
Strain	106.726	1	**<0.001**		
Environment	6.446	1	**0.01**		
Hatching type	6.891	1	**0.01**		
Activity					
Model 1				5754.552	0.737
Activity ~ 1 + Strain + Diet + Hatching type					
Strain	419.97	1	**<0.001**		
Diet	84.27	1	**<0.001**		
Hatching type	9.67	1	**0.01**		

Table 1.3. Alpha diversity results (Chao1 and Simpson's evenness) from the best models for fish (N=46) of both natural and artificially hatched groups using *glmulti* model method.

Parameters and Models	**LR Chisq**	**Df**	***P*-value**	**AICC**	**weight**
Chao1					
Model 1				574.79	0.168
Strain	10.133	1	**<0.01**		
Diet	3.392	1	0.066		
Hatching type	13.322	1	**<0.001**		
Diet:Strain	2.691	1	0.101		
Diet:Hatching type	11.227	1	**<0.001**		
Simpson's evenness					
Model 1				-102.431	0.203
Diet	8.475	1	**<0.01**		
Environment	0.012	1	0.913		
Hatching type	10.648	1	**<0.01**		
Diet:Environment	9.904	1	**<0.01**		

Table 1.4. Beta diversity results from fish both hatched groups (N=46) using *adonis2*

Diversity index and Experimental factors	**Df**	**Sum of Sqs**	**R^2**	**F- value**	***P*- value**
Bray-Curtis distance					
Strain	1	0.178	0.016	0.798	0.560
Diet	1	0.233	0.021	1.045	0.365
Environment	1	0.173	0.056	0.774	0.587
Hatching type	1	1.150	0.106	5.144	**<0.001**
Weighted Unifrac distance					
Strain	1	0.013	0.006	0.306	0.682
Diet	1	0.005	0.002	0.122	0.936
Environment	1	0.031	0.015	0.745	0.408
Hatching type	1	0.279	0.137	6.671	**0.01**

FIGURES

Figure 1.1. Environment and genetic strains influencing *K. marmoratus* responses.

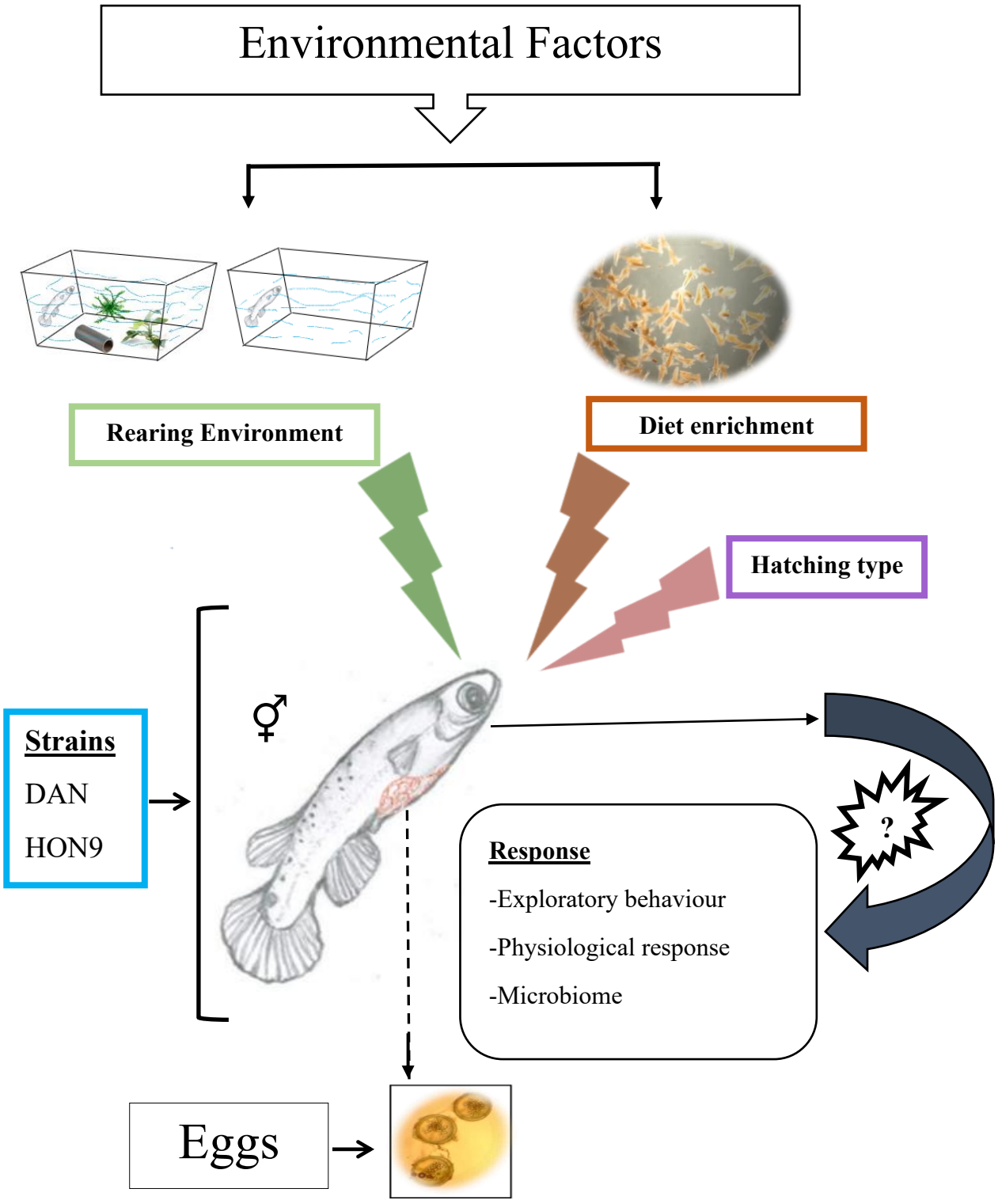


Figure 1.2. Inspections (frequency) by strain (DAN, HON9) and environment (Enriched, Poor), green colour showing fish from strain DAN and light orange from strain HON9.

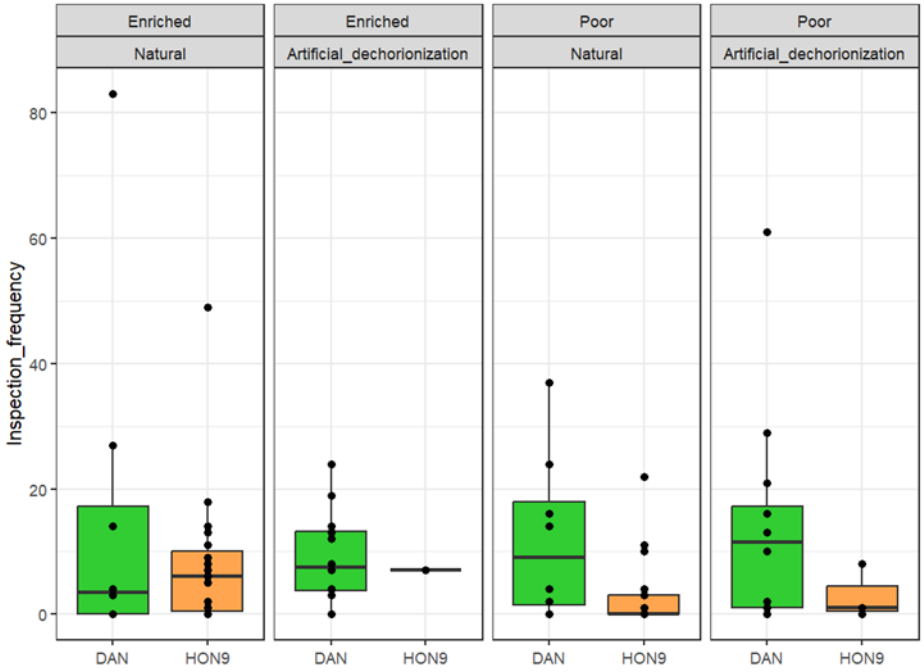


Figure 1.3. Activity by strains (DAN, HON9) from different diets (EDS, Probiotic) and hatching (Natural, Artificial dechorionization) groups, green and light orange colour representing strains DAN and HON9 respectively.

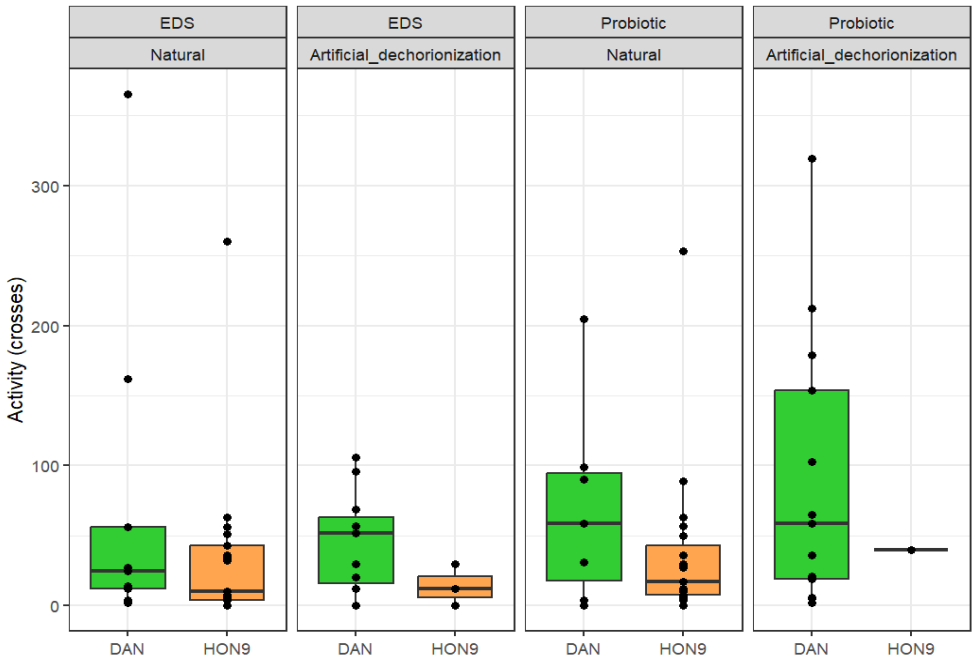


Figure 1.4. Most abundant 30 ASVs (family and genus level) observed across all samples (N=46), DF1= DAN Fish and HF1=HON9 Fish used to identify the fish individuals.

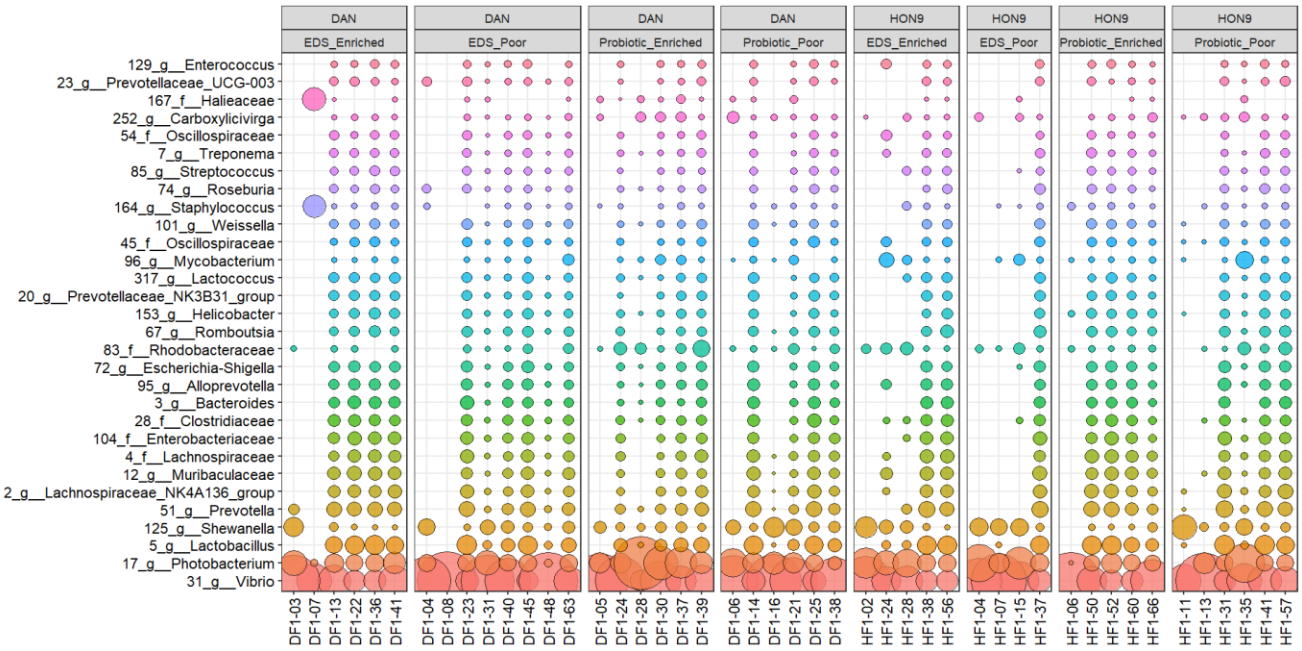


Figure 1.5. Alpha diversity results of gut microbiome (N=46)- (a) Chao1 richness, and (b) Simpson's evenness. Light green colour representing strain DAN and brown showing HON9.

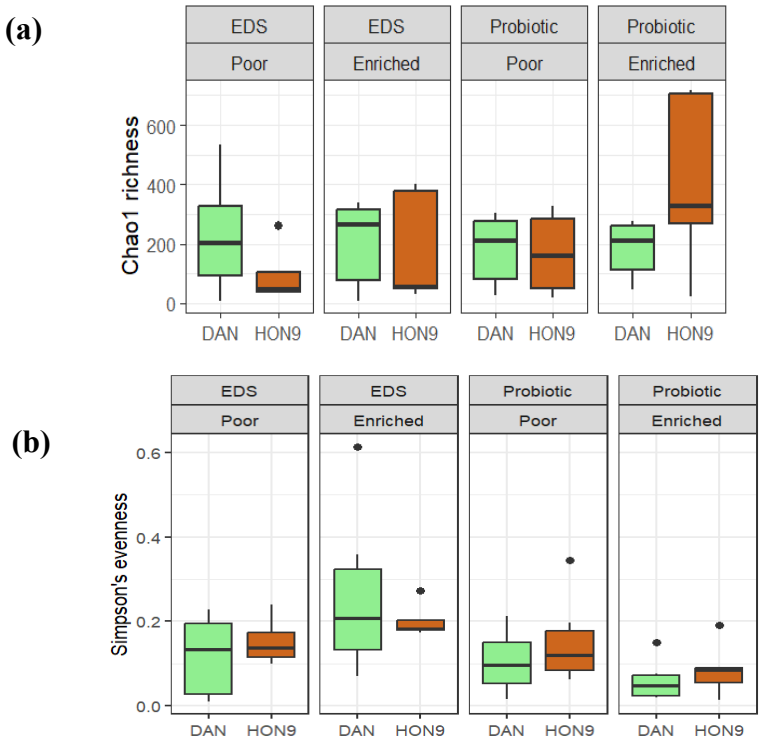
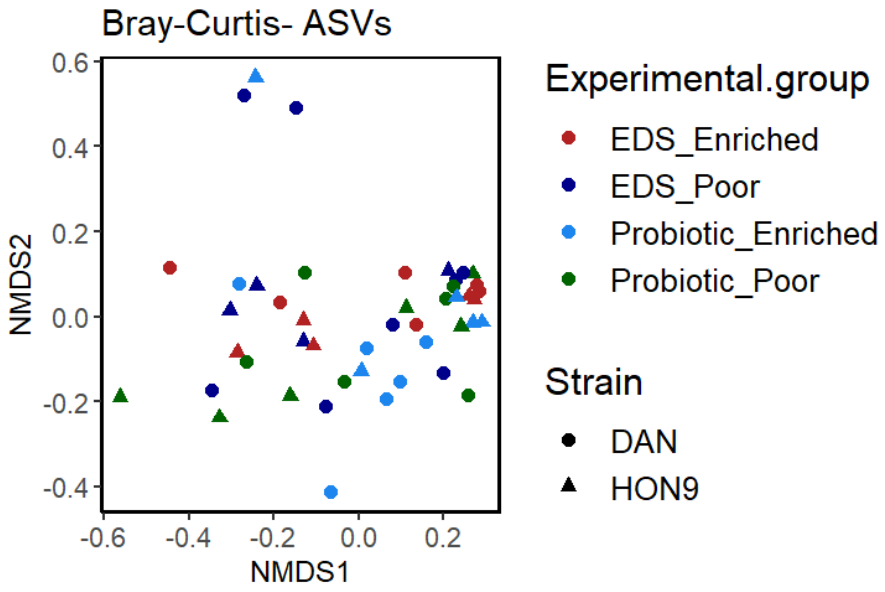


Figure 1.6. Beta diversity (ASVs) results plots from fish (N=46) hatched both naturally and artificially,

(a) Bray-Curtis and (b) weighted UniFrac distance

(a)



(b)

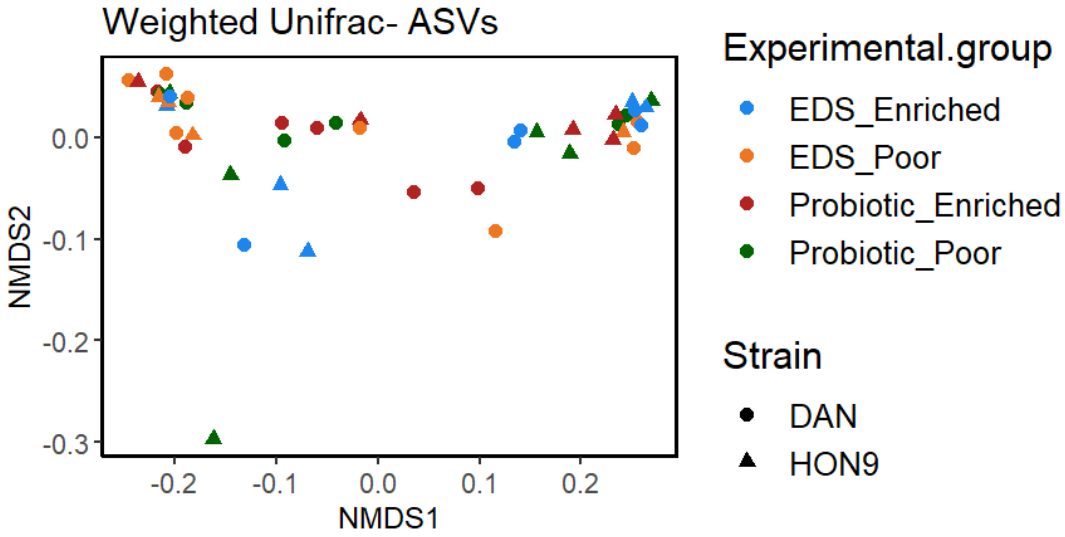
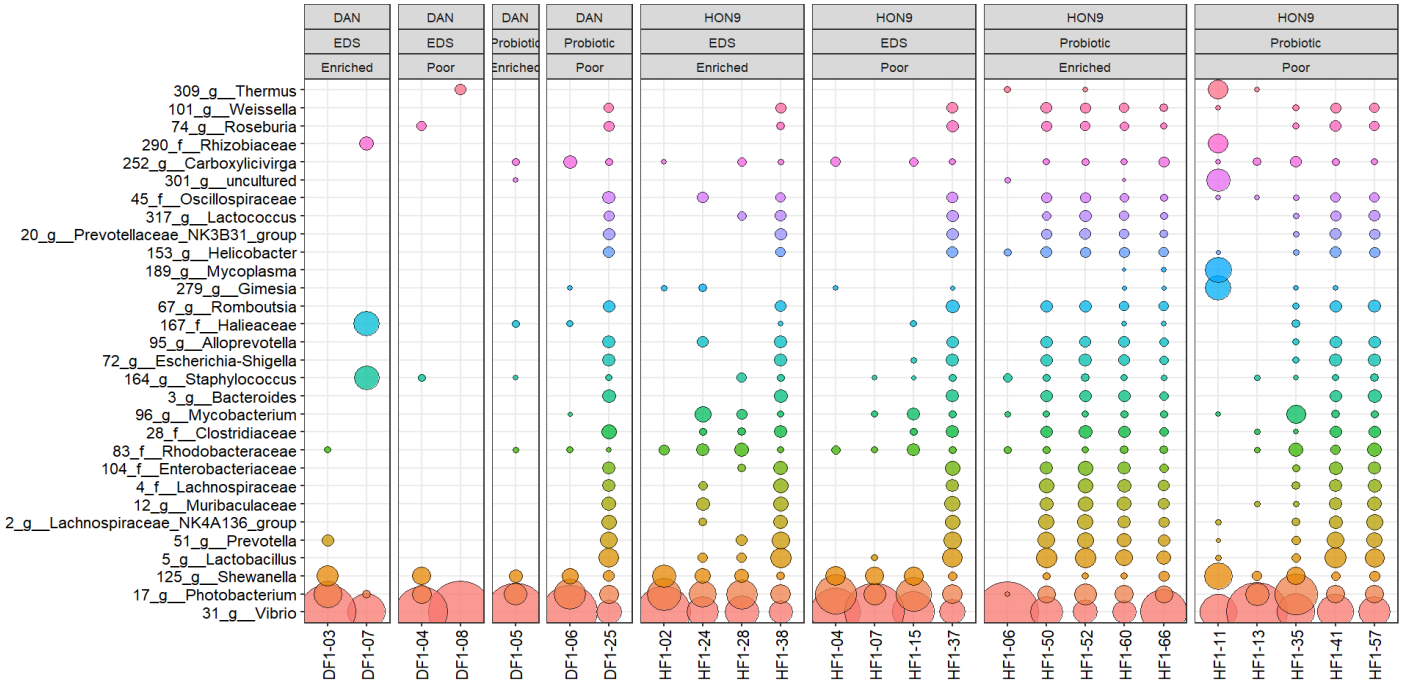


Figure 1.7. Most abundant 30 ASVs (family and genus level) in naturally hatched fish (N=25) from different environment and diet groups (analysed as a sub-sample of the previous analysis of all samples, N=46). DF1= DAN Fish and HF1=HON9 Fish used to identify the fish individuals.



CHAPTER 2: Intergenerational effects of early-rearing environment on inbred fish behaviour and basal metabolism

2.1. Abstract

Environmental changes influence fish physiology and behaviour with potential fitness consequences. Parental environment, behaviour, and stress can induce phenotypic changes over several generations. Consequently, understanding the influence of early rearing environment on behavioural changes is critical for captive breeding and restoration programmes. I investigated the role of genotype and environment (own and parental) in the behaviour and basal metabolic rate (BMR) of two generations of mangrove killifish (*Kryptolebias marmoratus*) from two naturally inbred lines, using a novel object test, and closed respirometry. There was a significant influence of the offspring rearing environment and its interaction with the parental environment on offspring's inspection and activity, while none of them influenced their BMR. A significant influence of parental activity on offspring's activity was also detected, although no significant difference was observed between the strains. The overall results highlighted a potential intergenerational influence of rearing environment on fish behaviour. These findings have further implications for understanding of behavioural adaptations in fish and for ensuring the optimum rearing environmental conditions for sustainable fish farming and management.

2.2. Introduction

Parents can influence both genetic and environmentally their offspring's survival, growth and phenotype (Cortese et al., 2022). These intergenerational effects can occur as a result of epigenetic changes in fish when parents and their offspring experience environmental challenges such as ocean acidification, increased temperature (Colson et al., 2019; Cohen-Rengifo et al., 2021), or changes in the rearing environment (i.e., captive rearing) (Venney et al., 2023). Environmental enrichment during early development has also been found to induce intergenerational effects on fish behaviour (Berbel-Filho et al., 2020).

Most studies have focused on understanding the non-genetic maternal effects (Zeender et al., 2023). For example, maternal effects such as egg mass or clutch size can affect early life social phenotypes (Reyes-Contreras et al., 2023) or anti-predatory behaviour (i.e., escape reflex studied in breeding cichlid, *Neolamprologus pulcher*) (Sharda et al., 2021) in the offspring. The impacts of maternal inbreeding on phenotypic variation such as offspring size and the mechanisms of sex determination have been observed in fish species, such as Alpine whitefish (de Guttry, 2021).

Parental effects on offspring fertilization and larval development have also been studied in relation to environmental stability (Guillaume et al., 2016) as environment can affect fish with unstable conditions (e.g., unusual seasonal fluctuations of temperature and excess nutrient loading), but in fish there is still very limited information on intergenerational effects related to parental environments and stress (mostly focused on zebrafish), and their impact on offspring's phenotype (behaviour and physiology) (Ord et al., 2020). Parental effects can further impact the reproductive potential and population dynamics in fish depending on the previous environmental experiences

(Domínguez-Petit et al., 2022), which can alter offspring's adaption to a different environment than their parents' (Jensen et al., 2014; Stein & Bell, 2014). Parental effects are not always positive, for example parental environmental instability can result in variation in spawning success (Mitterwallner & Shima, 2022) which can further affect negatively offspring's survival or fitness, along with some other negative influences such as pollutant effect on embryonic development (Lyons & Adams, 2015), or improper spawning conditions (i.e., site, timing) (Jones, 1981; Tillotson et al., 2019). These effects result from both parental genetics and phenotype (i.e., size as indirect genetic effect) (Marteinsdottir & Begg, 2002), and from parent's behaviour (i.e., parental care as a selection trait) (Goldberg et al., 2020). But there is still little understanding of the relative role of parents' environment and genotype on the offspring's phenotype and behaviour (intergenerational effects).

Parental stress such as predation has been found to shape offspring phenotypes (in guppy, *Poecilia reticulata*), where both parental and individual predation cues led to increased activity and anti-predatory behaviour in offspring in a sex specific manner (Stein & Hoke, 2022). Intergenerational effects of predation have also been observed in offspring (in mice) with a better adaptative behaviour (more active) against predator exposure in a predation-dense environment (Brass et al., 2020). In contrast, other aspects such as parental diet (overfeeding) do not seem to have significant intergenerational influence on fish, for example in zebrafish's anxiety-like behaviour (Anwer et al., 2022). At the individual level, a significant effect of rearing environment on risk taking behaviour was observed in rockfish, *Sebastes schlegelii* (Zhang et al., 2023). In terms of rearing environment, physical enrichment such as sand substrate in Senegalese sole, *Solea senegalensis* was used to promote fish welfare by lowering the occurrence of detrimental behaviour (i.e.,

aggression) developed by fish in captive environments (Almeida et al., 2023). Exposition to environmental enrichment compared to the standard environment in fathers and grandfathers can result in inherited changes in phenotypes (locomotion and morphology) in zebrafish larvae (Green & Swaney, 2023). But more specifically, early life environment can impact the subsequent generations in a more complex way than directly influencing the germline or parental physiology (Burton & Metcalfe, 2014), and can result in a "predictive adaptive response" (higher fitness in a matching environment) as a potential transgenerational effect (Massamba-N'Siala et al., 2014).

Different genotypes and environmental conditions produce ranges of organism's phenotypic variation (Sha & Hansson, 2022). One genotype can produce various phenotypes (plasticity) under different environmental conditions and, if affecting multiple generations (Pigliucci, 2001), can be maladaptive. For example, chronic oil exposure (in adults) can impact progeny feeding efficiency for as a minimum of two generations in Gulf killifish, *Fundulus grandis* (Little, 2020), and early life exposure to environmental pesticides in zebrafish can result intergenerational effects such as delayed hatching, offspring deformities, and increased latency like activity (Lamb et al., 2020; Marchand, 2023). The impact of rearing environment (social and structural enrichment) was tested on the boldness and exploratory behaviour in turquoise killifish (*Nothobranchius furzeri*), demonstrating that fish reared in an enriched environment were bolder (i.e., entered faster in the novel zone) compared to the fish from poor rearing environment (Thoré et al., 2020). However, most studies on the influence of the parental environment on the offspring have focused on environmental conditions with very limited consideration of genotypic influence. Intergenerational acclimation to extreme thermal conditions was observed in zebrafish as an indicator of complex inheritance of stress response in fish (Lim & Bernier, 2023). However, stress response due to

increased temperature can differ based on the stress physiology of fish (Alfonso et al., 2021) and their perception of internal or external environmental cues, and stressors (Schreck & Tort, 2016). Information on genotypic and environmental parental influence on fish behaviour and stress response is still scarce. On this basis, and given the increasing importance of captive rearing in fish (both for conservation and commercial purposes), I investigated the potential intergenerational influence of early rearing environment using different genotypes reared in contrasting environmental conditions.

As a model species I used the naturally inbred and self-fertilizing mangrove killifish, *Kryptolebias marmoratus*, which is being increasingly used to investigate the effect of environmental and genetic factors on phenotypic plasticity at different life stages and across generations (Berbel-Filho et al., 2020; Fortunato, 2023).

2.3. Materials and methods

2.3.1. Fish husbandry and experimental design

Parental fish (F0) tanks (details in materials and methods section in Chapter 1) were checked regularly for collecting the eggs to get the first experimental generation (F1). All collected eggs were incubated in the same conditions and with the same husbandry practices as their parents. Individual fish (F1) were obtained from parents of two different genetic strains - DAN and HON9 of *K. marmoratus* reared in two different environments [Enriched (E) and poor (P)], as described in Chapter 1. The parental generation consisted of 40 DAN and 40 HON9. For the F1, alevins (N=93; DAN n=49 and HON9 n=44) were allocated and reared individually in tanks either with the same environmental conditions as their parents (E-E and P-P) or different (E-P and P-E).

(Figure 2.1.). The environmental condition was considered as the rearing environmental enrichment which was termed as Enriched environment and lack of this was standard Poor environment in this study. Same controlled environment and husbandry was maintained and monitored throughout the experimental period of both parents and offspring. And, regarding the different rearing condition of the offspring, it means if parent was reared in Enriched environment, the offspring was reared in Poor environment and vice versa. The offspring were fed with commercial diet enrichment- Easy dry Seclo encapsulated artemia and reared under a standard 12:12 light regime. Sample sizes for the offspring were as follows: DAN E-E n=10, P-P n=13, E-P n=13, P-E n=13; and HON9 E-E n=13, P-P n=11, E-P n=11, P-E n=9 (Table S 2.1.).

2.3.2. Intergenerational effects (F0 vs F1) on behaviour and metabolism

F1 generation ~6 months old fish were individually assessed for behaviour (novel object exploration test) and basal metabolism rate (BMR) measurements in the same way as their parents (F0) (methods details and definition of behavioural patterns are in Table 1.1. in Chapter 1) and all the parameters were recorded to assess for potential intergenerational effects (behavioural metrics:- total exploration, inspections, and activity; and BMR with oxygen consumption).

2.3.4. Statistical analysis

For both behaviour and BMR, analyses were performed in R v. 4.2.2 (Team, 2013). Generalized Linear Models were carried out using *glm2* (Marschner, 2011) and *lme4* (Bates et al., 2014) to analyse the effects of fish size (length), genotype (strain DAN and HON9), and the intergenerational effects of the rearing environment by considering the effects of offspring's rearing environment, parental environment and the potential interaction between parent's and

offspring's environment. For all the studied behavioural parameters (total exploration, inspections, and activity); statistical models were run with and without including the parent of origin (Parent ID) as a random factor. Model comparisons (with and without Parent ID) were performed using analysis of variance (*anova*), and best models were chosen based on the lowest Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC). Then, a Generalized Linear Model was performed using *glm2* (Marschner, 2011) to check if there is any effect of parental activity on offspring's activity, including offspring's length as an additional predictor into the model.

For BMR analyses, a model comparison was performed between two different linear models with and without parental ID as a random factor. In both models, fixed factors included offspring's length, their own and parental environments and strain. The best model was selected based on the lowest AIC and BIC values and then the best model was run to get the final statistical results for BMR. An additional Generalized Linear Model was run using *glm2* (Marschner, 2011) to check the effect of parental basal metabolism on offspring's BMR including parental oxygen consumption and offspring's length as predictor variables.

2.4. Results

2.4.1. Intergenerational effects on behaviour

The intergenerational influence of rearing environment (Enriched and Poor) was tested in this study in two mangrove killifish strains (DAN and HON9). The average fish length of the offspring differed significantly between strains (t-test=-2.6393, p=0.009), with an average of 2.04 cm in strain HON9 (SD=0.109) and 1.98 cm in strain DAN (SD=0.095) (Table S2.1.1.). Fish from HON9

and DAN spent on an average of 846.80 seconds (SD=349.06) and 725.33 seconds (SD=406.50) total time exploring, respectively. The best model (run with "gaussian" family) for exploration included only the fixed factors, offspring's length, strain, environment, and their parental environment but indicated that the total exploration time spent by the offspring was not influenced by any of the factors, either strain (t-value=1.388, p=0.169), length (t-value=0.154, p=0.878), and the offspring's environment (t-value=1.434, p=0.155) or by their parental environment (t-value=-0.546, p=0.586) (Model comparison in Table S2.2.1. and the best model results in Table S2.2.2.). In the case of inspections (frequency to inspect: DAN average ± SD 7.37±7.47; HON9 average ± SD 8.39±13.76), the best model (run using "Poisson" distribution) included parental ID as a random factor and strain, offspring's length and environment, parental environment, as well as the interaction between offspring's own and their parental environment, as predictors. The offspring's own environment (z-value=2.84, p=0.004) and the interaction between their own and parental environment (z-value=-3.63, p<0.001) had a significant effect on offspring's inspections (fish reared in poor environment inspected the novel object less frequently), but strain (z-value=-1.22, p=0.22), offspring's length (z-value=0.41, p=0.68), and their parental environment (z-value=-1.241, p=0.214) had no significant effect (Figure 2.2., Table S2.3.1. and Table S2.3.2.).

Across all sample groups, only 10 DAN and 6 HON9 individual offspring made contact with the novel object, only once or twice maximum, so I decided to exclude this from further analyses due to the small number of contacts (Table S2.1.1.). Offspring's average activity was 45.59 crosses in strain HON9 (SD=69.21) and 35.20 crosses in strain DAN (SD=32.62). For activity, the best fitting model (run with "Poisson" family) included strain, length, offspring's environment, parental environment, and the interaction between offspring and parental environment as fixed factors, and

the parental ID as a random factor. Offspring's length (z-value=2.972, p=0.003), their own environment (z-value=3.822, p<0.001), parental environment (z-value=-4.180, p<0.001), and the interaction between their own and parental environment (z-value=-5.185, p<0.001) had a significant effect on offspring's activity (lower activity in fish reared in poor environment). There was no significant effect of strain on offspring's activity (z-value=-1.138, p=0.255) (Figure 2.3., Model comparison in Table S2.4.1. and the best model results in Table S2.4.2.). We also observed an effect of parental activity (z-value=3.265, p<0.001) and offspring's length (z-value=2.522, p=0.012) on offspring's activity (Table S2.5.).

2.4.2. Intergenerational effects on BMR

The basal metabolism of the offspring was measured and corrected with percent background respiration (average ± SD 18.05±9.41) using blank runs alongside individual BMR measurements. The average BMR of the offspring was 0.013 mg O_2 consumption/h/g in strain HON9 (SD=0.006) and 0.011 mg O_2 consumption/h/g in strain DAN (SD=0.005) (Table S2.1.2.). Models including and excluding parental ID as a random factor were compared. The best model included parental ID as a random factor and offspring's strain, length, own environment, parental environment, and the interaction between own and parental environment as fixed factors. We observed no effect of offspring's strain (t-value=1.165, Std. error=0.002, p-value=0.2560), length (t-value=-1.209, Std. error=0.005, p-value=0.2301), their own environment (t-value=-0.868, Std. error=0.001, p-value=0.3880), and parental environment (t-value=-1.441, Std. error=0.002, p-value=0.1625) on offspring's BMR in this study (Figure 2.4., model comparison in Table S2.6.1. and the best model results in Table S2.6.2.). In the Generalized Linear Model (run with "gamma" family) for assessing the effect of parental BMR on offspring's BMR, we observed no effect of offspring's length (t-

value=0.460, p=0.647), or parental BMR (t-value=-0.851, p=0.397) on offspring's BMR (model results in table S2.7.).

2.5. Discussion

The parental environment can play an important role in their offspring' behaviour, and we investigated its role and that of genotype in potential intergenerational effects on fish behaviour and physiology in highly inbred fish. We found that parental rearing environment affected fish behaviour either directly or through its interaction with offspring's own environment. Fish originated from a poor parental environment were less bold (inspections of a novel object) and had lower activity.

The effects of parental environment can be important at different levels, but have been rarely explored on both parents and offspring (Mir et al., 2023), and it is not clear how environmental change is influencing the behavioural activity and physiology of fish in the wild (Blewett et al., 2022). We found an effect of both offspring's own and parental environment on offspring's activity. The effect of the rearing environment had been previously observed on the activity of the of the *K. marmoratus*, with lower activity in individuals reared under poor conditions, similar to the ones in this experiment, but no effect of the parental or offspring's own environment on the offspring behaviour had been previously identified (Berbel-Filho et al., 2020).

Here, we found that parental activity had a significant influence on their offspring's activity but no difference between the inbred lines, suggesting that the influence could be more than just genetic, potentially epigenetic. In fact, Berbel et al. (Berbel-Filho et al., 2020) found that different

environment result in different epigenetic signatures in the brain, some of which could be transferred as for the behavioural patterns. In contrast, a previous study on the same killifish species, *Kryptolebias marmoratus* found no parental influence on offspring's developing behaviour (exploration, boldness, and aggression) from different rearing environmental factors such as food and predation risk (Edenbrow & Croft, 2013). The effect of genotype and environment has been observed in *K. marmoratus* in a recent study, where aggression varied significantly in juveniles (~40-43 days post hatching (dph)) compared to adults (~86-90 dph) among individuals and genotypes and the rapid changes of the tested aggression in juveniles was most likely through natural selection (Fortunato, 2023; Fortunato & Earley, 2023) with a further scope of behavioural changes throughout different life stages, but no intergenerational influence was tested in that study. The effect of genotype on feeding behaviour (nutrient intake) has also been detected in other fish species, for example selected genotypes of the European sea bass, *Dicentrarchus labrax* (Montero et al., 2023). Thus, both genotype and the environment could be potentially responsible for behavioural variation.

Environmental factors can cause positive or negative influence on fish behaviour. Environmental complexity can make fish either bold or shy in terms of exploration (Sales et al., 2023). We observed no effect of the rearing environment on the total exploration time of the offspring in our results. However, a previous study in Chinook salmon, *Oncorhynchus tshawytscha*, identified an effect of structural enrichment on exploratory and anti-predatory behaviour (Cogliati et al., 2023). Environmental enrichment can have beneficial effects at different life stages, and can be useful for fish to cope with diverse stressful events. This has made structural enrichment an emerging interest for rearing fish in captive environments (Arechavala-Lopez et al., 2022), especially during the

early life stage. Enriched rearing resulted in increased growth in Atlantic salmon (*Salmo salar*) in a semi-natural pond environment (Karvonen et al., 2023), and increased survival and more natural behaviour in Arkansas darter, *Etheostoma cragini* (Kopack, 2023). But enrichment can also influence (by inducing) the aggression and stress of captive fish differentially at different life stages, and therefore may not always be the best option for fish farming (Näslund & Johnsson, 2016). For example, in zebrafish, enriched environment in early rearing can reduce anxiety like behaviour and increased boldness (Gatto et al., 2022), although not in all cases an effect of environmental enrichment has been detected on aggression (Sarma et al., 2023). Some favourable effects of early life environmental enrichment were also observed in improved stress management and exploratory behaviour of sterlets, *Acipenser ruthenus* (Fazekas et al., 2023). However, such environmental manipulation related experiments can vary within and between experimental conditions among experimental animals throughout laboratories (Bayne & Würbel, 2014) not only at individual but also at intergenerational level.

Environmental factors can also affect sex determination (Pierron et al., 2021), antibiotic resistance (Yin et al., 2022), developmental neurotoxicity (Wan et al., 2022), and hyperactivity (Pandelides et al., 2023) in more than one generation of fish. Studies related to environment-induced intergenerational effects have been reported in zebrafish such as with elevated sex hormones and heart rate against aquatic toxicant (imidacloprid and thiamethoxam) (Zhang et al., 2023), and intergenerational toxicity due to ionic liquid exposures affecting natural fish movements (Lu et al., 2023). So, the key role of parental environment including nutrition, toxins, parental behaviour; and stress can shape offspring phenotypes throughout their life and can even pass persistent phenotypes on several generations (Lim & Brunet, 2013). We observed no influence of the environment or

genotype on the basal metabolic rate (BMR) of parental generation and of their offspring. We also observed no significant influence of the parental BMR on offspring's BMR in our findings. Individuals from strain HON9 compared to DAN had higher resting metabolic. The previously established positive correlation between cortisol (stress indicator) and BMR (Berbel-Filho et al., 2020) indicates that individuals with higher BMR in strain HON9 might have been more stressed. Consequently, BMR in fish could be a very important aspect to consider for both farmed and wild individuals and populations, as resting metabolic rate can affect fish swimming and feeding, which can further influence the ultimate survival of fish (Makiguchi et al., 2023). Therefore, environmental gradients and fluctuations can potentially influence fish physiology with fitness consequence. In fact, parental early-life temperature fluctuations had been previously found to influence significantly the metabolic rate of zebrafish offspring (Massey & Dalziel, 2023). Metabolism in fish can further contribute to energy budget for maintaining activities such as feeding latency (Lawrence et al., 2023), developing anti-predatory behaviour (Killen et al., 2015), or responding to environmental challenges (i.e., temperature) (Sandblom et al., 2014). The main hypothesis of this study was the parental environment can influence (positive or negative) their offspring behaviour and basal metabolism. I found an intergenerational influence of rearing environment (interaction between parental and offspring rearing environments) on activity (fish from poor environment were less active) but no significant influence on BMR was observed. Similar result was also observed in the parental generation, which still need further exploration.

Overall, our results demonstrated a potential intergenerational influence of the environment on fish behaviour. Individual genotype was observed as an additional important aspect to consider for the better understanding of the effect of parental behaviour on fish activity which would have

implications for subsequent prediction and optimization of early rearing environments in captive. Our findings would be useful for the further development of sustainable fish farming in aquaculture, from a welfare point of view, and to understand the fish behavioural adaptation to environmental change.

FIGURES

Figure 2.1. Experimental design for the intergenerational influence of environment and genotype basis on the behaviour and metabolism of mangrove killifish (*K. marmoratus*)

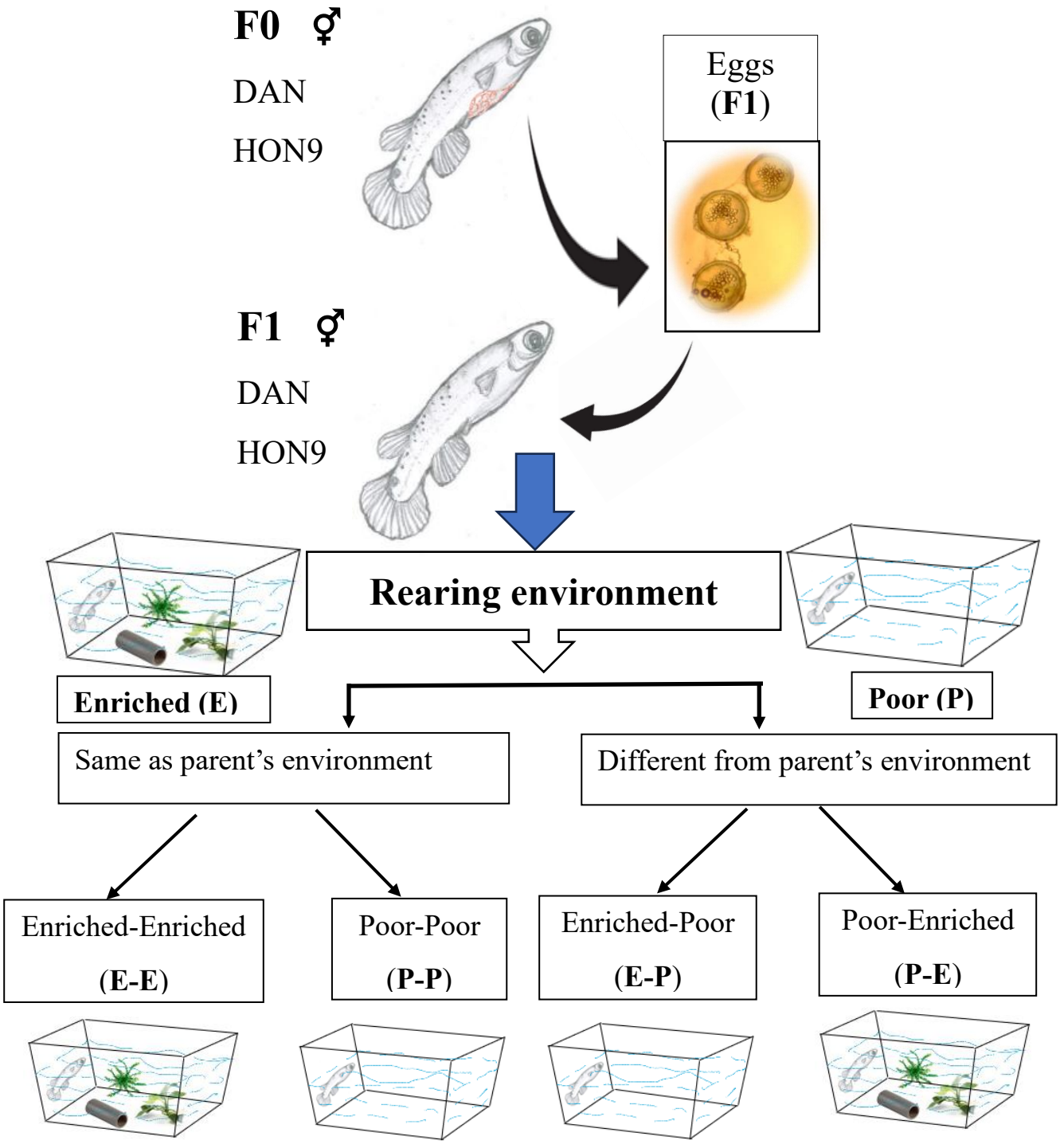


Figure 2.2. Inspections of *K. marmoratus* grouped by offspring's strain (DAN and HON9) and their environment (parental-own: E-E, E-P, P-E, and P-P; E=Enriched and P=Poor).

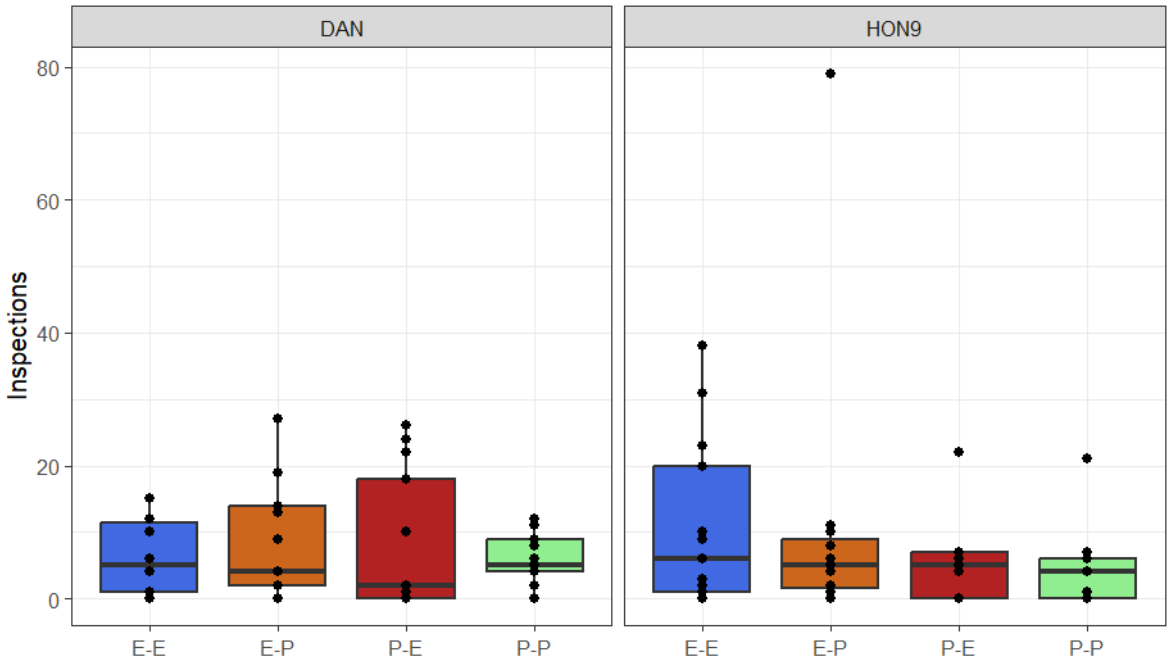


Figure 2.3. Activity of experimental F1 *K. marmoratus* in relation to their length from different strains (DAN and HON9) and grouped by their rearing environment (parental-own: E-E, E-P, P-E, and P-P; E=Enriched and P=Poor)

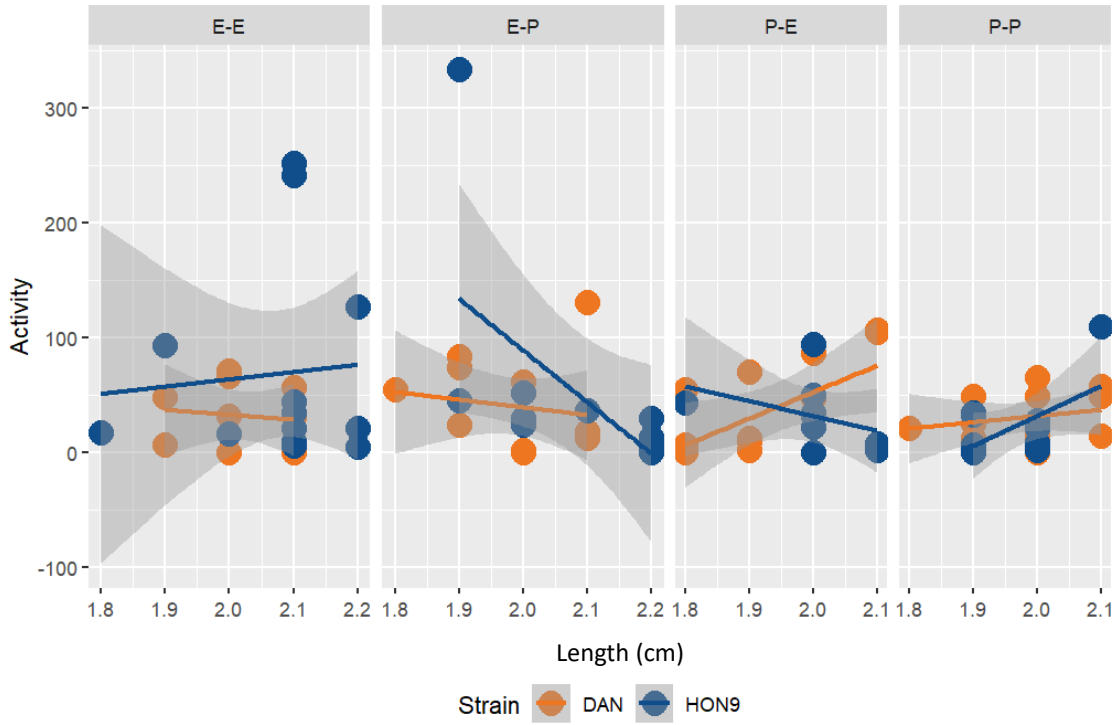
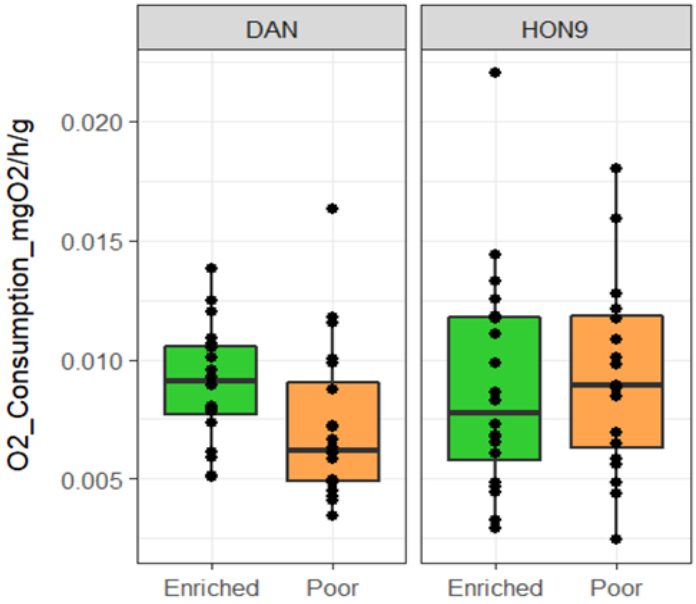


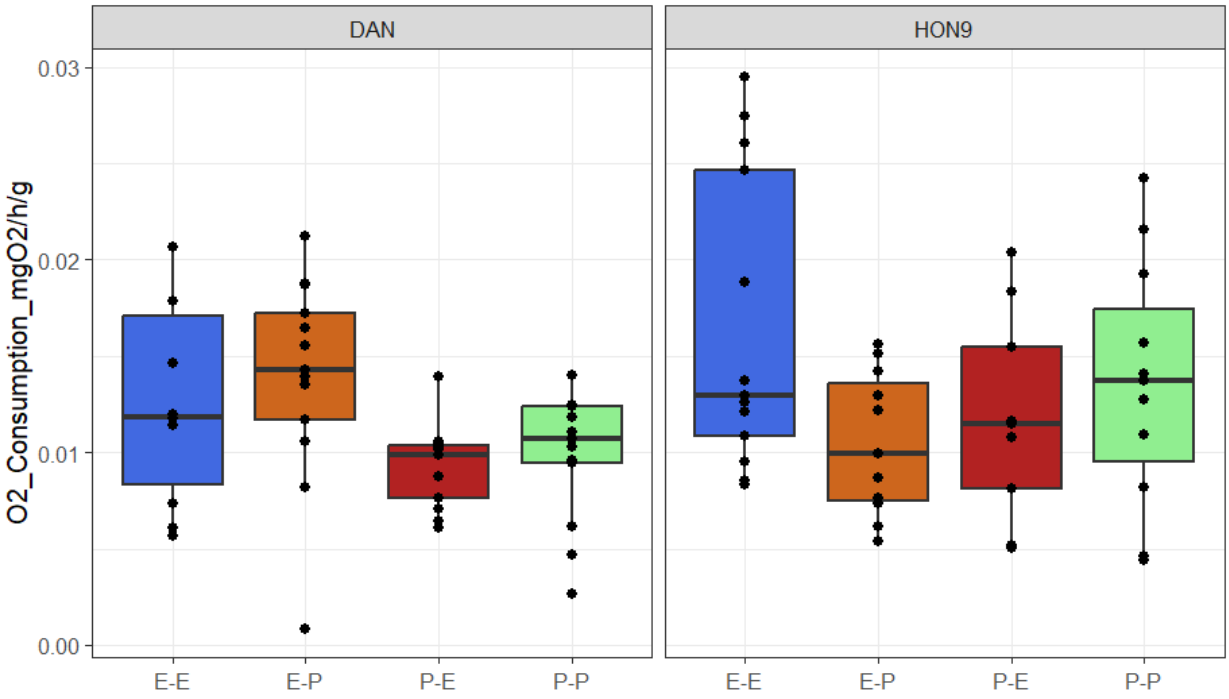
Figure 2.4. Basal metabolic rate (BMR) plots from parental mangrove killifish, F0 (A) and their offsprings, F1 (B).

In (B), parent-current environment: E-E, E-P, P-E, and P-P; E- enriched, P- poor.

(A) F0 BMR



(B) F1 BMR



CHAPTER 3: Intergenerational effects of the rearing environment on the microbiome of fish with low genetic diversity

3.1. Abstract

Gut microbiome plays an essential role in overall nutrient absorption, growth, and the immunity of fish though a good balance of healthy and beneficial microbial communities. Environmental factors and host genotype can modulate fish gut microbiome with consequences for host's physiology and behaviour. Understanding the factors affecting gut microbiome and its intergenerational stability is essential for maintaining good fish performance in captivity. The relative roles of fish genotype and environment on the establishment of gut microbiome in fish are still poorly explored. This study investigated the potential effect of fish genotype and early rearing environment (Enriched and standard Poor) on gut (whole) microbiome diversity (alpha) and composition (beta) in two selfing mangrove killifish (*Kryptolebias marmoratus*) strains, across two generations. *Vibrionaceae* was the most abundant bacteria across all gut samples regardless of strain and rearing environment. The results identified a dominant effect of the rearing environment (both parental and own) over the genotype (strains) on the alpha diversity (Simpson's evenness) of gut microbiome, but no influence of rearing environments on beta diversity was observed. Here, I found evidence of an interaction between parental and offspring rearing environments on microbiome alpha diversity indicating that origin, and environmental stability can have a long-term influence on the fish microbiome.

3.2. Introduction

The gut microbiome plays an important role in fish including feeding behaviour, growth (Butt & Volkoff, 2019), metabolism (Yukgehnaish et al., 2020), reproduction (Liu et al., 2021), health and immunity (A. R. Wang et al., 2018), population dynamics (Ghanbari et al., 2015) and host's overall fitness (Derome & Filteau, 2020). Understanding the factors affecting fish-gut microbial community composition is also essential for maintaining good fish performance in captivity (Sun et al., 2021). Diversity in the gut microbiome develops through the host's exposure to different environmental factors and can contribute to changes in host's physiology (Feng et al., 2018). Previous studies in rodents and humans suggested that the gut microbiome is essential to host's genetic and environmental influences and, in turn, influences directly or indirectly host's homeostasis (Rosenbaum et al., 2015). But there are significant differences between mammals and fish in terms of energy utilization (Sandrini et al., 2015), and in the interactions between host and environment in shaping the gut microbiome composition (Kuziel & Rakoff-Nahoum, 2022). Hence, understanding the relationship between the host's genetics and the environment in fish has become an important aspect to consider, particularly in breeding programs.

Generally, the microbiome has been defined as a set of genomes from numerous microorganisms present in an environment (Yukgehnaish et al., 2020). Among the multiple environmental factors that influence fish microbial communities, water and sediments have been the most commonly reported (Sylvain et al., 2016; Vestrum et al., 2018; Vestrum et al., 2020; Zeng et al., 2020). For example, gut microbial communities observed in *Trachinotus houdemeri* and in *Hemiculter leucisculus* were similar to the microbiome observed in the water, while the gut microbiome in *Oreochromis mossambicus* looked more like the microbial communities observed in the sediments

(Bi et al., 2021). But the development of gut microbiome in fish is a continuous process specially during the early life and the successive developmental stages, related to corresponding interactions with the dynamic environment either in the wild or under commercial aquaculture systems (Vadstein et al., 2004). Consequently, the early rearing environment can influence host's long-term fitness through gut microbiome inoculation from the surrounding environment (Deng et al., 2021), while differences in the rearing environment during growth may also change the gut microbiome profile with unpredictable health impacts (Legrand et al., 2020).

Environmental factors responsible for the establishment of gut microbiome in fish are still poorly explored (De Schryver & Vadstein, 2014). The analysis of the effect of water and rearing environment on gut microbiota in tilapia suggested that variations in bacterial composition were randomly dependent on different environmental factors over time (Giatsis et al., 2015), making it challenging to predict the effect of a particular environmental factor. No significant influence of salinity (3-7 ppt) on the abundance of different bacterial taxa was also recorded in the gut microbiome study of Pike Fry (*Esox lucius*) (Dulski et al., 2020). However, deleterious influence (e.g., growth inhibition, lowered beneficial bacteria, dominance of harmful bacteria) of higher salinity (6 ppt) on the gut microbiota of Grass carp (*Ctenopharyngodon idella*) has also been reported in (Liu et al., 2023). But again, the differences in the effects of salinity could be a result of the physiology and the origin of water of the fish and its overall rearing conditions. Rearing environment including different culture systems (e.g., reservoir, pond, and lake) has been reported to have a significant impact on the abundance and structural variability of gut microbiome in bighead carp, *Hypophthalmichthys nobilis* (Ye et al., 2023). The influence of environmental factors on fish-gut microbial community composition, diversity and richness has been observed in

previous studies in Nile tilapia (*Oreochromis niloticus*) (Bereded et al., 2021, 2022) and in various other fish species; such as in black sea bream, *Acanthopagrus schlegelii* (Sun et al., 2021) or the Japanese grenadier anchovy, *Coilia ectenes* (Duan et al., 2017). Gut microbiome studies in Atlantic salmon parr (*Salmo salar* L.) documented that both environmental and host related factors were substantially influencing the gut microbial composition (Dehler et al., 2017). Particularly among different host related factors, the effect of host's genetic variation on microbial differences is a crucial aspect for understanding the host-microbiome dynamics and their co-evolution (Koskella & Bergelson, 2020). But separating the relative role of host's genetic background from the environmental factors shaping the microbiome composition is difficult under most experimental conditions in fish (Small et al., 2023).

The genetic background of fish can further interact with environment through potential changes of microbiome across generations. For example, the gut microbiome composition and richness in medaka (*Oryzias latipes*) were significantly influenced by both genotype and the light intensity (Evangelista et al., 2023). The effect of genotypic differences on the composition and structure of the gut microbiome was also observed in a recent study in gibel carp (*Carassius auratus gibelio*) (Fan et al., 2023). Gut microbiome of the offspring can also be determined by their parents through vertical transmission of maternal microbes, for example in Nile tilapia *Oreochromis niloticus*, their early developmental conditions have been found to facilitate the gut microbial colonization across generations (Abdelhafiz et al., 2022). However, there is little information on the intergenerational effect of gut microbiome in fish due to differences in rearing environment and host's genotype.

The naturally inbred mangrove killifish, *Kryptolebias marmoratus* can be a good study species to investigate the relative roles of host-specific factors (i.e., genetic background) and the rearing environment on fish-gut microbiome, and its potential transmission from one generation to another. In this sense, this study investigated the potential effect of the genotype and rearing environment (environmental enrichment) on gut microbiome diversity and composition in two selfing lines of the mangrove killifish, across two generations.

3.3. Materials and methods

3.3.1. Experimental design, sample processing, sequencing, and bioinformatics

The offspring of the *K. marmoratus* from Chapters 1 and 2 were reared individually (until ~6 months old) in plastic tanks, same as their parents (F0) (Chapter 1), under controlled laboratory conditions (husbandry details in chapter 1 and 2). Eggs from parents reared in two different environments [Enriched (E) which means enriching the tank using artificial plants and plastic pipe and another environment was standard barren or Poor (P) which was lacking of the enrichments] were selected and maintained for the experiment with the offspring in either similar (E-E and P-P) or dissimilar environment (E-P and P-E) to their parental rearing environments (Figure 3.1.). Sampling of the whole gut for microbiome analysis was performed and processed for DNA extractions and sequencing following the same protocol as the parental generation using 16S rRNA metabarcoding, following the approach described in Chapter 1. For bioinformatics, Qiime2 (version: qiime2-2022.2) (Bolyen et al., 2019) was used. Sequenced data truncated at 200 forward and 147 reverse reads using DADA2 (Callahan et al., 2016) based on the quality check of the sequences. Filtering was done to remove any DNA sourced from the host. A sub-sampling (a normalization step) (Aguirre De Carcer et al., 2011) was then performed using a common sampling

depth of 2,116 reads based on the rarefaction (Figure S3.1.) and checking the filtered table in Qiime2, and 3.84% of amplicon sequence variants (ASVs) were retained from the samples. An extra-filtering step was carried out retaining only those ASVs which appeared at least twice in two samples and finally 1,404 ASVs were retained. Sample sizes for different experimental groups from the offspring (F1) were as follows: N=64; HON9 P-E n=8, P-P n=7, E-P n=9, E-E n=8; DAN P-E n=9, P-P n=9, E-P n=7 and E-E n=7 (offspring and parental details in Table 3.1.).

3.3.2. Statistical analysis

All analyses were done using R v. 4.2.2 (Team, 2010). The influence of strain, offspring's own rearing environment, and their parental environment on microbiome alpha (Chao1 richness and Simpson's evenness) and beta diversity (Bray-Curtis and weighted UniFrac distances) were analysed and compared with their parental microbiome alpha and beta diversity. For parents, microbiome alpha diversity was analysed with strain and environment as fixed factors using linear models with *lm*. For the offspring's alpha diversity data (Table S3.1.1.), statistical models were performed with and without the parent of origin (Parent ID) as a random factor. Generalized Linear Models included strain, offspring's own rearing environment, and their parental environment as fixed factors for both alpha diversity indices, and were run using *glm2* (Marschner, 2011). The mixed models included parental ID as a random factor and were run using *lme4* (Bates et al., 2014), including strain, offspring's own rearing environment, and their parental environment as fixed factors. Models with and without Parent ID were compared using *anova*, and the best models were chosen depending on the lowest Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC). For both the parents and offspring, beta diversity results were represented by non-metric multidimensional scaling (NMDS) and analysed using multivariate analysis of variance in

adonis2 (*vegan* package) (Oksanen et al., 2007) with 99,999 permutations in R. Differentially abundant ASVs were also detected using DeSeq2 (Love et al., 2014) and pheatmap in R based on False Discovery Rate (FDR)<0.05.

3.4. Results

The sequence resulted for the samples as the lowest >6,600 and the highest >66,000. After denoising, the lowest non-chimeric reads for the samples recorded as > 4,600 and the highest as > 52,000. Then, the features count for the samples was found as a total of 12,795. Then, after the removal of mitochondria or chloroplast the retained feature count was 12,555. Then checking the filtered table and rarefaction curves (Figure S3.1.), sub-sampling (2,116 sampling depth) was performed as a normalization step and left 7,422 features. Then, another extra filtering, sample contingency-based filtering was done, which left finally a total of 1,404 features or ASVs. Among the 30 most abundant ASVs across all the offspring samples (N=64), six different types of bacteria from the *Vibrionaceae* family were mostly detected in all the samples regardless of the differences in strains (DAN and HON9) and environments (parental-own environments: E-E, E-P, P-E, and P-P). *Shewanellaceae* was observed as the next most abundant bacteria in most of the samples, except DAN E-E where only one individual in that group had this bacterium. *Micobacteriaceae* was found to be most abundant in DAN P-E, P-P; and in HON9 E-E, P-E groups. Then *Cellvibrionaceae* and *Alteromonadaceae* were found to be the next most dominating bacteria in DAN P-P. Another *Vibrionaceae* ASV was only found in the individuals of HON9 E-E group. *Saccharospirillaceae* and *Rhodobacteraceae* were found mostly in individuals from DAN P-P and HON9 P-E. *Enterococcaceae* was abundant in one individual of HON9 E-E. Among the next most abundant bacteria, *Pseudoalteromonadaceae* was dominant only in some fish from DAN P-E group.

Rhizobiaceae was mostly observed in HON9 E-E individuals and *Parvibaculaceae* was dominant in HON9 E-P samples (Figure 3.2.). Thirty-seven more differentially abundant ASVs (DAA) were detected (Figure S3.1.2., Table S3.1.3.).

In the parental generation, neither genetic strain (DAN and HON9) nor environment (Enriched and Poor) and the interaction between strain and environment had any statistically significant influence on their microbiome alpha diversity (Chao1 richness and Simpson's evenness) (Figure 3.3., Tables S3.2.1. and S3.2.2.). In offspring, the model comparison for Chao1 indicated that the model including both the fixed factors and parental ID as a random factor (AIC=542.75, BIC=557.86) (model comparison in Table S3.3.1.) fitted the data better than the one with only the fixed factors. This analysis indicated no significant effects of genotype (Strain HON9 Estimate=3.438e-04, Std. Error=6.121e-03, t-value=0.056, and *P*-value=0.955), offspring's own environment (Enriched environment Estimate=-9.693e-06, Std. Error=5.254e-03, t-value=-0.002, and *P*-value=0.999), or their parental environment (Enriched environment Estimate=-1.852e-03, Std. Error=6.845e-03, t-value=0.270, and *P*-value=0.787), or any interaction between own and parental environments (Offsprings own environment Enriched : Parental environment Enriched Estimate= 1.299e-02, Std. Error=7.412e-03, t-value=1.753, *P*-value=0.080) on offspring's microbiome alpha diversity parameter Chao1 richness (Figure 3.4.a., Table S3.3.1. with model comparison and Table S3.3.2. with the best model results).

The evenness of offspring microbiome was tested using Simpson's evenness index, where the best model included the random effect of parents (AIC=12.06, BIC=25.01) (Table S3.4.1. including model comparison). Simpson's evenness was only found to be influenced by the interaction

between offsprings' own and their parental environment (offspring's environment enriched : parental environment enriched Estimate=0.313, Std. Error=0.126, t-value=2.478, and *P*-value=0.02) but not by strain (Strain HON9 Estimate=-0.004, Std. Error=0.06, t-value=-0.062, and *P*-value=0.95), by their own environment (Enriched Environment Estimate=-0.12, Std. Error=0.09, t-value=-1.33, and *P*-value=0.19), or by their parental environment (Enriched Environment Estimate=-0.164, Std. Error=0.089, t-value=-1.84, and *P*-value=0.07) (Figure 3.4.b., Table S3.4.1. including model comparison and Table S3.4.2. with the best model outcomes). No influence of strain or environment on microbiome beta diversity (Bray-Curtis distance and weighted UniFrac distance) was observed in the parental generation (Figure 3.5., Table S3.5. and Table S3.6.) as well as in their offspring in this study (Figure 3.6., Tables S3.7. and Table S3.8.).

3.5. Discussion

The environment and host genetics can regulate the gut microbiome composition of fish. The main objective of the present study was to explore the relative role of host genotype and environment as well as any potential impact of parental environment on fish-gut microbiome diversity and composition. Mangrove killifish (*K. marmoratus*) were used for the experiment due to the possibility of controlling the genetic background through their capability to produce genetically homogeneous individuals by selfing, to disentangle the relative role of host's environmental and genotypic factors on their gut microbiome.

Among all the detected ASVs in this study in offspring, some *Vibrionaceae* were found to be the most abundant bacteria across all the gut samples. The abundance of *Vibrionaceae* in the offspring was not significantly influenced by the strain or by the rearing environment, with the parental

generation displaying a similar distribution (Chapter 1). High abundance of *Vibrio* species has been previously reported in cultured marine fish and in the estuarine environment (the natural habitat of this fish group) (Amalina et al., 2019; Mohamad et al., 2019; Sohn et al., 2019), but here we used artificially made saline water with ~14 ppt, and temperature ~20-26°C, so the origin should not be marine. Most of the fish gut bacteria are regulated by several factors like the developmental stages of fish, followed by their diet, and the trophic level in the surrounding environment (Uma et al., 2020). Additionally, a closed rearing system can also affect the abundance of certain dominant taxa due to the less scope of microbial variations in their corresponding environment (Lorgen-Ritchie et al., 2023). The microbiome composition here contrasts with the wild populations of the other two species of mangrove killifish analysed in Chapter 4 (*Kryptolebias ocellatus* and *K. hermaphroditus*), where *Vibrio* was not one the most abundant bacteria. Yet, the fact that they are different species, and a different organ was examined (skin instead of gut), makes them not directly comparable. Several species of *Vibrio* can cause fish disease outbreaks (Triga et al., 2023). However, although we did not carry out any pathogenicity test, none of the fish displayed any symptoms of disease and their growth and survival was normal, so we conclude that the *Vibrio* species we found are normal colonizers of the gut microbiome in our closed system.

We observed no direct influence of genotype (strain) and rearing environment on the alpha diversity index (Chao1 richness and Simpson's evenness) of the fish gut microbiome in the parental generation. The offspring from strain HON9 reared in poor environment (originated from enriched parental environment) had a wider (but not significant) range of Chao1 richness than the other three groups (DAN poor and enriched and HON9 enriched). A predominant influence of the environment instead of genetic factors on fish gut microbiome has been reported recently (Kim et

al., 2021), and our results indicate that the rearing environment (including water composition, temperature and diet which were common among all the experimental groups) may have a stronger effect than the host genome (strain) on the composition and structuring of the mangrove killifish, although environmental enrichment did not show any effect on that. Relatively insignificant influence of host genetics has been also identified in Atlantic salmon (*Salmo salar*) (Dvergedal et al., 2020), compared to the effect of the environment (Uren Webster et al., 2018). Aquaculture practices (e.g., selective breeding, feeding) can potentially modify the relative role of genetics and environment on microbiome composition (Lorgen-Ritchie et al., 2023).

As environmental factors play a crucial role on the gut microbiome composition through the initiation of the microbiome colonization, the early hatchery rearing conditions play an essential role to determine the diversity and richness of the microbial community (Minich et al., 2020) with potential scope of microbial transmission across generations. Further, microbiome from one generation to another can also be modified depending on the stability of the environment and the capability of the host's adaptation towards the dynamic environmental stimuli (Llewellyn et al., 2014). We identified an influence of the interaction between parental and offspring's environment on offspring's gut microbiome alpha diversity (Simpson's evenness), where the offspring originated and reared in an environment similar as their parents (P-P, E-E) displayed more even distribution of their microbiome. This could indicate an indirect influence of the rearing environment on microbiome diversity across generations. However, environmental factors have considerably more influence on the microbiome in early rearing developmental phases (Stephens et al., 2016), and the diversity of fish-gut microbiome at later developmental stages displays a lower influence of their surrounding environment (Li et al., 2017).

Although microbial modulation in fish throughout their life depends on multiple factors (e.g., developmental stages, diet, microbiome composition of surrounding water or environmental components), it has been observed that fish can develop adaptations that facilitate some favourable microbial colonization, as in mammals (Wang et al., 2018). But the differences observed among different fish microbiome studies indicate that the influence of different factors on the gut microbiome of different fish species is very variable and cannot be generalized, likely needing a more targeted approach depending on the research question (Pan et al., 2023). Considering this, the measurements of the microbiome in most of the environmental components (e.g., water, sediments) and diet along with the targeted microbiome in fish in several or particular organs of fish could give us more detailed information in this regard. In this study, the ranges of microbiome richness in offspring were reported lower than that was observed in their parents, which could be an effect of developmental age (some parents experienced natural hatching, and some had varied diapause, while all offspring experienced the diapause).

In summary, the results indicate a likely dominant effect of the rearing environment (but not physical enrichment) over the genetic differences between strains on the microbiome composition and distribution, which was not significantly different among the different groups. The effect was not so clear at the level of environmental enrichment, where there was only evidence of a potential effect of interaction between rearing environments (parental and offspring) on microbiome alpha diversity. This contrasts with the results of Chapter 1 and Chapter 2, where the different strains and environmental enrichment influenced the behaviour of both parents and their offspring, and the microbiome of parental generation with a prominent effect of diet over the environmental enrichment. Statistically significant influence of strain (higher microbiome diversity in HON9)

and environment (higher microbiome evenness was observed in EDS fed and reared in enriched environment) on microbiome alpha diversity was observed in the parents (**Chapter 1**), which is not the case in the offspring in this study (**Chapter 3**), where no significant influence of strain or environment was observed. These results could have been influenced by the captive origin of the fish, the stability of the rearing environment and the fact that we were comparing two strains of the same species. Species can play an important role in the microbiome composition (as in Chapter 4 for example), although the environment seems to have a dominant effect in most fish species, as seen here.

TABLES

Table 3.1. List of experimental killifish from parental and offspring's generation

Offspring_ID	Offspring's Rearing Environment	Offspring's Genotype (Strain)	Parent_ID	Parental Environment
HF2-32	Enriched	HON9	HF1_17	Poor
HF2-34	Enriched	HON9	HF1_11	Poor
DF2-35	Poor	DAN	DF1_09	Enriched
DF2-24	Enriched	DAN	DF1_28	Enriched
HF2-36	Enriched	HON9	HF1_22	Enriched
HF2-38	Enriched	HON9	HF1_17	Poor
HF2-37	Poor	HON9	HF1_22	Enriched
DF2-52	Enriched	DAN	DF1_21	Poor
DF2-72	Poor	DAN	DF1_38	Poor
DF2-109	Poor	DAN	DF1_16	Poor
DF2-56	Poor	DAN	DF1_38	Poor
DF2-65	Poor	DAN	DF1_38	Poor
DF2-59	Poor	DAN	DF1_25	Poor
DF2-58	Enriched	DAN	DF1_06	Poor
DF2-108	Enriched	DAN	DF1_02	Poor
DF2-67	Enriched	DAN	DF1_38	Poor
DF2-68	Enriched	DAN	DF1_38	Poor
DF2-60	Enriched	DAN	DF1_25	Poor
HF2-79	Poor	HON9	HF1_41	Poor
HF2-59	Poor	HON9	HF1_41	Poor
HF2-61	Poor	HON9	HF1_41	Poor
HF2-74	Poor	HON9	HF1_13	Poor
HF2-75	Poor	HON9	HF1_13	Poor
HF2-66	Enriched	HON9	HF1_13	Poor
HF2-60	Enriched	HON9	HF1_41	Poor
HF2-76	Enriched	HON9	HF1_13	Poor
HF2-69	Enriched	HON9	HF1_57	Poor
DF2-114	Poor	DAN	DF1_09	Enriched
DF2-116	Poor	DAN	DF1_09	Enriched
DF2-86	Poor	DAN	DF1_28	Enriched
DF2-80	Poor	DAN	DF1_30	Enriched
DF2-79	Enriched	DAN	DF1_30	Enriched
DF2-115	Enriched	DAN	DF1_28	Enriched
DF2-81	Enriched	DAN	DF1_30	Enriched
DF2-83	Enriched	DAN	DF1_09	Enriched
HF2-72	Poor	HON9	HF1_12	Enriched
DF2-71	Enriched	DAN	DF1_24	Enriched

Table 3.1. Continued

Offspring_ID	Offspring's Rearing Environment	Offspring's Genotype (Strain)	Parent_ID	Parental Environment
HF2-86	Poor	HON9	HF1_22	Enriched
HF2-87	Poor	HON9	HF1_22	Enriched
HF2-88	Poor	HON9	HF1_22	Enriched
HF2-89	Poor	HON9	HF1_52	Enriched
HF2-91	Enriched	HON9	HF1_52	Enriched
HF2-92	Enriched	HON9	HF1_52	Enriched
HF2-93	Enriched	HON9	HF1_52	Enriched
DF2-128	Poor	DAN	DF1_39	Enriched
HF2-94	Poor	HON9	HF1_50	Enriched
HF2-95	Poor	HON9	HF1_06	Enriched
HF2-96	Poor	HON9	HF1_66	Enriched
HF2-97	Enriched	HON9	HF1_60	Enriched
HF2-105	Enriched	HON9	HF1_50	Enriched
HF2-106	Enriched	HON9	HF1_22	Enriched
HF2-90	Enriched	HON9	HF1_52	Enriched
DF2-06	Poor	DAN	DF1_06	Poor
HF2-22	Enriched	HON9	HF1_11	Poor
DF2-21	Poor	DAN	DF1_05	Enriched
DF2-22	Enriched	DAN	DF1_05	Enriched
DF2-23	Enriched	DAN	DF1_21	Poor
DF2-39	Poor	DAN	DF1_14	Poor
DF2-43	Poor	DAN	DF1_06	Poor
DF2-36	Poor	DAN	DF1_02	Poor
DF2-40	Enriched	DAN	DF1_14	Poor
DF2-44	Enriched	DAN	DF1_14	Poor
HF2-35	Poor	HON9	HF1_11	Poor
HF2-31	Poor	HON9	HF1_17	Poor

FIGURES

Figure 3.1. Experimental design using mangrove killifish from different genotypes and environments

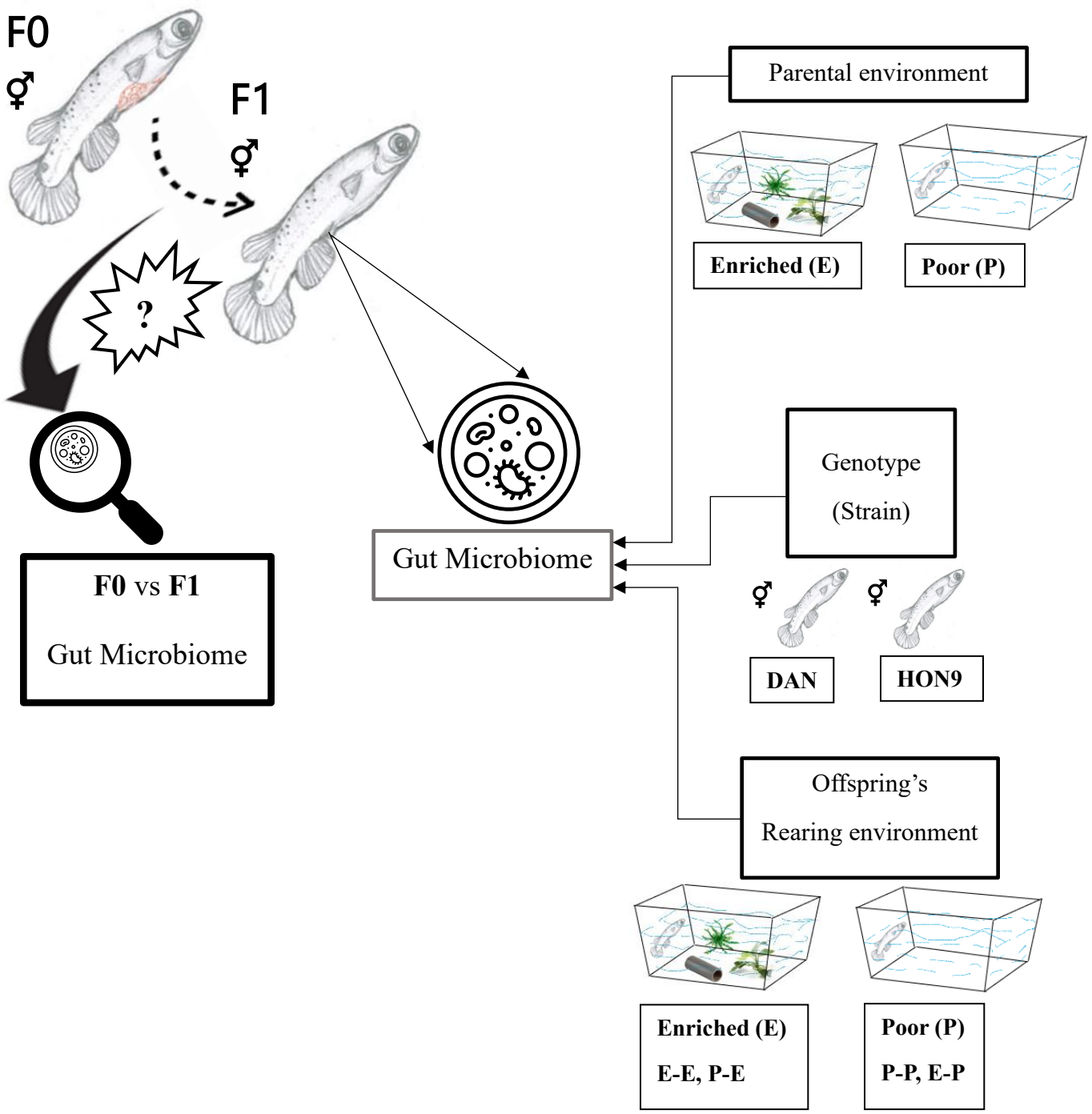


Figure 3.2. Most abundant 30 ASVs across all gut samples (N=64) of offspring, DF 2= DAN Fish, HF 2= HON9 Fish used to identify the fish individual.

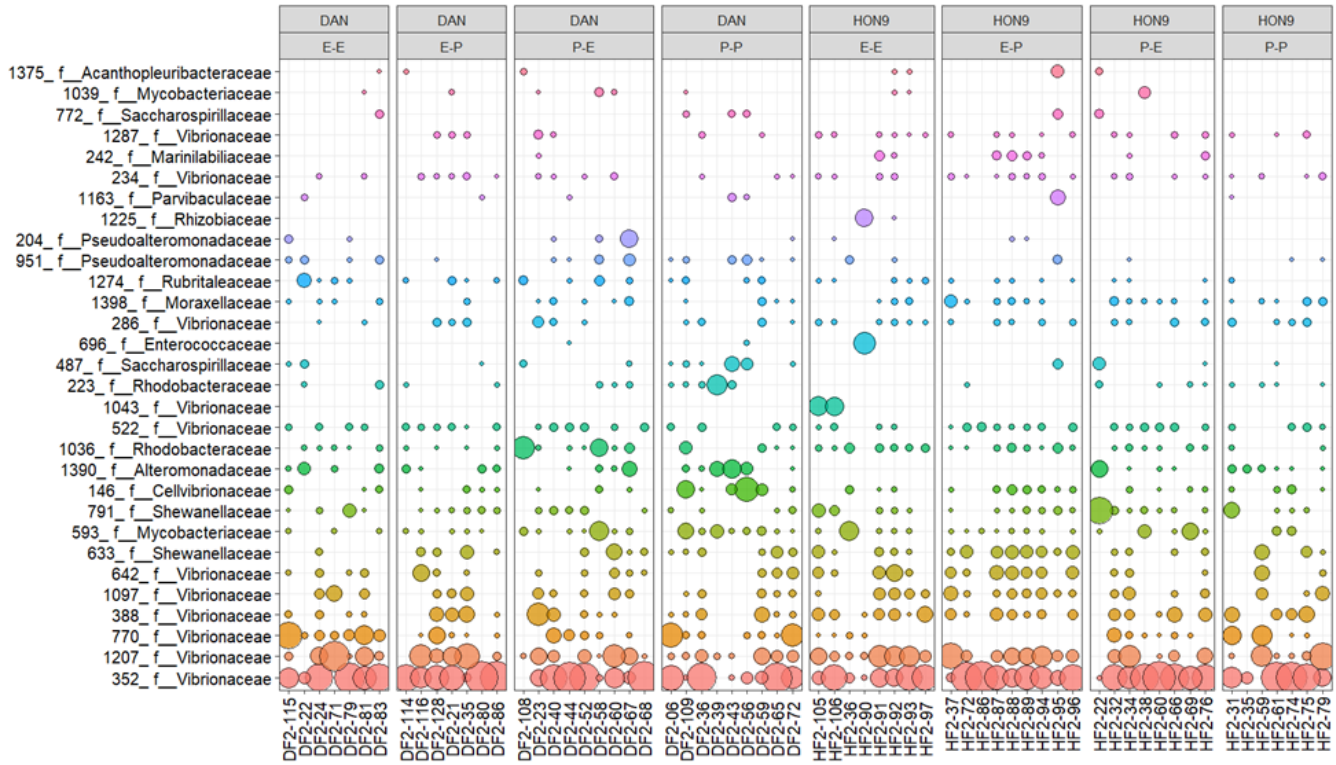
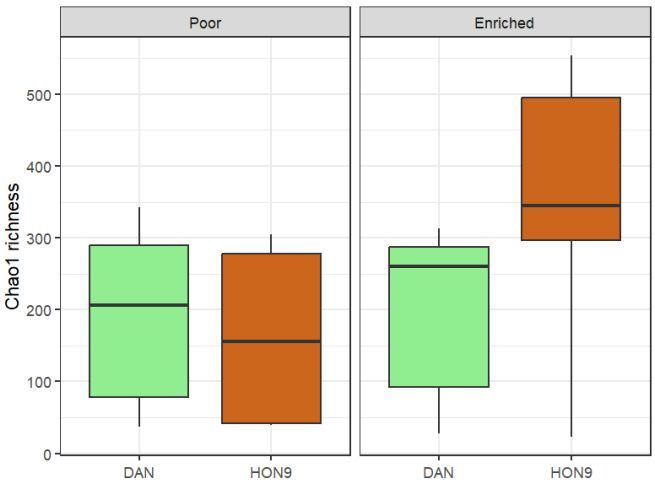


Figure 3.3. Parental (F0) microbiome alpha diversity plots - (a) Chao1 richness and (b) Simpson's evenness, P=Poor and E= Enriched. Green and brown plots- strain DAN and HON9 respectively.

(a) **F0** Chao1 richness



(b) **F0** Simpson's evenness

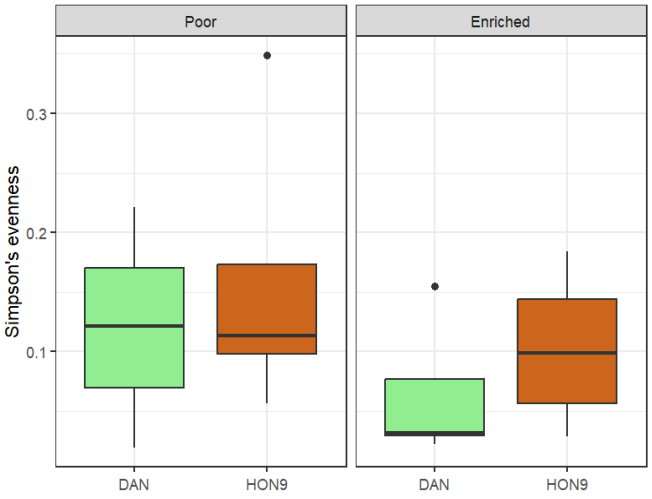
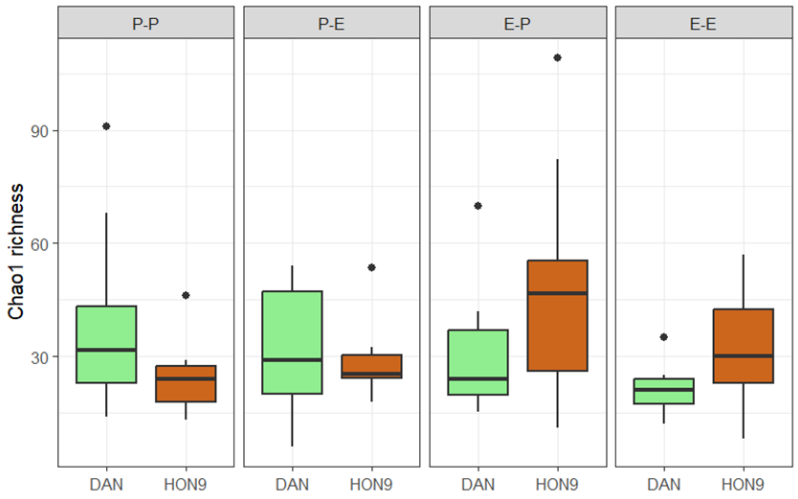


Figure 3.4. Microbiome alpha diversity plots for offspring (F1)- (a) Chao1 richness and (b) Simpson's evenness, different sample groups are, Parental-offspring's rearing environment: P-P, P-E, E-P, and E-E where P=Poor and E= Enriched. Green and brown colour representing strain DAN and HON9 respectively.

(a) **F1** Chao1 richness



(b) **F1** Simpson's evenness

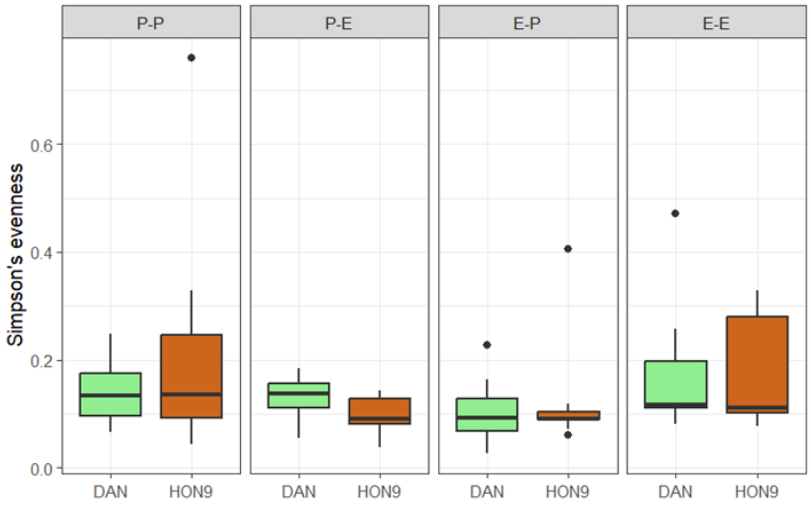


Figure 3.5. Parental (F0) microbiome beta diversity (ASVs) NMDS plots: (a) Bray-Curtis distance and (b) weighted UniFrac distance, from DAN and HON9 strains under Poor (P) and Enriched (E) environments.

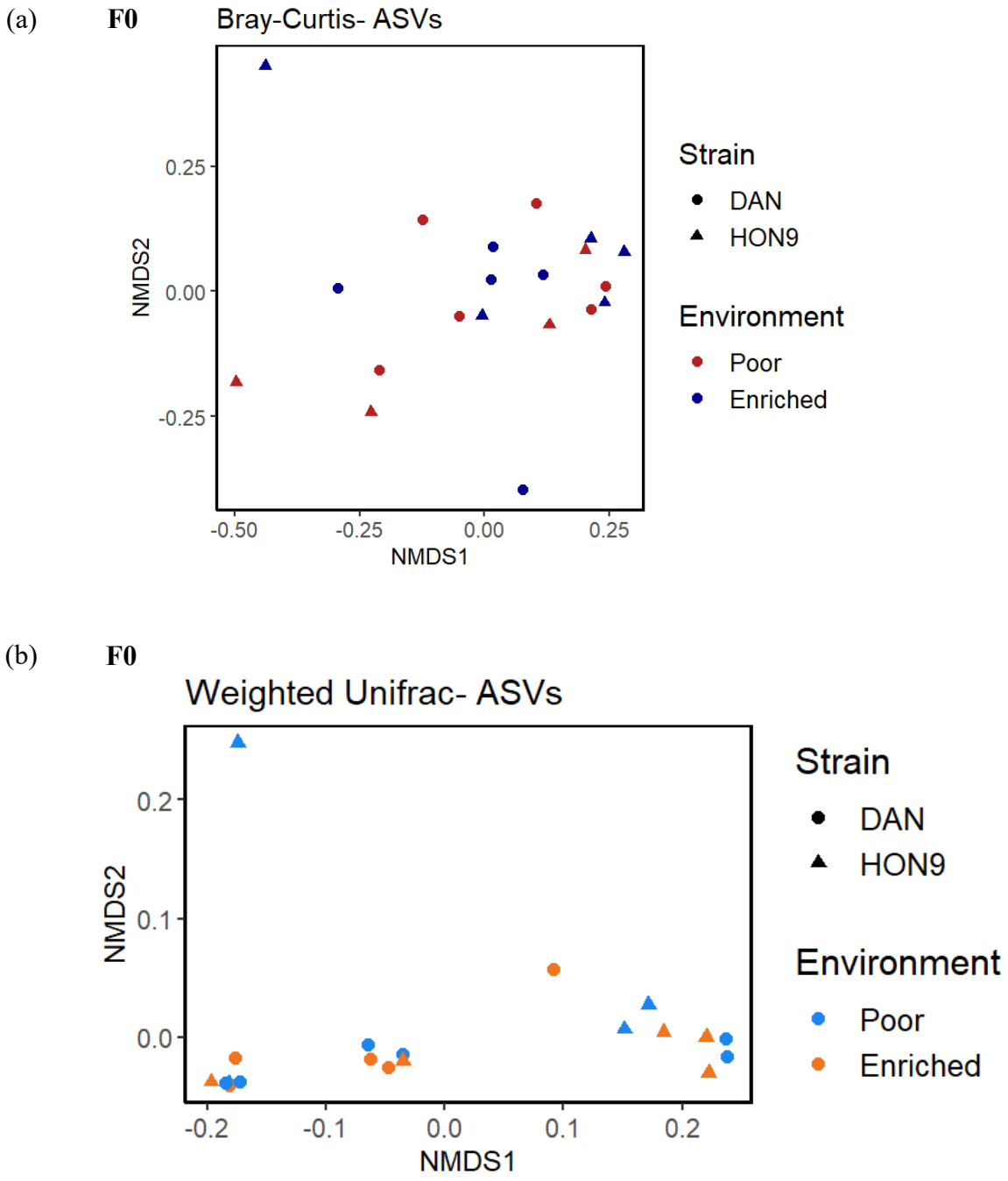
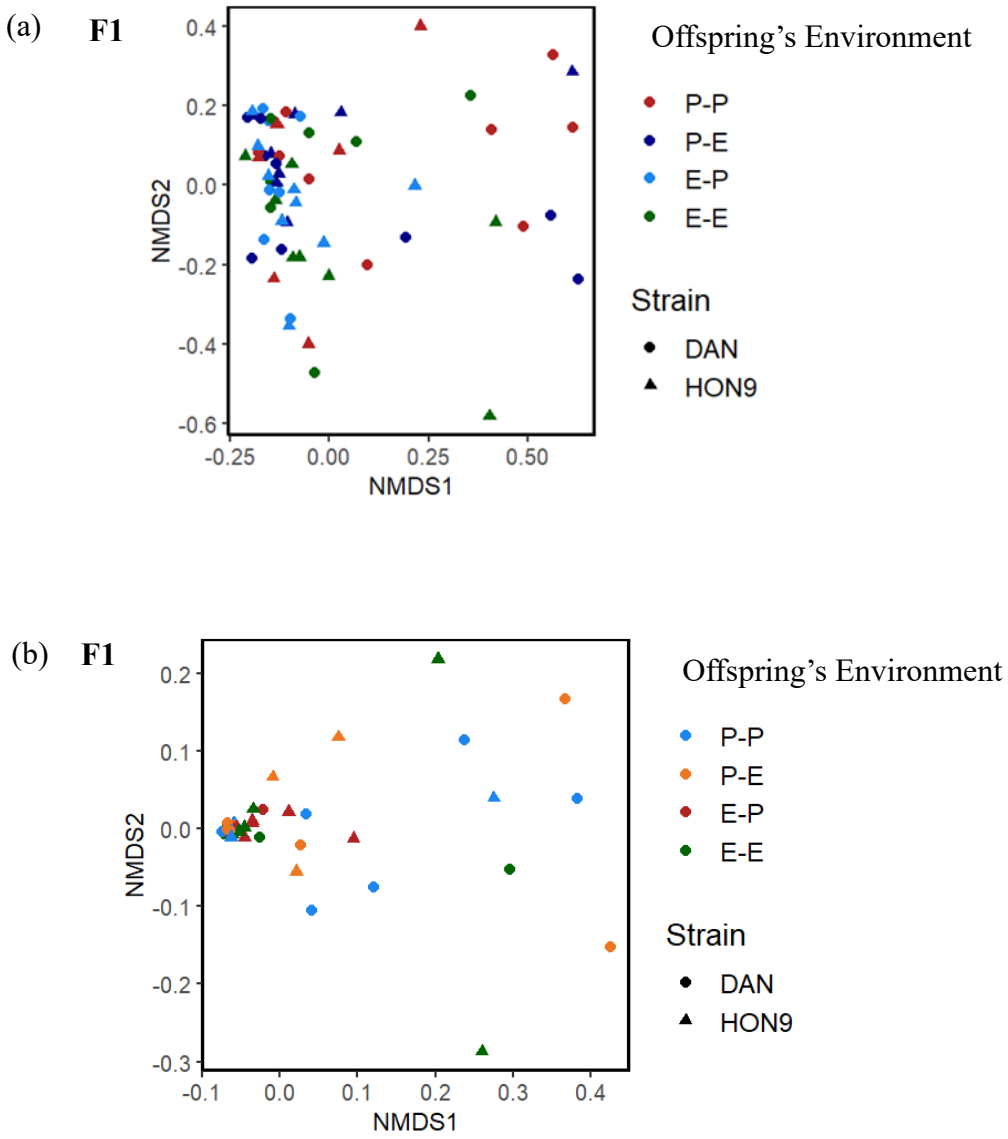


Figure 3.6. Offspring's (F1) microbiome beta diversity (ASVs) NMDS plots: (a) Bray-Curtis distance and (b) weighted UniFrac distance, from different environments (Parental-offspring's rearing environment: P-P, P-E, E-P, and E-E where P=Poor and E= Enriched) and strains (DAN and HON9).



CHAPTER 4: Microbiome and epigenetic variation in wild fish with low genetic diversity*

*A version of this work has been published

4.1. Abstract

Non-genetic sources of phenotypic variation, such as the epigenome and the microbiome, could be important contributors to adaptive variation for species with low genetic diversity, but little is known about the complex interaction between them and the genetic diversity of the host, particularly in wild populations. We examined the skin microbiome composition of two closely related mangrove killifish species with different mating systems (self-fertilising and outcrossing) under sympatric and allopatric conditions, to partition the influence of the genotype and the environment on their microbiome and (previously described) epigenetic profiles. The diversity and community composition of the skin microbiome were strongly shaped by the environment and, to a lesser extent, by species-specific influences. Heterozygosity and microbiome alpha diversity, but not epigenetic variation, were associated to the fluctuating asymmetry of traits related to performance (vision) and behaviour (aggression). Our study identified that a proportion of the epigenetic diversity and microbiome differentiation was unrelated to genetic variability, and we found the first evidence for an associative relationship between microbiome and epigenetic diversity in these wild populations, suggesting that both mechanisms could potentially contribute to variation in species with low genetic diversity.

4.2. Introduction

Species with low genetic diversity have limited capacity for genetic-based adaptation under environmental change and have a higher risk of extinction (Ørsted et al., 2019), yet some can persist over many generations, suggesting that non-genetic sources of phenotypic variation (such as epigenetics or the microbiome) could also be involved in their adaptation to change (O'Dea et al., 2016; Aagaard et al., 2022). Epigenetic modifications (i.e., DNA methylation, histone modifications, non-coding RNAs) modulate changes in gene expression that can occur in response to environmental variation but do not involve changes in DNA sequence (Bossdorf et al., 2008; Richards et al., 2017), are mitotically and/or meiotically heritable and result in phenotypically plastic responses within genotypes (Bossdorf et al., 2010; Verhoeven & Preite, 2013). DNA methylation plays a role on the regulation of biological processes, such as cell differentiation and genomic imprinting, can be affected by selection (Koch et al., 2016) . Many plants and animals display high levels of genome-wide DNA methylation (Massicotte et al., 2011; Richards et al., 2012; Liebl et al., 2013) despite having low heterozygosity, suggesting that epigenetic variation might compensate for low genetic diversity and/or asexually reproduction (Castonguay & Angers, 2012; Verhoeven & Preite, 2014; Douhovnikoff & Dodd, 2015), particularly when the variation is not under genetic control (Rey et al., 2020; Wang et al., 2020). Like the epigenome, the microbiome, can also increase host fitness by either increasing phenotypic variation and the ability to respond to wider selective pressures, but also by buffering the host against environmental perturbations (Henry et al., 2021). The interaction between the microbiome and the host genome results in changes in gene expression without modifying the underlying DNA sequence, is strongly influenced by environment, and can respond to selective pressures (Gilbert et al., 2010), therefore could be considered an additional epigenetic mechanism of the host (Collens et al., 2019).

Both the microbiome and epigenome can influence host gene expression, and it is likely that there is a degree of interaction between them, but current understanding of the cross-talk between the microbiome and the genome and epigenome of the host, and their potential contribution to host plasticity is still in its infancy (Angers et al., 2020). In mammals, the host-gut microbiome interaction seems to be primarily mediated by microbiota-produced metabolites, such as short chain fatty acids (SCFAs), that modify the epigenome of gastrointestinal host cells through DNA methylation and histone acetylation, thereby altering the host cells' function (Alenghat, 2015). Thus, changes in the microbiota composition or diversity can alter the production of metabolites that regulate the host DNA and histone modifications (Oliveira, 2021). Microbiome composition and function are influenced by the environment and by intrinsic host factors such as age, sex, immunocompetence and genotype, although their relative influence varies (Nichols & Davenport, 2021). Host genetics tends to play a relatively small part on microbiome composition and involves few genes (Sanna et al., 2022), influencing some tissues more than others (Schommer & Gallo, 2013; Kolde et al., 2018). However, population bottlenecks can reduce the diversity of the host and its microbiome, decreasing host fitness and its evolutionary response to stress (Ørsted et al., 2022), which makes the study of the interaction between the host and its microbiome very relevant for conservation (Littleford-Colquhoun et al., 2022).

The fish microbiome consists of a diverse community of bacteria, viruses, eukaryotes and protists associated to mucosal tissues in the gut (Banerjee & Ray, 2017), skin (Chiarello et al., 2018) and gills (Merrifield & Rodiles, 2015). Its composition differs between organs, all of which have specialized microbiota (Zhang et al., 2019). The skin microbiota is strongly influenced by environmental factors including water chemistry and the bacterioplankton (Sylvain et al., 2020).

Yet, the microbiome of the fish skin is distinct from that of the surrounding water and although it lacks a set of core taxa, it is mostly dominated by Proteobacteria (Gomez & Primm, 2021). The composition of the fish gut microbiome varies between fish genotypes, as does that of the skin and gills' (Boutin et al., 2014; Pratte et al., 2018), but the evidence for phylosymbiosis (higher intraspecific than interspecific similarity in the structuring of the microbial communities) is generally weak (Sevellec et al., 2018; Escalas et al., 2021). For example, host phylogenetics (but also diet) influence the skin microbiome composition of coral reef fishes (Chiarello et al., 2018) but not of Amazonian fishes (Sylvain et al., 2020), and an analysis of teleosts and elasmobranchs only found a consistent phylosymbiotic pattern in the latter (Doane et al., 2020). Given that the fish skin mucus and its microbiome constitute the first barrier against infection (Gomez et al., 2013), the influence of host genotype and the environment in its composition are likely to be important for the persistence of natural populations, potentially through epigenetic modulation. However, the influence of the environment and host genetics on skin microbiome and its relationship with host epigenetics has not yet been explored, and is particularly challenging in wild populations, where the ability to control for environmental conditions and genetic background is very limited. Studying closely related taxa under sympatric and allopatric conditions could help overcome this limitation, as this would allow to control for environmental influences (shared under sympatry) and examine genotype by environment interactions with microbial communities under natural conditions (Rennison et al., 2019).

To examine the impact of environmental and genetic variability on the microbiome of species with low genetic diversity, as well as the association with host epigenetics and fluctuating asymmetry (a proxy for phenotypic fitness), we compared the skin microbiome composition of two closely

related killifish species, *Kryptolebias ocelatus* and *Kryptolebias hermaphroditus*, with different mating systems (outcrossing and self-fertilization respectively), that result in varying levels of genetic variation. *K. hermaphroditus* is one of the only two known self-fertilising hermaphrodites in vertebrates (Tatarenkov et al., 2017b) and its populations consists mainly of self-fertilising hermaphrodites with males at very low frequencies (Berbel-Filho et al., 2016). Outcrossing rarely occurs between *K. hermaprhoditus* males and hermaphrodites, which are typically inbred with very high homozygosity levels (Berbel-Filho et al., 2019). In contrast, *K. ocellatus* populations consist of males and hermaphrodites in approximately equal ratio and only reproduce via outcrossing (Berbel-Filho et al., 2020). We sampled geographic locations where both species coexisted (sympatry) and where only one of them was present (allopatry), to control for environmental conditions, and assessed (a) the relative roles of the environment, species and host genetic variability in the skin microbiome diversity and community structuring and (b) the relationship among microbiome diversity and host genetics, epigenetics (DNA methylation patterns) and fluctuating asymmetry.

4.3. Methods

4.3.1. Species selection and sampling

Kryptolebias hermaphroditus and *Kryptolebias ocellatus*, two closely related mangrove killifish, were sampled (under license ICMBio/SISBIO 57145-1/2017) from six sites in south and southeast Brazil (in August, 2017), two sites where both species coexisted in sympatry (Guaratiba and Fundão; GUA and FUN), two sites only inhabited by *K. ocellatus* (Florianópolis and São Francisco do Sul; FLO and SFR) and two sites only inhabited by *K. hermaphroditus* (Picinguaba and Aracruz; PIC and ARA) as described in (Berbel-Filho et al., 2020; Berbel-Filho et al., 2022)

(Figure 4.1.). Skin swabs of the left flank of the fish (between the operculum and caudal fin) were collected for this study from forty-two mangrove killifish: 8 *K. ocellatus* and 14 *K. hermaprhoditus* coexisting in the two common sampling locations, and 12 *K. ocellatus* and *8 K. hermaprhoditus* collected from locations only inhabited by each species respectively (Table 4.1.; Figure 4.1.). The swabs were stored in molecular grade ethanol at -80°C until analysis. Fish standard length (SL, mm) was measured in the field (FUN and GUA) or from ethanol-stored specimens using the following empirical relationship SL fresh = 0.9246 * SL ethanol + 3.012 ($R^2 = 0.96$). We used our previous data on single nucleotide polymorphism (SNP) diversity and DNA methylation patterns of *K. ocellatus* and *K. hermaprhoditus* sampled (same samples were genotyped for genome and epigenome (Berbel-Filho et al., 2022), and subset of these samples were used for the microbiome study) in sympatry at GUA and FUN locations (Berbel-Filho et al., 2022) to assess the potential relationship between individual genetic and microbiome diversities, as well as the potential relationship between the microbiome community structure and epigenetic differentiation.

4.3.2. DNA extraction, library preparation, and sequencing

The DNeasy PowerLyzer PowerSoil Kit (QIAGEN) was used to extract the microbial DNA from the skin swab samples (Uren Webster et al., 2018). Amplification of the 16S rRNA-V4 region (Klindworth et al., 2013) was performed using the 515F-806R primers (Caporaso et al., 2012) with updated sequences 515F:GTGCCAGCMGCCGCGGTAA (Parada et al., 2016) and 806R:GGACTACHVGGGTWTCTAAT (Apprill et al., 2015). PCR_1 consisted of a total volume of 22.5 µL incorporating 12.5 µL of Platinum™ II Hot-Start PCR Master Mix (2X) (Thermo Fisher Scientific), 0.5 µL of Forward (FP) and Reverse (RP) primers (10 uM), 9 µL of Ultra-pure water (UPW) and 2.5 µL of DNA. The PCR began with a 3 min denaturation step at 95°C followed by

28 cycles of 95℃ for 30 seconds, 55℃ for 30 seconds and 72℃ for 30 seconds, then a final elongation step at 72°C for 5 minutes. During PCR_2, indexing with Nextera ® XT Index Kit v2 (Illumina, Inc., San Diego, CA, 92122 United States) was performed. PCR_2 was made with a total volume of 27.5 µL per sample, containing 2.5 µL of PCR_1 product, 1.25 µL of each index, 12.5 µL of Platinum™ taq and 10 µL of UPW. The reaction conditions were as above but with 12 cycles. Final PCR products were pooled based on agarose gel band intensity and cleaned using AMPure XP beads (Beckman Coulter Genomics, Brea, CA, United States). Final library quantification was performed using qPCR (NEB Illumina quantification kit), prior to sequencing on a MiSeq Illumina platform (300 bp, paired end). Blanks were sequenced alongside the samples and yielded 206 reads.

4.3.3. Bioinformatics analysis

Sequence analysis was performed as described in (Uren Webster et al., 2021) using Qiime2 (version: qiime2-2022.2) (Bolyen et al., 2019). Briefly, based on quality filtering, DADA2 (Callahan et al., 2016) was used to trim leading primers and truncate forward (220 bp) and reverse (180 bp) reads, denoise and merge reads, remove chimeras and assign amplicon sequence variants (ASVs). Filtering of mitochondrial, chloroplast and unclassified reads was carried out before sub-sampling a total of 12,844 reads (10.20% ASVs retained) and further removal of ASVs with total abundance of less than 2 across all samples leaving a total of 9,598 ASVs. Classification was then performed using the Silva reference taxonomy (v138) (Quast et al., 2012). Qiime2 (Bolyen et al., 2019) was used to estimate alpha diversity (Chao1 richness, Shannon diversity, Faith's phylogenetic diversity and Simpson's evenness). Beta diversity between sample pairs was calculated using Bray-Curtis and weighted Unifrac distances.

Statistical differences in ASV abundance were examined using DeSeq2 (Love et al., 2014). Individual DeSeq models were constructed to identify differentially abundant ASVs occurring between fish species (KO and KH) present in the same environment (FUN and GUA), and, for each fish species separately, to assess the effect of different locations on ASV abundance. Low coverage ASVs were independently filtered within DeSeq2, and default settings were applied for outlier detection and moderation of ASV dispersion. ASV abundance was considered significantly different at False Discovery Rate (FDR) <0.05. ASV relative abundance was visualised using Pheatmap (Kolde & Kolde, 2015), based on Euclidean distance clustering. We also used the function *multipatt* in IndicSpecies to identify ASVs significantly associated with species and location. *Multipatt* uses the function Indval.g to correct for unequal sample sizes and 9999 permutations to estimate statistical significance. Samples were grouped for the analyses (a) by species considering all the locations, (b) by species only in shared locations (FUN and GUA) and (c) by habitat only in shared locations. Parameters A (specificity) and B (fidelity) were used to assess the predictive value of the ASVs for the location or species, respectively, and their sensitivity as indicators of the group.

Predicted community metagenomic profiling was performed using PICRUSt2 v2.5.2 (Markowitz et al., 2012). Briefly, employing HMMER (Eddy, 2011), EPA-NG (Barbera et al., 2019) and GAPPA (Barbera et al., 2019), ASVs were aligned with the reference Integrated Microbial Genomes database (Markowitz et al., 2012) and a phylogenetic tree constructed. Hidden state prediction, employing Castor (Louca & Doebeli, 2018), was then used to predict gene family abundance. ASVs with nearest-sequenced taxon index (NSTI) values > 2 were filtered from the analysis. Metagenome predictions, accounting for 16S copy number and ASV relative abundance,

were then generated. Whole community enzyme classification (EC) number abundances were calculated and subsequently used to infer MetaCyc pathway abundances using MinPath (Ye & Doak, 2009). Differential analysis of predicted functional pathway representation was performed using ALDEx2 v1.30.0 (Gloor et al., 2016), using the *glm* tool with a Holm-Bonferroni FWER correction to identify differences between fish species in different locations and between species in sympatry.

4.3.4. Fluctuating asymmetry

To assess the potential relationship between genetic, epigenetic and microbiome variation and phenotypic variation, we measured fluctuating asymmetry on three morphometric traits potentially related to fitness (area of the caudal ocellus, distance between the eye and the snout and pupil diameter) in 21 fish (11 *K. ocellatus* and 10 *K. hermaphroditus*), all analysed for microbiome and genetic diversity, including those with epigenetic information as well (Supplementary material Table S4.1.). For the ocellus, digital photographs were taken on both sides of the fish against a scale and the area of the ocellus was measured using Image J. Pupil diameter and distance between the tip of the snout and the outermost part of each eye were measured on preserved specimens using a microscope at 2 x magnification. Two measurements were carried out by the same observer, separated 2-4 weeks apart to reduce observer bias.

4.3.5. Statistical analysis

All statistical analysis were carried out in R v4.2.2 (R Core Team, 2019). We used linear models to examine the influence of species, sampling location and fish size on measures of alpha diversity (Shannon diversity, Chao1 richness, Simpson's evenness and Faith's phylogenetic diversity).

Model comparison was carried out by examining changes in AIC using the *anova* command. As sample sizes were relatively small and unequal among sampling sites, we run non-parametric bootstrapping regressions with 1,000 replicates for all linear models, using the *Tidymodels* package (Kuhn & Wickham, 2023). Nonparametric bootstrapping involved the random sampling with replacement from the dataset to generate a set of new distributions, linear models were run on each one of these data sets.

Structural analysis (microbial beta diversity) was based on community distance matrices calculated using the Bray-Curtis dissimilarity index and the weighted UniFrac distance to take into account phylogenetic relationships among taxa. Non-metric multidimensional scaling ordination was performed using the vegan package (Oksanen et al., 2007). To examine the influence of fish species, sampling location and fish length on community structure, multivariate analysis of variance (PERMANOVA) was performed using *adonis* (Oksanen et al., 2007) with 99,999 permutations.

To assess the influence of genetic variation on microbiome diversity, individual heterozygosity was calculated in GeneAlex v.6.5.1b (Peakall & Smouse, 2006) from 28 fish (14 *K. ocelatus* and 14 *K. hermaphroditus*) from FUN and GUA locations previously genotyped for 5,477 SNPs (Berbel-Filho et al., 2021b). We applied linear models on alpha diversity measures with heterozygosity, species and sampling location as predictors, controlling for size differences. The *drop1* function was used to perform variable selection by comparing the full model to reduced models. We compared the full model to each of the reduced models using AIC values and used the likelihood ratio test to compare model fits. We also analysed the relationship between genetic

differentiation (pairwise Euclidean genetic distance) and microbiome dissimilarity (based on Bray-Curtis and weighted UniFrac distance) using a Mantel test implemented in the *ecodist* package (Goslee & Urban, 2007).

Finally, to assess the relationship between the microbiome, genetic and epigenetic patterns we used DNA methylation pairwise distances between individuals previously estimated in (Berbel-Filho et al., 2022) for 18 fish (14 *K. hermaphroditus* and 4 *K. ocellatus*) from the sympatric locations (GUA and FUN). We assessed the relationships among the epigenetic pairwise distance (Bray-Curtis), Euclidean genetic distance (based on SNPs) and microbiome dissimilarity (weighted UniFrac and Bray-Curtis distances) with multiple regression on distance matrices (MRM), using the function MRM in the *ecodist* package (Goslee & Urban, 2007). For this analysis we carried out 1,000 permutations. In this way, we evaluated all the explanatory variables while accounting for the non-independence of distance matrices.

As a measure of individual epigenetic diversity we also estimated the coefficient of variation (CV) of the counts per million of 64,152 methylated sites (Berbel-Filho et al., 2022) and fitted linear models including alpha diversity, individual heterozygosity, size and species as predictors. Models were checked for various assumptions using the package *performance* (Lüdecke et al., 2021) and, after removing collinearity, model selection was carried out using the *drop1* function as above.

For FA analyses we followed the steps recommended in Palmer (1994) (Palmer, 1994) (version updated in 1996). We first tested the assumptions that (a) the difference in size between left and right measurements (L-R) was not different from zero, using one sample t-tests and (b) that side

differences (L-R) were normally distributed, using Shapiro-Wilk's tests. After these tests, only ocellus area and pupil diameter were retained for further analyses, as the snout distance distribution was not normal, indicative of antisymmetry. Linear regression was then used to test if the absolute difference between left and right trait measurements (|L-R|) was dependent on trait size, and the relationship was found insignificant in all cases (Supplementary material Figure S4.14.d.). A two-way ANOVA (sides x individuals) was used for testing the significance of FA relative to measurement error, while simultaneously testing for the presence of directional asymmetry (DA) and for trait-size differences among individuals. The results of the significance tests for the various components of variation derived from the two-way ANOVA of the ocellus and pupil measurements indicated that FA was large relative to measurement error as well as a minor contribution of DA (Supplementary material Table S4.7.). Corrections for DA and error measurements (between replicates) were carried out as in Tocts *et al.* (2016) (Tocts et al., 2016), and these corrected FA estimates were used for the rest of the analyses (Supplementary material Table S4.8.). Total fluctuating asymmetry was estimated as the sum of both ocellus and pupil diameter corrected FA, and we assessed its relationship with microbiome (alpha diversity), genetic (heterozygosity) and epigenetic (CV) diversities using linear regressions. Pearson correlation probabilities were tested using Monte-Carlo simulations with 1000 permutations, to account for the small sample sizes.

4.3.6. Ethical statement and funding

Sampling was carried out under license ICMBio/SISBIO 57145-1/2017 and approved by Swansea University Ethics Committee reference SU-Ethics-Student-250717/245. Fieldwork was supported by the National Geographic/Waitt program [W461-16] and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (WMB-F).

4.4. Results

Skin swabs were collected from two closely related mangrove killifish *Kryptolebias hermaphroditus* (N= 22; mean standard length =28.27 mm SD= 4.90) and *Kryptolebias ocellatus* (N= 20; mean standard length =28.80 mm SD= 7.17; Supplementary material Table S4.1.), from six sites in Brazil, two sites where both species coexisted in sympatry (Guaratiba and Fundão; GUA and FUN), two sites only inhabited by *K. ocellatus* (Florianópolis and São Francisco do Sul; FLO and SFR) and two sites only inhabited by *K. hermaphroditus* (Picinguaba and Aracruz; PIC and ARA) as described in (Berbel-Filho et al., 2020; Berbel-Filho et al., 2022) (Figure 4.1.).

4.4.1. Differences in microbial composition between locations and fish species

The sequence for the samples were recorded as lowest >23,000 and the highest was >3,70,000. After denoising, the lowest non-chimeric reads recorded as >20,000 and the highest as > 3,30,000. Total features were recorded as 13,010, and after filtering mitochondria or chloroplasts the total features retained was 11,936. After that, sub-sampling (12,844 sampling depth) and another extra filtering (total abundance of less than 2 across all samples) left 9,598 ASVs in total from the samples. The relative abundances of different families differed between sampling locations, as shown by amplicon sequencing of the 16S rRNA region. In sympatry, *Phycisphaeraceae, Arcobacteraceae, Sulfurovaceae* were the three most abundant families for both species at location GUA, while *Arcobacteraceae, Moraxellaceae* and *Vibrionaceae* were the most abundant at FUN. Under allopatry, the five most abundant families in *K. ocelatus* were *Phycisphaeraceae, Rhodobacteraceae*, *Desulfosarcinaceae, Pirellulaceae* and *Anaerolineaceae* at SFR and *Wohlfahrtiimonadaceae, Sphingomonadaceae, Rhodobacteraceae, Anaerolineaceae* and *Flavobacteriaceae* at FLO. For *K. hermaphroditus* the most abundant families were *Vibrionaceae,*

Pseudoalteromonadaceae, Comamonadaceae, Moraxellaceae and *Arcobacteraceae* at PIC and *Rhodobacteraceae, Thermaceae, Solimonadaceae, Moraxellaceae* and *Vibrionaceae at* ARA (Figure 4.2.a.).

The two species also displayed significant differences in ASV composition at the sympatric locations, with 46 ASVs being significantly different in abundance at GUA and 20 at FUN. The highest differences at location GUA corresponded to *Sulfurovum* (represented by at least three different ASVs with different abundance in both fish species) (P<0.001), *Arcobacteraceae* (P<0.001) and *Sulfurimonas* (P<0.001 (all more abundant in *K. ocellatus*). At FUN, the main differences corresponded to *Anoxybacillus* (P=0.010), *Meiothermus* (P=0.030) and *Intrasporangiaceae* (P=0.008) (more abundant in *K. hermaphroditus*) and *Desulfosarcina* (P=0.010) and *Rhodobacteriaceae* (P=0.025) (more common in *K. ocellatus*) (Figure 4.2. b,c).
Some ASVs were significantly associated with certain species and locations. In sympatry, 13 and 26 indicator taxa were identified at GUA and FUN, respectively (Supplementary material, Table S4.2.a), whereas 3 and 19 were identified for *K. hermaphroditus* and *K. ocellatus* (Supplementary material, Table S4.2.b). A similar pattern was found between species when all the locations were pooled (Table 4.2.), with 61 indicative taxa for *K. ocellatus* compared to 25 for *K. hermaphroditus*. Indicative taxa for locations displayed a higher relative abundance than those from different species (Supplementary material Table 4.2. a,b). Indicative species-specific ASVs included different ribotypes of *Sulfurovum*. Indicative ASVs related to the location included *Phycisphaeraceae* (Supplementary material Table S4.3.).

The predicted community metagenomic profiling based on MetaCyc pathway data for prokaryotes identified 17 and 2 functional traits that differed between locations within *K. ocellatus* and *K. hermaphroditus* populations, respectively, when all locations were compared. In contrast, no functional differences were identified between species in the shared locations, indicating that species-indicative ASVs in those habitats had likely redundant functions. Functional traits enriched in the different locations included the mevalonate pathway I and isoprene biosynthesis II (engineered) for *K. ocellatus* and L-arginine degradation II (AST pathway) and cob(II)yrinate a,c-diamide biosynthesis II (late cobalt incorporation) for *K. hermaphroditus* (Supplementary material Figure S4.9.).

4.4.2. Species and sampling location both influence microbiome alpha and beta diversity

We assessed the influence of species, sampling location and fish size on measures of alpha diversity (Shannon diversity, Chao1 richness, Simpson's evenness and Faith's phylogenetic diversity) (Supplementary material Table S4.1.). Body size (standard length) did not have a significant effect on Chao1 ($F=0.486$, $P=0.49$), Faith ($F=0.022$, $P=0.88$) or Simpson ($F=2.481$, $P=0.12$) diversity measures. Species, but not location, had a significant effect on Faith PD (phylogenetic diversity) and Chao1 (richness) measures of skin microbiome diversity (Faith: $F=0.005$, $P=0.006$; Chao: $F=6.771$, $P=0.013$), with *K. ocellatus* displaying higher diversity than *K. hermaphroditus* in both cases (Faith KOce mean=31.32 SD=13.10; KHer mean=17.86 SD=8.81; Chao: KOce mean =422.69 SD=263.91, KHer mean =213.68 SD=130.69). Only location influenced Simpson's evenness, which measures species' dominance, (Species: $F=0.301$; $P=0.58$; Location $F=8.039$, $P<0.0001$), which was also higher in *K. ocelatus* than in *K. herpmaphroditus* (KOce mean =0.23 SD=0.15, KHer mean =0.19 SD=0.13). Shannon diversity (which takes

abundance and evenness into account) was only significantly influenced by size (Species F=0.440, P = 0.512; Location F=1.586, P=0.191; Size F=5.851, P=0.021) (Figure 4.3.). Bootstrapping analyses, with 1,000 dataset replicates, supported the results of these models (Supplementary material Table S4.6., Figure S4.10.). Multivariate analysis of community separation (PERMANOVA) indicated that species, location and their interaction had a significant effect both on Bray-Curtis dissimilarity and weighted UniFrac distance, with location explaining the highest percentage of the data in both cases (Bray-Curtis: Location R^2= 0.265 P <0.001; Species R^2= 0.037 P =0.002; Location*Species R^2=0.004 P=0.004; UniFrac: Location R^2= 0.397 P <0.001; Species R^2= 0.039 P =0.026; Location*Species R^2=0.039 P=0.028). Group visualization by NDMS using Bray-Curtis distance revealed the influence of location and species in the structural diversity of skin microbiome, as both species were intermingled in the shared locations (FUN and GUA) but tended to group by species when originated in separate locations (Figure 4.4.a.). NMDS structuring based on weighted UniFrac distance was less clear apart from the samples from GUA that clustered together and more distant from the rest (Figure 4.4.b.).

Chao1 diversity was significantly influenced by species and sympatry (Species: F= 10.819 P= 0.002; Sympatry: F= 4.575 P=0.038), and so was Faith PD (Species: F= 17.118 P= 0.0002; ~Sympatry: F= 5.780 P=0.02), unlike Shannon diversity (Species: F= 3.486 P= 0.069; Sympatry: F= 2.177 P=0.148) or Simpson's evenness which was influenced only by sympatry (Species: F= 0.641 P= 0.428; Sympatry: F= 11.638 P=0.002). Chao1 and Faith_PD diversities were higher in *K. ocellatus* than in *K. hermaphroditus* and higher for both species when they were in sympatry compared to allopatry, while the latter trend was the opposite for Simpson's evenness (Figure 4.3., Supplementary material Figure S4.11.).

4.4.3. Individual genetic diversity influences microbiome diversity

To assess the influence of genetic variation on microbiome diversity, individual heterozygosity (He) was calculated from fish from FLO, SFR, FUN and GUA locations previously genotyped for 5,477 SNPs (Berbel-Filho et al., 2021b) (Supplementary material Table S4.1.). *K. hermaphroditus* (N=14) had an average individual heterozygosity of 0.04 (SD=0.01) while *K. ocellatus'* average individual heterozygosity (N=14) was 0.08 (SD=0.01), the latter being higher than expected from their respective mating systems, self-fertilising *K. herpmaphroditus* and outcrossing *K. ocellatus*. Comparisons between sympatric and non-sympatric populations indicated that species ($F= 186.571$ $P<0.0001$) and sympatry (shared or non-shared location; $F=28.101$ $P<0.0001$) both influenced individual heterozygosity, with *K. ocellatus* displaying higher heterozygosity when coexisting with *K. hermaphroditus* than in locations where it lived in isolation (there was no data available on He for *K. hermaphroditus* in isolation) (Figure S4.12).

Full models of microbiome alpha diversity included species, location, size and individual heterozygosity as predictors. Model checks carried out using the *performance* package indicated collinearity between species and heterozygosity, and species was removed from the model. Stepwise model selection using the *drop1* command indicated that individual heterozygosity ($F=6.192$, $P=0.020$) and location ($F=4.353$, $P=0.014$) significantly affected Chao1 richness and Faith phylogenetic diversity (heterozygosity $F=8.338$ $P=0.008$ and $F=6.573$ $P=0.22$). Fish size ($F=12.451$ $P=0.002$) and location ($F=6.191$ $P=0.003$) significantly influenced Shannon diversity and only location significantly influenced Simpson's evenness ($F=7.88$ $P=0.0009$) (Figure 4.5a-d). Non-parametric bootstrapping regressions based on 1,000 repetitions supported these results (Supplementary material Table S4.6.). Mantel tests between genetic (based on SNPs) and

microbiome distance matrices, carried out using 10,000 permutations, indicated a weak but significant positive correlation between Euclidean genetic distance and weighted UniFrac microbiome dissimilarity (which considers microbiome phylogenetic distance) (Mantel R= 0.155 P=0.047) but no significant correlation with Bray-Curtis microbiome dissimilarity (Mantel R= -0.134 P=0.108) (Supplementary material Table S4.4., Figure S4.13.).

4.4.4. Host microbiome and genetic differentiation are associated with DNA methylation

To assess the relationship between microbiome and host DNA methylation patterns, we used data on genetic (SNP-based) and epigenomic (DNA methylation) pairwise distances between individuals, previously estimated in (Berbel-Filho et al., 2022) for 18 fish occurring in sympatry. Multiple regression analysis (MRM) was carried out using 10,000 permutations to assess the relationship between epigenetic distance and genetic and microbiome distance matrices, and the results indicated a significant relationship (R^2=0.435 P=0.001), with methylation dissimilarity being significantly and positively correlated to both genetic Euclidean (P=0.001) and microbiome Bray-Curtis (P=0.001) distance (Supplementary material Table S4.5., Figure 4.6.). When the analysis was run with weighted Unifrac distance instead of Bray-Curtis for the microbiome dissimilarity, the relationship was still significant (R^2=0.131 P=0.001) but only the Euclidean genetic distance was significantly correlated to the methylation Bray-Curtis dissimilarity (Euclidean: P=0.001; Unifrac P=0.921). As a measure of individual epigenetic diversity we also estimated the coefficient of variation (CV) of the counts per million of 64,152 methylated sites (Berbel-Filho et al., 2022). Simpson's evenness index was the only alpha diversity metric significantly associated with methylation CV, with the best model including just Simpson_e (F=25.12 P<0.001).

4.4.5. Fluctuating asymmetry correlates with host microbiome and genetic differentiation

Fluctuating asymmetry (FA; i.e., the random deviation from symmetry in bilateral organisms) (Palmer, 1994) is a phenotypic indicator of developmental instability often associated with environmental or genetic stress, although its relationship with fitness is unclear (Lens et al., 2002). We measured FA in three traits: pupil diameter and distance from the eye to the snout, previously shown to exhibit FA in fish (Allenbach et al., 1999) and area of the caudal ocellus, a dark spot present in *Kryptolebias hermaphrodites* and secondary males which has been associated with aggressive behaviour (Luke & Bechler, 2010). Only two traits (ocellus area and pupil diameter) displayed FA and were retained for the analyses, while the eye-snout distance displayed antisymmetry. Total FA was higher in *K. ocellatus* than in *K. hermaphroditus* ($t = -2.0886$, $df = 19$, $P = 0.05$) (Supplementary material Figure S4.14.a-c.) and positively correlated with Faith phylogenetic distance ($R=0.55$, Pearson $P = 0.010$, Permutation-based $P=0.006$), chao1 diversity ($R=0.51$, Pearson $P = 0.019$, Permutation-based $P=0.020$) and individual heterozygosity ($R=0.45$, Pearson $P = 0.039$, Permutation-based $P=0.044$) (Figure 4.7.) but not with methylation coefficient of variation ($R=-0.24$, Pearson $P = 0.422$, Permutation-based $P=0.414$) (Supplementary material Figure S4.14.d.).

4.5. Discussion

Current understanding of how the microbiome and the host (epi)genome contribute to host phenotypic plasticity is still limited, despite their potentially important influence on adaptation (Baldassarre et al., 2022), particularly in populations with low genetic diversity. We assessed the potential association of microbiome and epigenetic variation of two closely related fish species with contrasting mating systems and variable levels of genetic diversity, living in sympatry and in

allopatry, to analyse their potential contribution to variability in wild populations with low genetic diversity.

Species and location as drivers of skin microbiome composition and diversity

The skin microbiome of the mangrove killifishes was dominated by *Proteobacteria* at the phylum level, followed by *Campilobacterota*, *Planctomycetota*, *Actinobacteriota*, *Bacteroidota* and *Firmicutes*, all of these commonly present in the fish microbiota (Ghanbari et al., 2015, Givens et al., 2015). Differences between sympatric and allopatric populations highlighted the influence of the environment on microbiome composition and diversity, more pronounced in *K. ocellatus* than in *K. hermaphroditus*, but differences between both species in sympatry also indicated a species-specific effect on the skin microbiome. These results suggest that location played a more important role than species in microbiome differentiation, and that *K. ocellatus* (the outcrossing species) displayed a higher proportion of unique taxa than *K. hermaphroditus* (selfing species). Fish species and location affected alpha diversity in different ways. While species seemed to influence more microbial ASV richness and phylogenetic diversity, location had a stronger influence on ASV evenness and dominant community members. The interaction between location and species was observed as well in the microbiome population structuring, measured by beta diversity, but the environment explained more of the differentiation, as for other fish species like Atlantic salmon (Uren Webster et al., 2018). A similar pattern is found in the gut microbiome of mangrove crabs, that displays species-specific microbiome metagenomic profiles but also influence of the environment, resulting in a large amount of OTUs shared between species (Tongununui et al., 2022). In general, the fish skin supports a very diverse microbiome community, different from the surrounding water and variable at different levels, from species to individuals and organs

(Chiarello et al., 2015) and the influence of species-specific factors and location on the microbiome seems very variable among fish groups (Riiser et al., 2020; Sylvain et al., 2020; Escalas et al., 2021), probably resulting from the large fish diversity and long evolutionary history (Pan et al., 2022). We found strong differences in ASV composition but also functional redundancy between the microbiome of both species living in sympatry. Functional redundancy of the fish microbial community occurring at different scales (e.g., local communities or habitats) acts as an spatial ecological insurance within ecosystems, ensuring the maintenance of key ecological processes within and across habitats (Escalas et al., 2017). Thus, the functional redundancy observed between both species in sympatry could also be a reflection of their different colonization histories, with *K. hermaphroditus* having only recently colonized those locations (Berbel-Filho et al., 2020; Lira et al., 2021). Indicative species-specific ASVs included different ribotypes of *Sulfurovum*, which oxidises sulphur and thiosulfate and is found in the gut and gill microbiome of marine invertebrates (like sea cucumber and snails) where it could be providing detoxification and nutritional intake for the host (Yang et al., 2022). Indicative ASVs related to the location included *Phycisphaeraceae*, a member of the scarcely studied class *Phycisphaerae*, common in the marine environment (Lage & Bondoso, 2014; Kopprio et al., 2021) and in the microbiome of freshwater fish and is related to low dissolved oxygen levels (Krotman et al., 2020), such as those found in mangrove killifish habitats (Turko et al., 2012).

Relationship between microbiome, host genetic diversity and epigenetics

The role of genetics in determining the microbiome composition has been mainly discussed in terms of species specificity, in fish and other taxa (Brucker & Bordenstein, 2012; Larsen et al., 2013), evidenced by the concordance between host phylogenies and microbiome assemblages

(Brooks et al., 2016). Population genetic divergence has also been shown to influence microbiome differentiation in fish (Smith et al., 2015). The populations we analysed displayed a natural gradient of individual heterozygosity which correlated positively with both microbial phylogenetic diversity and richness. *K. ocellatus* (the outcrossing and genetically more variable species), displayed more microbiome differentiation and diversity within and between locations than its self-fertilising counterpart, reflecting the strong relationship between microbiome and genetic diversity. Given the influence of environment and species on microbiome composition, the observed relationship between heterozygosity and the microbiome could also reflect co-variation between species and genetic diversity, driven by the selective pressures imposed by environmental heterogeneity on population sizes and genetic diversity of both species (Vellend, 2005; Vellend & Geber, 2005) . This result highlights the importance of considering microbiome measurements for conservation (Redford et al., 2012), particularly in the face of rapid environmental change, which also affects the microbiome. The key role of both microbiome and genetic diversity in host fitness, and the implications that low genetic diversity and inbreeding have in reducing host immunocompetence (Bahrndorff et al., 2016), mean that reduced genetic and microbial host diversity could interact to reduce host resilience to environmental change (Ørsted et al., 2022). However, despite the lower genetic and microbial diversity of the self-fertilising *K. hermaphroditus*, these populations are stable or even expanding across their range (Berbel-Filho et al., 2020), suggesting that alternative sources of plasticity could also play a role in their adaptation to environmental change.

It has recently been suggested that the microbiome, which is influenced by the host genetics and environmental selective pressures (Gilbert et al., 2010), could be considered as an additional

epigenetic mechanism of the host (Angers et al., 2020), and that the holobiont (host and microbiome with their respective genomes) (Gilbert et al., 2010) could be the target of selection. Our results indicate an association between the host genetics and skin microbiome with the host epigenetics (DNA methylation). Epigenetic pairwise distance between individuals was positively correlated with microbiome differentiation and genetic dissimilarity. In addition, fish with higher coefficient of variation in DNA methylation (used here as a rough estimation of epigenetic individual variability) displayed higher alpha diversity (evenness) in their skin microbiome distribution. At least part of this association could reflect the close relationship between epigenetic and genetic diversity (Fargeot et al., 2021), which we have previously observed in the sister species of *K. hermaphroditus*, *K. marmoratus*, also self-fertilising, reared under different environmental conditions (Berbel et al., 2020). Our previous data also indicated that an interaction between parasite loads (including gill bacterial cysts) and genetic diversity influenced DNA methylation patterns in wild *K. hermaphroditus* populations (Berbel-Filho et al., 2019). However, variation in epigenetic diversity (CV) was not explained by heterozygosity and, of all measurements of alpha diversity, only Simpson's evenness (unrelated to heterozygosity) had an influence on its distribution. This suggests that a proportion of epigenetic diversity is not directly related to host genetic diversity. In the closely related *K. marmoratus*, we had previously found that there was a small proportion of epigenetic diversity associated with the rearing environment, but not with the genotype, that might be maintained in the next generation (Berbel-Filho et al., 2019; Berbel-Filho et al., 2020). Stochastic and pure epigenetic epimutations (driven by the environment) which can persist over generations (Beltran et al., 2020) have been suggested as a potential bet-hedging strategy, particularly relevant for populations with low genetic diversity (Rey et al., 2020). Microbiome dissimilarity (both Bray-Curtis and UniFrac differentiation) was also uncorrelated

with genetic differentiation, indicating that the more genetically similar fish (in this case those originating from *K. hermaphroditus* self-fertilisation) did not share a more similar microbiome, which instead was more related to the sampling location.

As with most field studies, our data is observational and this makes it difficult to determine the direction or causality of the observed relationships or draw inferences about the adaptive response. We attempted to overcome this challenge, by measuring fluctuating asymmetry in traits related to fish performance. Fluctuating asymmetry in phenotypic traits is often used as a proxy for fitness (Allenbach, 2011), based on its relationship with heterozygosity, stress and inbreeding, but the significance and strength of this association is inconsistent among studies (Lens et al., 2002). We identified a positive association between FA and heterozygosity, which does not fit the 'heterozygosity theory', according to which more heterozygous individuals should be developmentally more stable than their more homozygous counterparts, due a higher metabolic efficiency (Clarke, 1993). This relationship would, however, fit the 'genomic coadaptation theory', according to which developmental stability can decline if coadapted gene complexes are disrupted, for example by gene flow or introgression (Clarke, 1993). *K. hermaphroditus* is naturally highly inbred and displays no evidence of inbreeding depression (Gresham et al., 2021), thus its self-fertilising reproduction could result in a purge of deleterious alleles and in more balanced coadapted gene complexes, which would increase developmental stability and therefore decrease asymmetry (Markow, 1995). Despite their different mating systems, both species are able to hybridise when occurring in sympatry, and we previously found evidence of backcrosses of the hybrids with *K. ocellatus* (Berbel-Filho et al., 2021a). Although none of the fish analysed here were classified as hybrids, the hybridisation history among mangrove killifishes (Berbel-Filho et

al., 2022) means that there is potential for gene flow between both species, with introgression likely affecting mostly *K. ocellatus*. This asymmetric hybridisation could be a source of genetic stress reducing developmental stability in *K. ocellatus*. In addition to heterozygosity, both faith and chao1 measures of microbiome diversity, but not epigenetic diversity, were associated with FA.

There are some limitations of this study. The main limitation was the study would have been more detailed and complete if it could also analyse the water samples of the respective study sites and if that would compare with the skin microbiome data of this study. Also, another limitation comparing this study with laboratory reared killifish was about the lack of gut samples of these wild killifish.

In summary, we found that both environment and species play a role on shaping the microbiome diversity and community composition of the mangrove killifish. Genetic, epigenetic and microbiome diversity displayed a complex relationship, where heterozygosity and microbiome alpha diversity, but not epigenetic variation, were associated to the fluctuating asymmetry of traits related to fish performance (vision) and behaviour (aggression). We also identified the occurrence of epigenetic diversity and microbiome differentiation independent of host heterozygosity or genetic differentiation, and associated to each other. We cannot ascertain whether this association could be due to the production of microbial metabolites regulating the epigenome (as in mammals (Alenghat, 2015; Oliveira, 2021)), the result of the influence of the host epigenome on the microbiome, or co-variation in response to environmental pressures (Vellend & Geber, 2005). Yet, irrespective of its origin, the proportion of epigenetic and microbiome diversity unrelated to host genetics could provide an additional source of variation, potentially very important for fish with low genetic diversity.

TABLES

Table 4.1. Species and sampling locations

Species	**Species code**	**Location code**	**Sampling location**	**Sample size N=42**
Kryptolebias ocellatus	KO	FLO	Poço das Pedras, estuário do rio Ratones, Florianópolis, SC	7
Kryptolebias ocellatus	KO	SFR	Manguezal no canal do Linguado, São Francisco do Sul, SC	5
Kryptolebias hermaphroditus	KH	PIC	Manguezal do rio da Fazenda, P. E. S. M. Picinguaba, Picinguaba, SP	5
Kryptolebias hermaphroditus	KH	ARA	Alagado na praia de Coqueiral, Aracruz, ES	3
Kryptolebias ocellatus	KO	GUA	Manguezal do rio Piracao, Guaratiba, RJ	5
Kryptolebias hermaphroditus	KH	GUA	Manguezal do rio Piracao, Guaratiba, RJ	10
Kryptolebias ocellatus	KO	FUN	Manguezal da Ilha do Fundao, Rio de Janeiro, RJ	3
Kryptolebias hermaphroditus	KH	FUN	Manguezal da Ilha do Fundao, Rio de Janeiro, RJ	4

Table 4.2. Pairwise comparison of ASV abundances between localities for both mangrove killifish species in sympatric (GUA, FUN) and allopatric locations. GUA=Guaratiba, FUN=Fundão, Florianópolis=FLO, São Francisco do Sul=SFR, Picinguaba=PIC, Aracruz =ARA.

Species	**Locations**	**No ASV differences**
K. hermaphroditus	FUN v ARA	46
	FUN v GUA	147
	FUN v PIC	79
	GUA v ARA	104
	PIC v ARA	60
	PIC v GUA	170
K. ocellatus	FUN v FLO	314
	GUA v FLO	321
	SFR v FLO	286
	FUN v GUA	147
	FUN v SFR	201
	GUA v SFR	347

FIGURES

Figure 4.1. Sampling locations for *Kryptolebias ocellatus* and *Kryptolebias hermaphroditus* (fish photos from Waldir Berbel-Filho) indicating locations with single (FLO, SFR, PIC and ARA) and both species present (FUN and GUA). GUA=Guaratiba, FUN=Fundão, Florianópolis=FLO, São Francisco do Sul=SFR, Picinguaba=PIC, Aracruz =ARA.

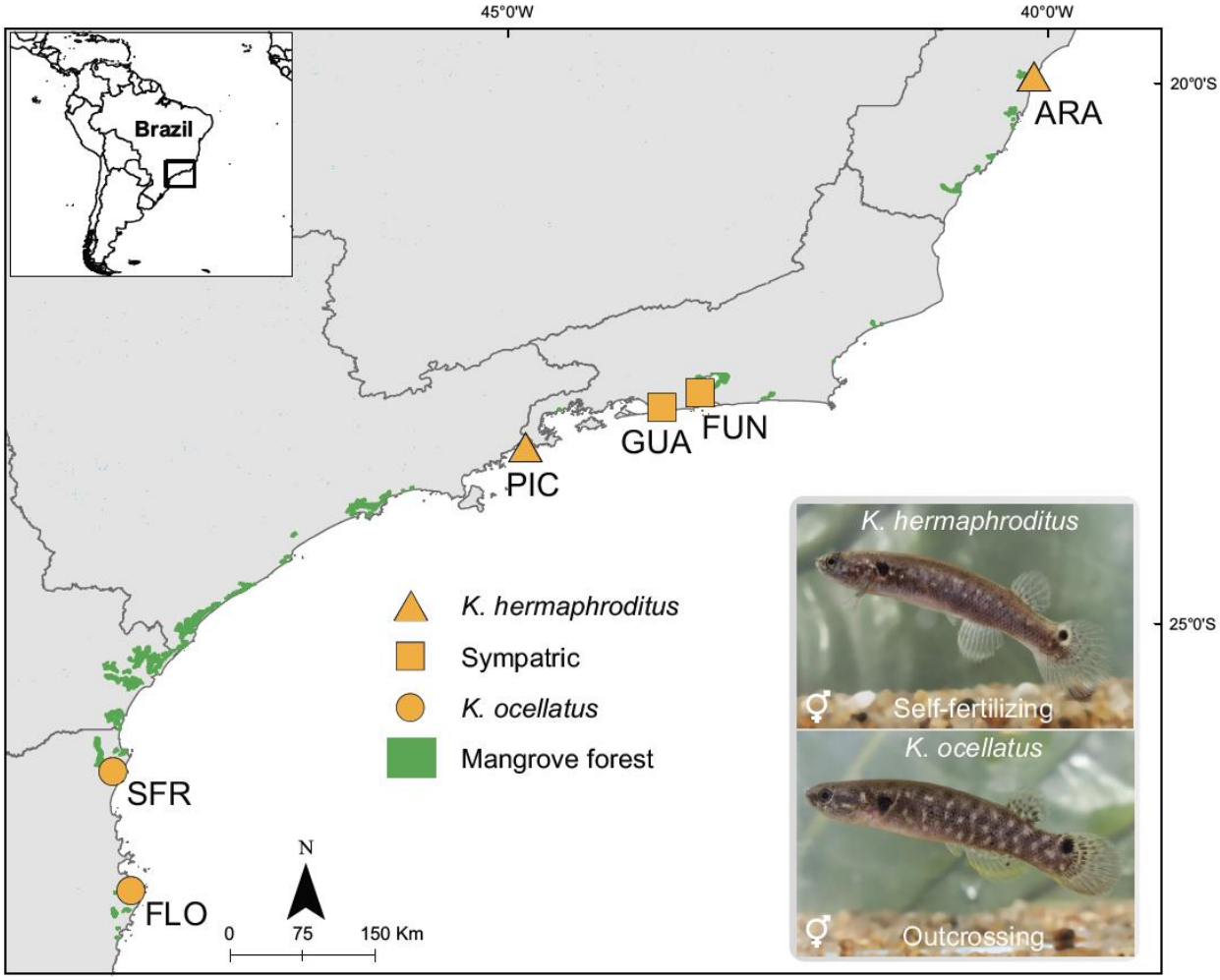
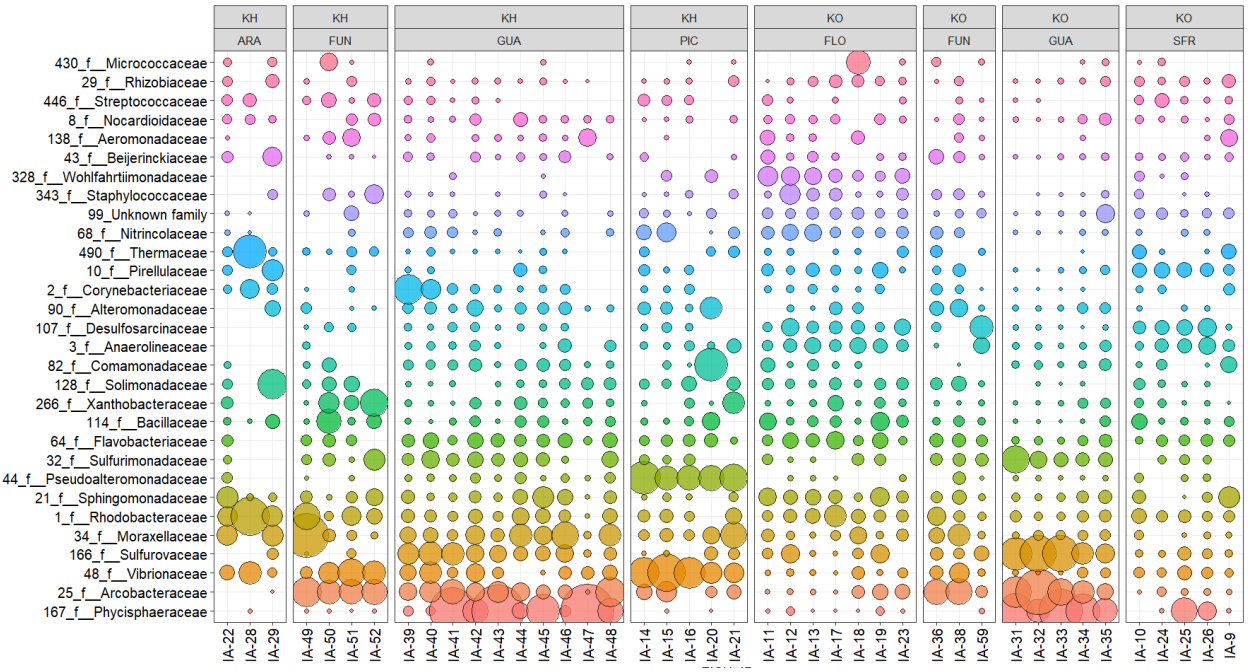
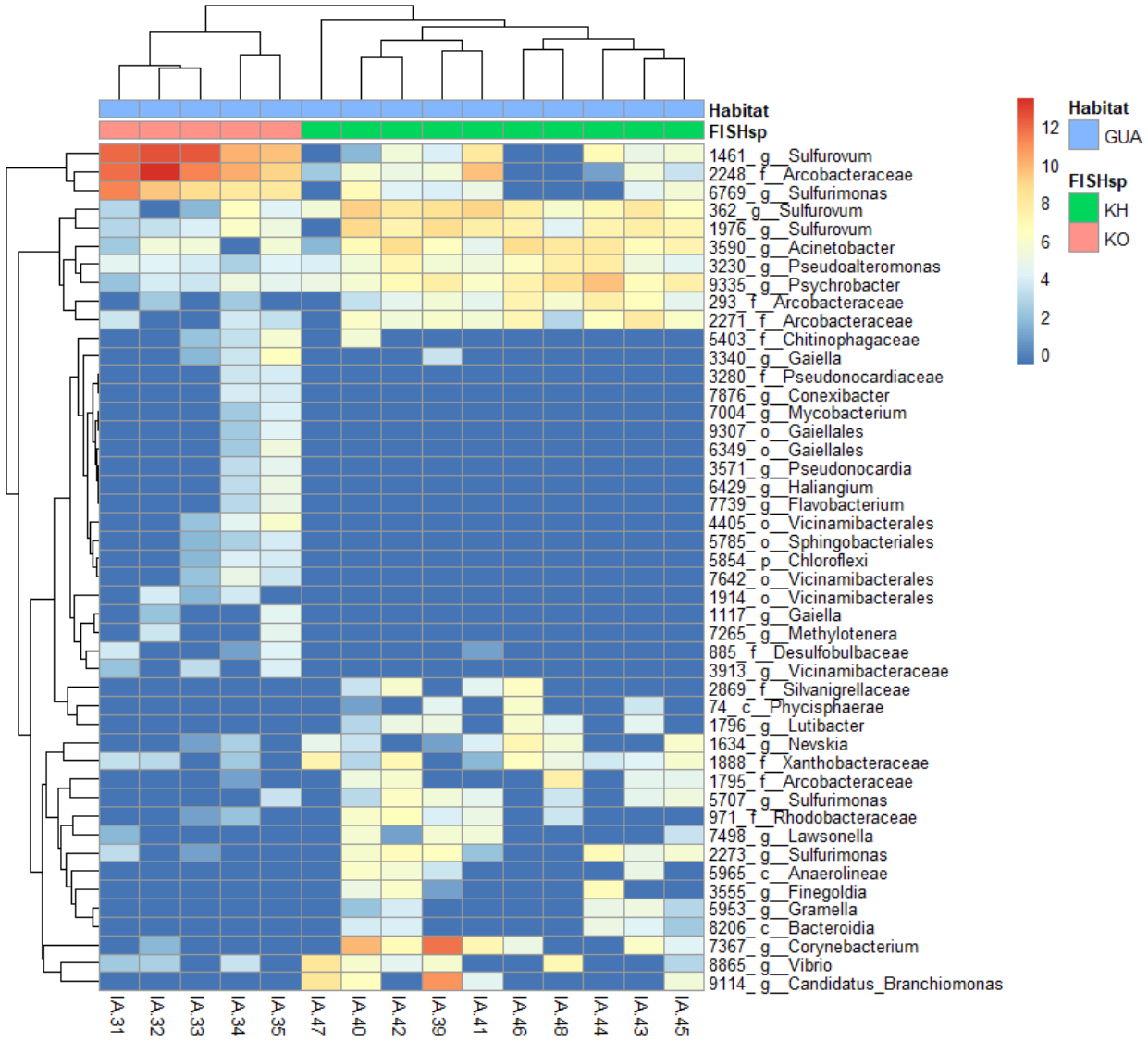


Figure 4.2. (a) Most abundant 30 families based on 12,844 subsampled reads, separated by species and location (single or shared). Columns represent individual fish. **(b)** Significant differences in ASV composition between species in sympatry in GUA sampling location and **(c)** in FUN. GUA=Guaratiba, FUN=Fundão, Florianópolis=FLO, São Francisco do Sul=SFR, Picinguaba=PIC, Aracruz =ARA. KO= *Kryptolebias ocellatus*, KH= *Kryptolebias hermaphroditus*, and IA= Identifying As used to identify individual fish with an identical number in this study.

(a)



(b)


Habitat
FISHsp
1461_g__Sulfurovum
2248_f__Arcobacteraceae
6769_g__Sulfurimonas
362_g__Sulfurovum
1976_g__Sulfurovum
3590_g__Acinetobacter
3230_g__Pseudoalteromonas
9335_g__Psychrobacter
293_f__Arcobacteraceae
2271_f__Arcobacteraceae
5403_f__Chitinophagaceae
3340_g__Gaiella
3280_f__Pseudonocardiaceae
7876_g__Conexibacter
7004_g__Mycobacterium
9307_o__Gaiellales
6349_o__Gaiellales
3571_g__Pseudonocardia
6429_g__Haliangium
7739_g__Flavobacterium
4405_o__Vicinamibacterales
5785_o__Sphingobacteriales
5854_p__Chloroflexi
7642_o__Vicinamibacterales
1914_o__Vicinamibacterales
1117_g__Gaiella
7265_g__Methylotenera
885_f__Desulfobulbaceae
3913_g__Vicinamibacteraceae
2869_f__Silvanigrellaceae
74_c__Phycisphaerae
1796_g__Lutibacter
1634_g__Nevskia
1888_f__Xanthobacteraceae
1795_f__Arcobacteraceae
5707_g__Sulfurimonas
971_f__Rhodobacteraceae
7498_g__Lawsonella
2273_g__Sulfurimonas
5965_c__Anaerolineae
3555_g__Finegoldia
5953_g__Gramella
8206_c__Bacteroidia
7367_g__Corynebacterium
8865_g__Vibrio
9114_g__Candidatus_Branchiomonas
IA.31
IA.32
IA.33
IA.34
IA.35
IA.47
IA.40
IA.42
IA.39
IA.41
IA.46
IA.48
IA.44
IA.43
IA.45
Habitat
GUA
FISHsp
KH
KO
12
10
8
6
4
2
0

(c)

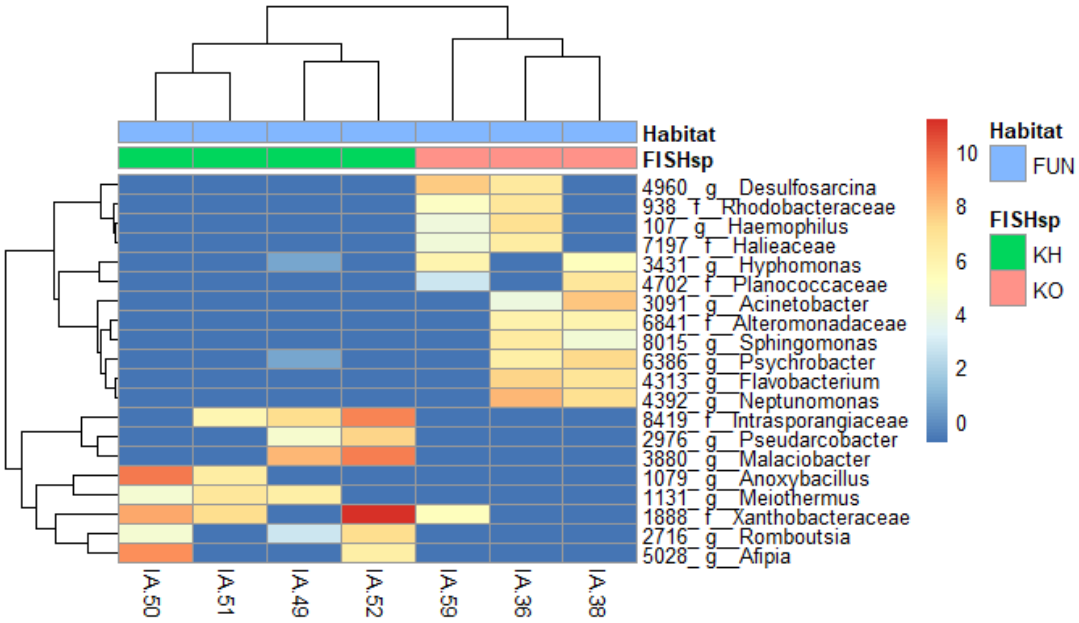

Habitat
FISHsp
4960_g__Desulfosarcina
938_f__Rhodobacteraceae
107_g__Haemophilus
7197_f__Halieaceae
3431_g__Hyphomonas
4702_f__Planococcaceae
3091_g__Acinetobacter
6841_f__Alteromonadaceae
8015_g__Sphingomonas
6386_g__Psychrobacter
4313_g__Flavobacterium
4392_g__Neptunomonas
8419_f__Intrasporangiaceae
2976_g__Pseudarcobacter
3880_g__Malaciobacter
1079_g__Anoxybacillus
1131_g__Meiothermus
1888_f__Xanthobacteraceae
2716_g__Romboutsia
5028_g__Afipia
IA.50
IA.51
IA.49
IA.52
IA.59
IA.36
IA.38
10
8
6
4
2
0
Habitat
FUN
FISHsp
KH
KO

Figure 4.3. Alpha diversity measures of the skin microbiome of *Kryptolebias ocellatus* (blue) and *Kryptolebias hermaphroditus* (red) from shared (GUA and FUN) and separate (SFR, FLO, PIC and ARA) locations.

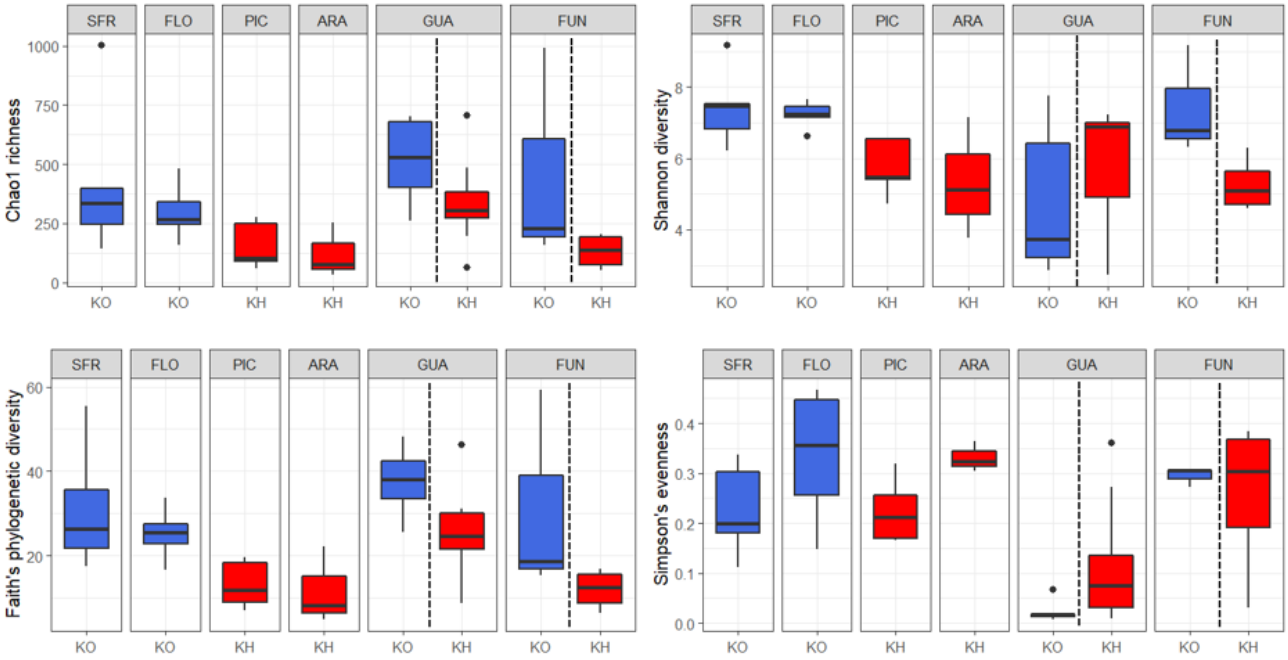
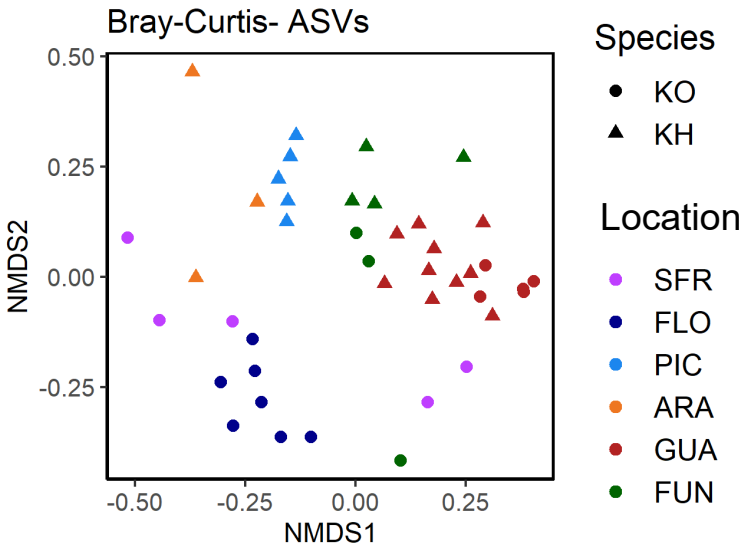


Figure 4.4. Non-metric multidimensional scaling (NMDS) ordination of the microbial skin community of *Kryptolebias ocellatus* (circles) and *Kryptolebias hermaphroditus* (triangles) from shared (GUA and FUN) and separate (SFR, FLO, PIC and ARA) locations based on (a) Bray-Curtis distance and (b) weighed UniFrac distance.

(a)



(b)

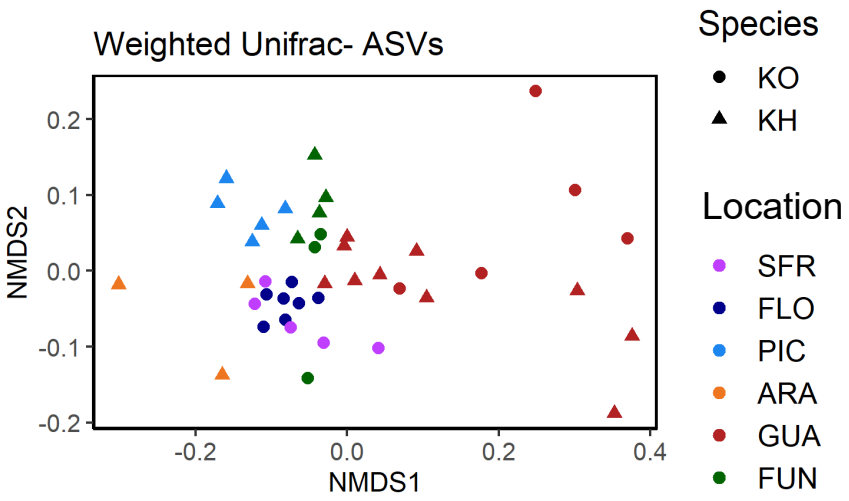


Figure 4.5. Relationship between individual heterozygosity and estimates of skin microbiome alpha diversity (a) Chao1, (b) Shannon index, (c) Faith phylogenetic distance and (d) Simpson's evenness index for *Kryptolebias ocellatus* (blue) and *Kryptolebias hermaphroditus* (orange) in the locations where both species coexisted (FUN, GUA).

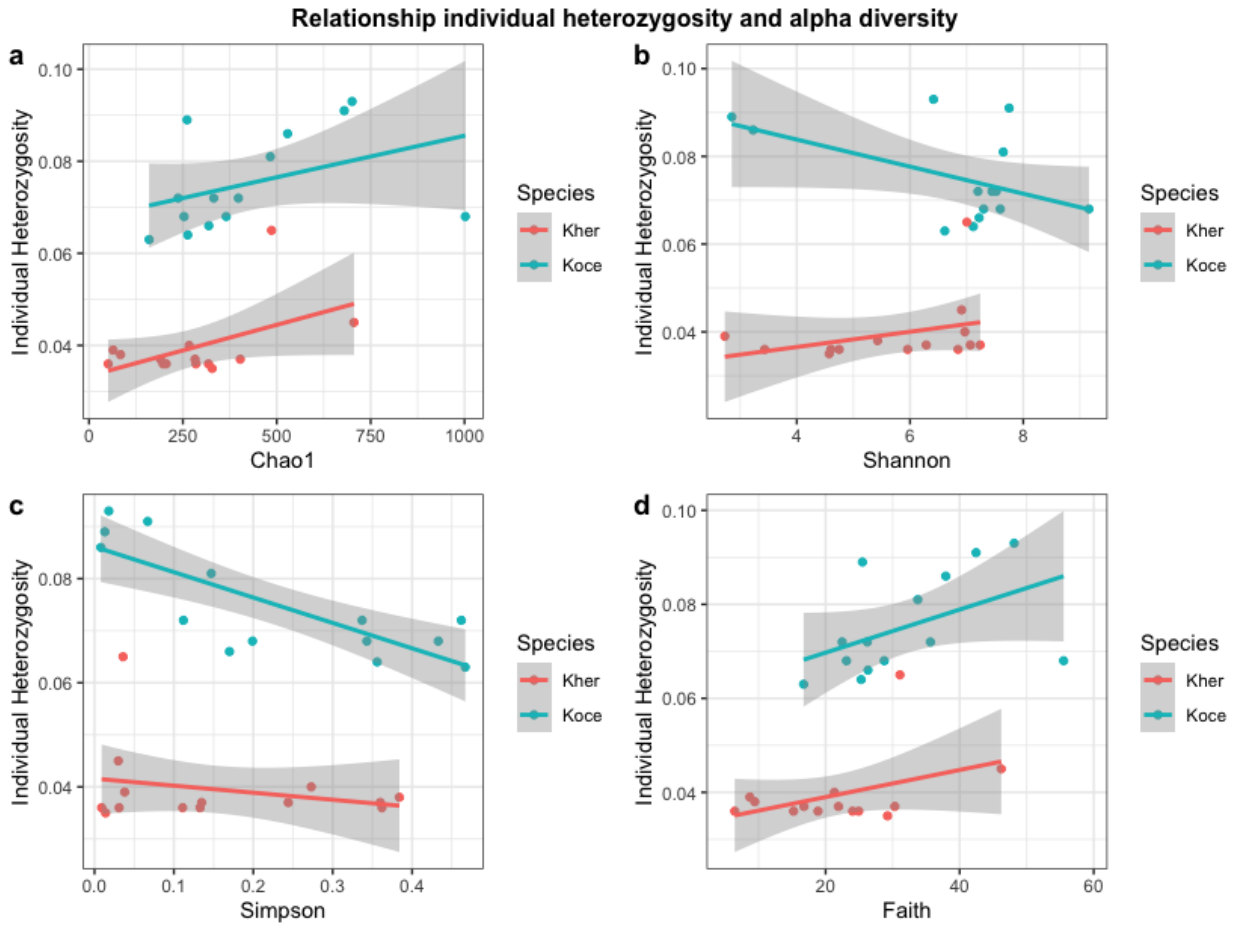


Figure 4.6. Relationship between epigenetic (DNA Methylation, Bray-Curtis), genetic (SNPs, Euclidean) and microbiome (Bray-Curtis) pairwise distances between 18 individuals (14 *Kryptolebias hermaphroditus* and 4 *K. ocellatus*) in sympatry (GUA and FUN sampling locations), including, variables distribution, value of the correlation and significance based on Pearson tests (see main text for MRM analysis on distance matrices).

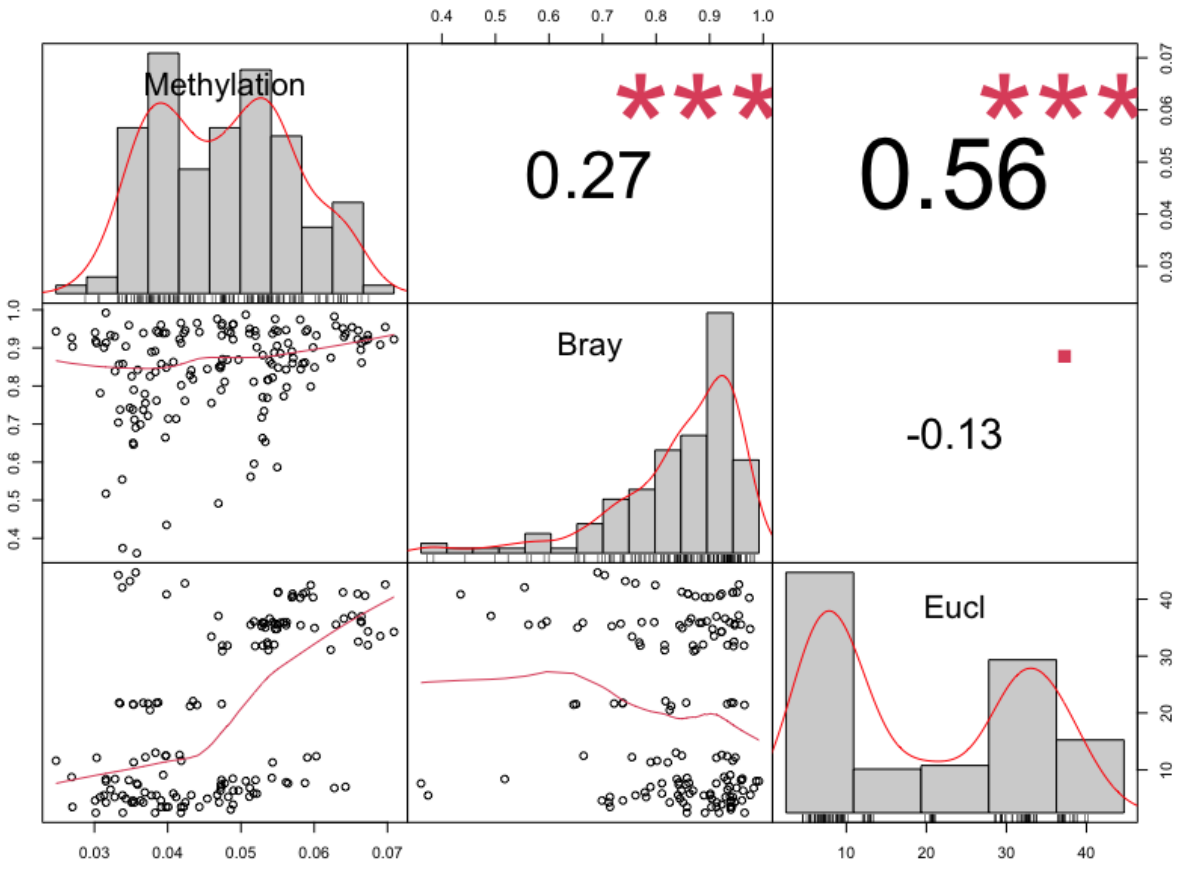
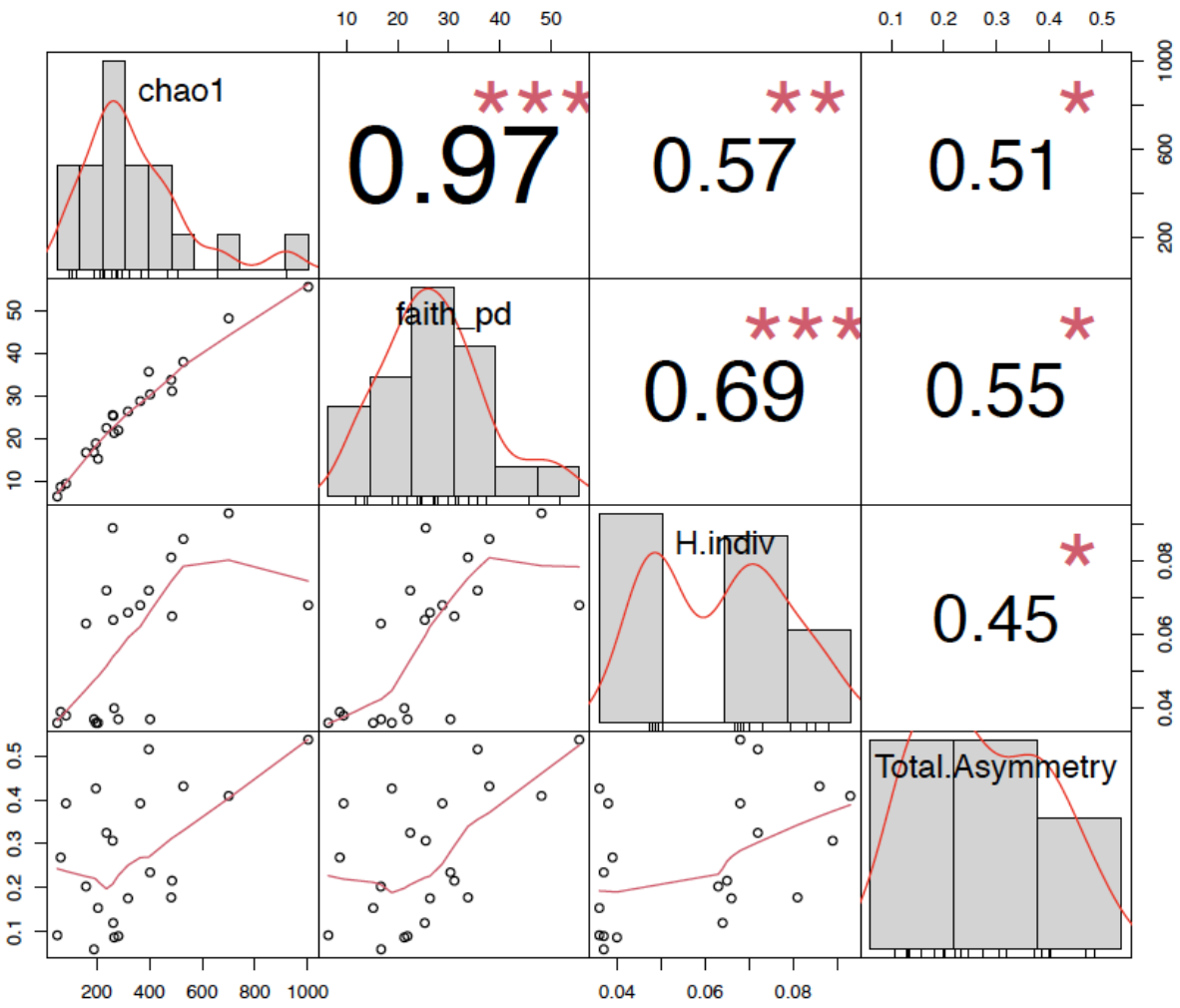


Figure 4.7. Relationship between total fluctuating asymmetry, genetic diversity (individual heterozygosity) and microbiome alpha diversity pairwise distances between 21 individuals (10 *Kryptolebias hermaphroditus* and 11 *K. ocellatus*), including variables distribution, value of the correlation and significance based on Pearson tests (see main text for additional probabilities based on 1,000 permutations).



VII. GENERAL DISCUSSION

The genetic diversity and environment of fish affect their fitness and survival by influencing both behaviour and microbiome, and understanding the complex interaction between genetics and environment is essential for managing fish performance under environmental challenges. The mangrove killifishes (*Kryptolebias* sp.) are considered effective models to disentangle the effects of the environment from fish genotype, due to their distinctive mating modes. The global aim of this thesis was to explore phenotypic (behaviour and basal metabolism) and microbiome (gut) responses to changes in rearing conditions in the selfing mangrove killifish species (*Kryptolebias marmoratus*) under laboratory conditions, and to assess the microbiome (skin) composition in two other closely related mangrove killifish species (*K. ocelatus* and *K. hermaphroditus*) with different mating systems (self-fertilising and outcrossing) in their natural environment, to understand combinedly how the environment and genotype are contributing to fish response and their microbiome overall.

The first three chapters (**Chapter 1-3**) contain the results from the experiments conducted with self-fertilising *K. marmoratus*, where I tested the environmental factors (diet and physical rearing conditions) on two closely related genetic strains (DAN and HON9) of this species. The findings of **Chapter 1** suggest that the differences in individual fish behaviour depend on several important aspects such as the incubation length, genetic variability, and the physical enrichment of the rearing environment. I found an important influence of genetic background (strain), diet (as an environmental factor) and hatching type on fish activity, possibly reflecting behavioural differences with incubation time. Individuals that hatch earlier can be more active compared to the later hatchers. In zebrafish, hatching time has resulted in behavioural differences in anxiety (early hatched fish showed lower anxiety compared to the lately hatched ones) and avoidance behaviours

in a novel environment (Silva et al., 2022). Hatching time has also been previously reported as an important marker to predict some other behavioural response such as alcohol sensitivity in zebrafish (Leite-Ferreira et al., 2019). The results obtained in **Chapter 1** depict a significant influence of hatching time on the activity of mangrove killifish (*K. marmoratus*), where earlier hatched fish were more active compared to the later hatchers, which had a longer embryonic diapause that could have an impact on their lower activity after hatching. Activity is an important aspect to consider for fish survival and reaction to environmental changes. However, behavioural differences can also be determined by several others associated factors such as early life experiences (Menezes et al., 2020) and, genetics.

Chapter 1 also evidenced the influence of host's diet and genotype (strain) on their microbiome alpha diversity, as well as the influence of hatching type on both their microbiome alpha and beta diversity. The interaction between diet and hatching type influenced the alpha diversity (Chao1) of the gut microbiome both in the naturally and artificially hatched fish groups, with an influence of experimental probiotic diet on the microbiome alpha diversity, more prominent in the earlier hatched individuals. A significant influence of genetic strain was also observed on the diversity of microbiome of both hatching groups. The influence of genetic background on fish microbiota has been documented before. However, the reason for this is still unclear (de Bruijn et al., 2018). Ultimately, it suggests that apart from the environmental factors that have been considered mostly as a major diver to shape the gut microbiome, it is also essential to consider the role of host's genetics on the diversity and composition of their microbiome (Savard et al., 2023). This is because the gut microbiome (diversity and composition) can be shaped by the larger effects of host's genotype and diet (Sullam et al., 2012) but also by additional effects of the environment on the

host, which can result in strong differences from the surrounding environment (Kashinskaya et al., 2018). Thus, both host's intrinsic (genotype) and extrinsic (diet and environment) factors and their interactions can play a key role to the phenotypic response and microbiome in fish. On this basis, I moved to the next phase of this research (**Chapter 2**), which analysed the impact of the rearing environment across generations.

The environment can induce trans or inter-generational phenotypic plasticity in fish through a range of phenotypic responses (including behaviour) which can be either adaptive or non-adaptive for the individuals. Phenotypic plasticity (inter or transgenerational) in a rapidly changing environment is the ability of the parents to influence the phenotypes of their offsprings with no changes in the genetics of the offsprings (Roy et al., 2023). In **Chapter 2**, I investigated the intergenerational influence of the rearing environment in two different genotypes (strains) of mangrove killifish, *K. marmoratus* and both the effect of parental and own rearing environment on the offspring's activity was identified. The observed effect of parental activity on offspring's activity suggests an epigenetic basis for the examined behaviours. Previous evidence suggested that epigenetic inheritance (environmentally induced) could also play some role in the coping mechanism and different biological aspects (e.g., stress response) of the offspring against environmental stressors from one generation to another (Shen et al., 2023), and can impact the physiology and ultimate fitness of the individuals. I have found an evidence of intergenerational influence of the rearing environments (interaction between parental and offspring's environments) on fish activity. In terms of physiology, we analysed the resting or basal metabolic rate (BMR) as a proxy to stress in **Chapter 2**. No significant influence of rearing environment or genotype was observed on offspring's BMR, similar to what was found in their parents (**Chapter 1**). However,

basal metabolism (oxygen consumption over time) being an important physiological biomarker in fish can depend on multiple factors such as cortisol (stress hormone) levels, rearing unit, and the biological condition and size of the fish (Samaras, 2023).

The comparison between parents and offspring in **Chapter 2** indicated a potential intergenerational influence of the rearing environment on fish behaviour, and this research was then expanded in **Chapter 3** by exploring whether the rearing environment could have any intergenerational influence on the fish microbiome. The microbiome is influenced by both host genetics and the environmental factors experienced by the individuals (Kokou et al., 2018). Fish microbiome comprises of *Bacteria*, viruses, protists and *Archare* that can be colonised in fish skin, gill, and gastrointestinal tract (i.e., gut) (Merrifield & Rodiles, 2015). The environment can cause microbial modulations in fish in a species-specific manner. For example, altered gut microbiome and physiology (growth) was observed in salmonids (chinook salmon, *Oncorhynchus tshawytscha*) due to different water temperatures (8-20 ℃) (Steiner et al., 2022), whereas no influence of temperature variation (8-12 ℃) on gut microbiome diversity or abundance was found in large yellow croaker (*Larimichthys crocea*) (Lv et al., 2021). So, environmental changes can potentially impact fish microbiome, yet the actual underlying process is unclear (Morshed & Lee, 2023) and the relative role of host genetics and the intergenerational influence of their rearing environment (enriched vs poor) on fish gut microbiome is still largely unexplored.

In **Chapter 3**, environmentally induced intergenerational influence on fish-gut microbiome diversity was investigated. The host's genotype had no significant influence on microbiome alpha (Chao1 richness, Simpson's evenness) and beta (Bray-Curtis and Weighted UniFrac distances)

diversity. Neither direct nor significant influence of both host's own and parental rearing environments on microbiome diversity (Chao1) of the offspring's gut microbiome was observed at their early life stage (~6 months old individuals compared with parental matched or mismatched environments). However, there was an influence of the interaction between own and parental environment on the microbiome Simpson's evenness. This evidence of intergenerational environmental influence on microbiome alpha diversity (Simpson's evenness) could indicate that a similar rearing environment to the parents (i.e., intergenerational environmental stability) can result in a more even distribution of diverse gut microbiome in mangrove killifish, *K. marmoratus*.

The finding that microbiome diversity can be determined by the environment of fish at its very early rearing stages has a broader implication for the management of the fish rearing environment. But the relative role of host's genotype and the environment can vary in different fish species. The association between host's genetics and gut microbiome was observed through the among-individual variation of major histocompatibility complex class (MHC IIb) polymorphism correlated with fish gut microbiome in stickleback (*Gasterosteus aculeatus*), where the presence of certain MHC was found to alter the increase or decrease of some microbial Families relative abundance (Bolnick et al., 2014). However, it does not say anything about epigenomic changes in that observation. It can be a new field to explore whether certain MHC could regulate or not the expression of certain genes (i.e., epigenomic change) which might regulate the feeding, immune status or activity of fish. Host's overall lifespan (duration) can be impacted by their gut microbiome colonization at early life stage (young-middle age), as reported previously in African turquoise killifish (*Nothobranchius furzeri*) (Smith et al., 2017). A large abundance of common microbes can be observed in the individuals from same species regardless of different rearing environments,

for example wild or farmed (Merrifield & Rodiles, 2015). In this study, *Vibrio* was the most abundant bacteria across the individuals from both parental and offspring generations (**Chapter 3** and **Chapter 1**). In the offspring, several bacteria of the *Vibrionaceae* family were observed. *Shewanellaceae* was another abundant bacterium in both parents and offspring's gut samples from both genotypes (DAN and HON9) of *K. marmoratus* in this study. Although there is no information on the microbiome of the wild *K. marmoratus*, these results contrast with the composition of their closely related species *K. ocellatus* and *K. hermaphroditus*, sampled in the wild in Brazil (**Chapter 4**). This difference is likely due to the influence of the rearing environment (the strains have been laboratory based for many generations) which has made them diverge from the wild, and it is likely that their original microbiome also differed due to the species-specific characteristics as well as their original location (Strains DAN and HON9 was originated from Belize and Honduras respectively). Sometimes, fish display the same microbial composition in both natural and artificially maintained environments. For example, in zebrafish, similar microbiome was detected from both laboratory reared and wild fish individuals (Roeselers et al., 2011), although the findings was from freshwater origin. So, the diversity and the composition of gut microbiome in fish is not a reflection always of their surrounding environment, still the contribution of environment into this mechanism is very important subject to consider not only to understand host-microbiome functions but also to explore the potential interactions between host and environment (**Chapter 4**).

Given the important role of host's genetic and environmental factors on fish microbiome (Arun & Midhun, 2023), their influence was further assessed for the first time in *Kryptolebias* sp. from natural habitats, focusing on skin microbiome in **Chapter 4**. When the skin microbiome of two

different wild mangrove killifish species, *K. ocelatus* (outcrossing) and *K. hermaphroditus* (self-fertilizing) were compared, higher microbial alpha diversity was observed from the outcrossing species. A positive correlation between microbiome alpha diversity indices (richness and phylogenetic diversity) and species heterozygosity was also observed in this study, indicating a potential species-specific effect (Sadeghi et al., 2023) but also a correlation between genetic and microbiome diversity. Microbiome beta diversity was significantly influenced by the interaction of both species and their locations. In addition, a correlation between the skin microbiome and host's epigenetics (DNA methylation) variation indicated that both variables could be important for the response of fish with low genetic diversity to the environmental changes.

Overall, this thesis revealed that both genetic and environmental factors play an important role in the behaviour and microbiome composition in mangrove killifishes, both critical aspects for fish adaptation to environmental change. Despite the strong environmental differences between the laboratory and the natural conditions, our analysis on both types of environments highlighted the interaction between genotype and the environment in shaping fish microbiome composition and diversity and suggested potential role of epigenetics on influencing the microbiome diversity (which warrants further exploration).

Future research scopes

In **Chapter 1**, probiotic fed fish from strain HON9 were more active, HON9 was also reported with higher microbiome alpha diversity, while incubation time influenced activity and microbiome at different level which needs further research to explore the influence of incubation time differences on shaping the microbiome and behaviour in fish considering the comparison of microbiome between environment and fish (Eichmiller et al., 2016). **Chapter 2** found an intergenerational influence of rearing environment on killifish; it requires further research on fish to examine associated other environmental challenges (Feng et al., 2024) and the relative role of individual's genotype on the pattern of fish behaviour depending on parental experiences. In **Chapter 3**, observed intergenerational effect of rearing environments on killifish gut microbiome needs further research to test whether multiple environmental changes (e.g., climate change) would impact the immune response (Franke et al., 2024) across generations in a specific fashion or not. Moreover, the role of location over fish species on skin microbiome in different wild killifish species in **Chapter 4** would lead further research to examine how the environmental stimuli (an important fact for wild dynamic environment) would play a major role in shaping the microbiome of a particular fish species by comparing the microbiome of multiple fish organ (e.g., gut, gill) with their environmental (e.g., soil, water) microbiome (Diwan et al., 2022) and also by incorporating a metagenomic approach this time followed by possible exploration of functional analysis (Legrand et al., 2020) of the metagenomes from the findings.

VIII.CONCLUDING REMARKS

- There is a significant influence of hatching type (incubation time) on the activity and microbiome composition of the mangrove killifish, *Kryptolebias marmoratus*, at least under experimental conditions. The genetic background (strain) plays a significant role in the behaviour (activity and inspections) of *K. marmoratus*.
- Diet and genetic strain also have significant influence on the diversity (alpha) of *K. marmoratus* gut microbiome, while microbiome beta diversity was mainly influenced by incubation time.
- The rearing environment (both parental and offspring) has an influence on offspring's activity in *K. marmoratus*, and the interactions between parental and the offspring's own rearing environment significantly influenced offspring's gut microbial alpha diversity, but not beta diversity. Influence of parental activity (but no influence of genotype) on their offsprings activity, suggesting an epigenetic basis for this behaviour.
- Parental and offspring basal metabolic rate (BMR) were not influenced by the environment or strain in the laboratory reared *K. marmoratus*.
- Bacteria from *Vibrionaceae* and *Shewanellaceae* families were the most abundant gut microbiome of *K. marmoratus* both in parents and their offspring.
- Results from comparisons between wild mangrove killifish species *K. ocelatus* and *K. hermaphroditus* suggest that a complex interaction between host's genetics, microbiome (skin), and host's epigenetics, which could be important for fish with low genetic diversity.

IX. APPENDIX I: SUPPLEMENTARY MATERIALS AND INFORMATION

CHAPTER 1:

Influence of early rearing environment and genetic background on fish behaviour and microbiome

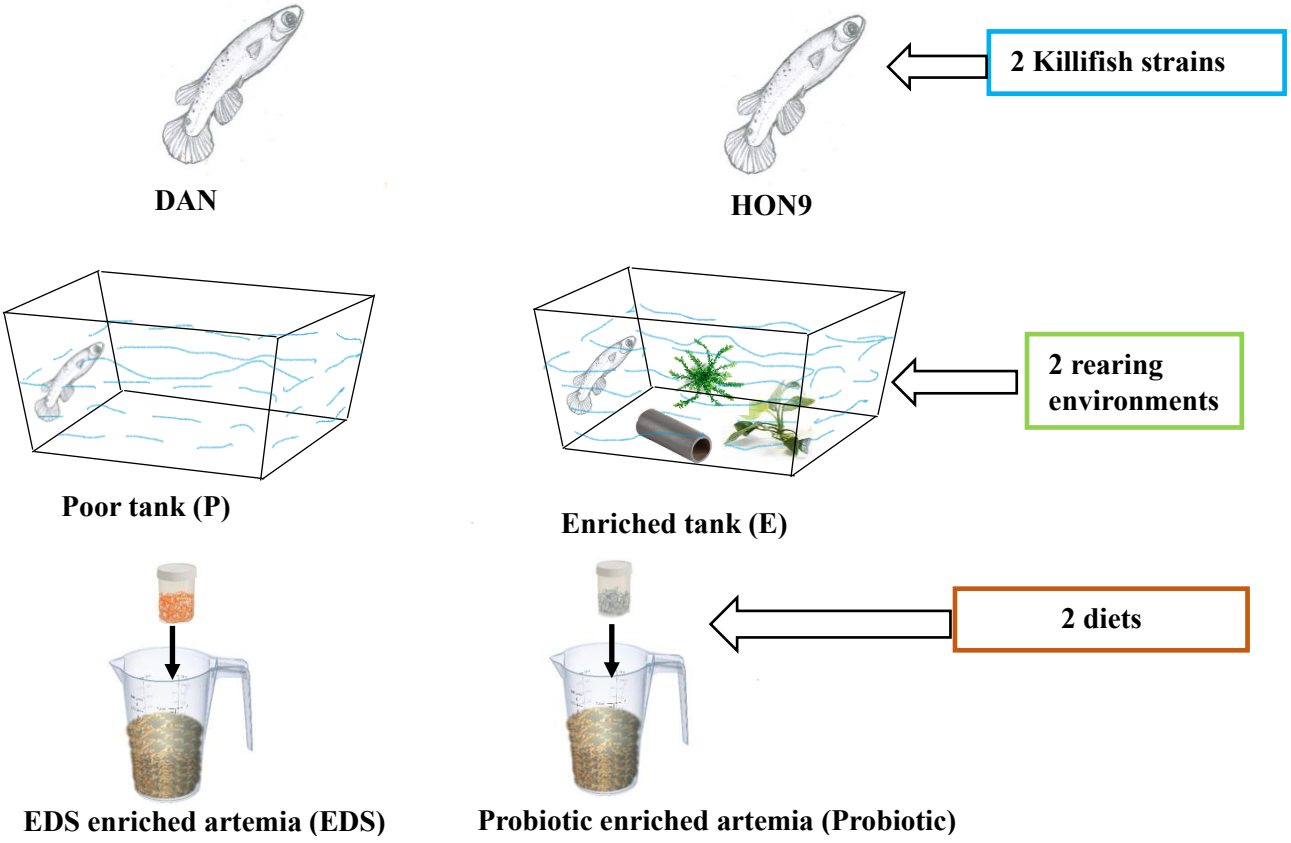
Table S1.1. List of experimental mangrove killifish, *K. marmoratus* from genetic strain HON9

SL. No./ Group	Fish ID	Diet Enrichment (EDS/Probiotic)	Rearing Environment (Enriched/Poor)
1.	HF1-02	EDS	Enriched
2.	HF1-20	EDS	Enriched
3.	HF1-24	EDS	Enriched
4.	HF1-28	EDS	Enriched
5.	HF1-38	EDS	Enriched
6.	HF1-56	EDS	Enriched
7.	HF1-08	EDS	Enriched
8.	HF1-10	EDS	Enriched
9.	HF1-16	EDS	Enriched
10.	HF1-36	EDS	Enriched
Subtotal = 10			
1.	HF1-04	EDS	Poor
2.	HF1-07	EDS	Poor
3.	HF1-15	EDS	Poor
4.	HF1-29	EDS	Poor
5.	HF1-37	EDS	Poor
6.	HF1-27	EDS	Poor
7.	HF1-33	EDS	Poor
8.	HF1-43	EDS	Poor
9.	HF1-63	EDS	Poor
10.	HF1-19	EDS	Poor
Subtotal = 10			
1.	HF1-06	Probiotic	Enriched
2.	HF1-12	Probiotic	Enriched
3.	HF1-22	Probiotic	Enriched
4.	HF1-50	Probiotic	Enriched
5.	HF1-52	Probiotic	Enriched
6.	HF1-60	Probiotic	Enriched
7.	HF1-66	Probiotic	Enriched
8.	HF1-14	Probiotic	Enriched
9.	HF1-18	Probiotic	Enriched
10.	HF1-26	Probiotic	Enriched
Subtotal = 10			
1.	HF1-11	Probiotic	Poor
2.	HF1-13	Probiotic	Poor
3.	HF1-17	Probiotic	Poor
4.	HF1-31	Probiotic	Poor
5.	HF1-35	Probiotic	Poor
6.	HF1-41	Probiotic	Poor
7.	HF1-57	Probiotic	Poor
8.	HF1-23	Probiotic	Poor
9.	HF1-30	Probiotic	Poor
10.	HF1-45	Probiotic	Poor
Subtotal = 10			
Total = 40			

Table S1.2. List of experimental mangrove killifish, *K. marmoratus* from genetic strain DAN

SL. No./ Group	Fish ID	Diet Enrichment (EDS/Probiotic)	Rearing Environment (Enriched/Poor)
1.	DF1-03	EDS	Enriched
2.	DF1-07	EDS	Enriched
3.	DF1-22	EDS	Enriched
4.	DF1-41	EDS	Enriched
5.	DF1-13	EDS	Enriched
6.	DF1-36	EDS	Enriched
7.	DF1-17	EDS	Enriched
8.	DF1-26	EDS	Enriched
9.	DF1-32	EDS	Enriched
10.	DF1-46	EDS	Enriched
Subtotal = 10			
1.	DF1-08	EDS	Poor
2.	DF1-04	EDS	Poor
3.	DF1-23	EDS	Poor
4.	DF1-31	EDS	Poor
5.	DF1-40	EDS	Poor
6.	DF1-45	EDS	Poor
7.	DF1-48	EDS	Poor
8.	DF1-63	EDS	Poor
9.	DF1-19	EDS	Poor
10.	DF1-27	EDS	Poor
Subtotal = 10			
1.	DF1-05	Probiotic	Enriched
2.	DF1-09	Probiotic	Enriched
3.	DF1-24	Probiotic	Enriched
4.	DF1-28	Probiotic	Enriched
5.	DF1-30	Probiotic	Enriched
6.	DF1-37	Probiotic	Enriched
7.	DF1-39	Probiotic	Enriched
8.	DF1-20	Probiotic	Enriched
9.	DF1-47	Probiotic	Enriched
10.	DF1-58	Probiotic	Enriched
Subtotal = 10			
1.	DF1-02	Probiotic	Poor
2.	DF1-06	Probiotic	Poor
3.	DF1-15	Probiotic	Poor
4.	DF1-16	Probiotic	Poor
5.	DF1-21	Probiotic	Poor
6.	DF1-14	Probiotic	Poor
7.	DF1-25	Probiotic	Poor
8.	DF1-38	Probiotic	Poor
9.	DF1-42	Probiotic	Poor
10.	DF1-43	Probiotic	Poor
Subtotal = 10			
Total = 40			

Figure S1.3. Experimental design considering two killifish (*K. marmoratus*) strains and environmental factors (rearing environments and diets) for the experiments.



Experimental design

DAN

EDS [P (n=10), E (n=10)] and Probiotic [P (n=10), E (n=10)]

HON9

EDS [P (n=10), E (n=10)] and Probiotic [P (n=10), E (n=10)]

S1.4. Probiotic supplement, preparation of diets and feeding protocol

A commercial probiotic, Sanolife® PRO-F supplied by the industrial partner INVE AQUACULTURE, Belgium (from product range of Health) and a common commercial diet, Easy Dry Selco® (EDS) were tested in this experiment. The probiotic product was a combination of three *Bacillus* bacteria (*Bacillus subtilis*, *B. licheniformis* and *B. pumilus*) and especially formulated ($1x10^{10}$ cfu/g) for fish farming. This probiotic supplement has been used in aquaculture (fish and shrimp) for several years to prevent and control disease, gut microflora colonization, and to improve feed utilization through inhibiting pathogenic bacteria. By contrast, EDS is a dry enrichment formulation that boosts *Artemia* with balanced levels of fats, proteins, vitamins, key minerals, immunostimulants, essential amino acids, and flavonoids.

Newly hatched *Artemia* sp. were used as bio-encapsulates to incorporate the diets in our experiment. According to the manufacturers' instructions, the inclusion of EDS and Probiotic was performed throughout the experimental diet trial. Freshly hatched *Artemia* nauplii was harvested and rinsed carefully prior to divide in 2 portions- one portion to enrich with EDS (~0.6-0.8 g/L) and the rest to enrich with probiotic supplement (~0.5 ml probiotic suspension/L, suspension was made of ~0.05 g dry probiotic powder and 0.5 ml distilled water). Continuous aeration was provided in the *Artemia* enrichment jar to ensure continuous agitation of the nauplii. In the following morning, enriched *Artemia* was filtered out and washed thoroughly prior to feed the fish. For both diets, *Artemia* was enriched for at least 18 hours to ensure the encapsulation of supplied enrichments. All fish under experimental conditions were fed daily once at a rate of 0.5 ml/fish for the newly hatched alevins for 1 month, 1 ml/fish for 1-2 months old fish, and 1.5 ml/fish for the fish from 3 months old and onwards.

Figure S1.5. A. Representative parents including male and self-fertilizing hermaphrodite *K. marmoratus.*

Male (♂)

Hermaphrodite (⚥)

Self-fertilizing killifish (⚥) laying fertilized eggs

Table S1.5. B. Different embryonic developmental stages were checked, and following stages were recognized based on previous developmental record references -

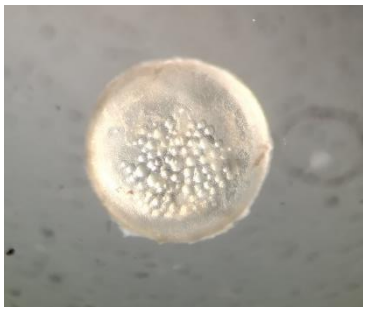
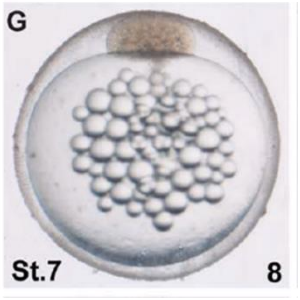
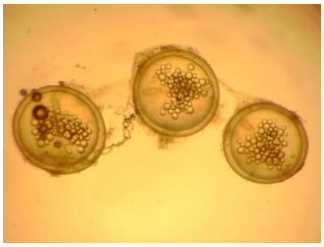
Photo from this experiment	**Reference photos from (Mourabit et al., 2011)**	**Recognized stages**
		**Stage 7. Early blastula** Dividing blastomeres seems to aggregate near the edge of slightly less rounded part of blastodisc, the separate cells in blastomeres can hardly be seen.
		**Stage 12. Mid-gastrula** Gastrulation begins and the divergence line of blastoderm is visible at this stage, very clear germ ring can be defined along with the mobile and aggregating oil droplets.

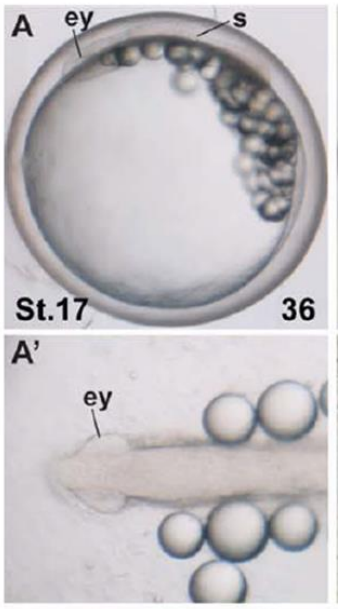
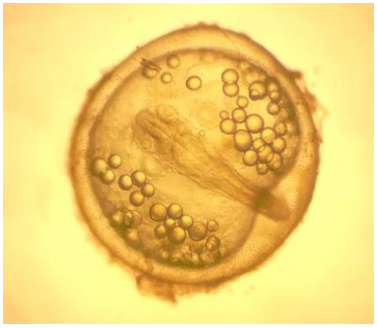
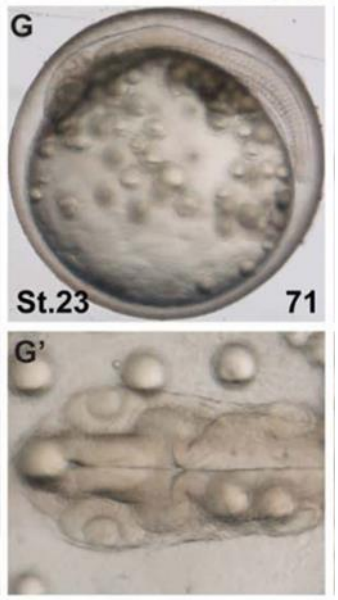
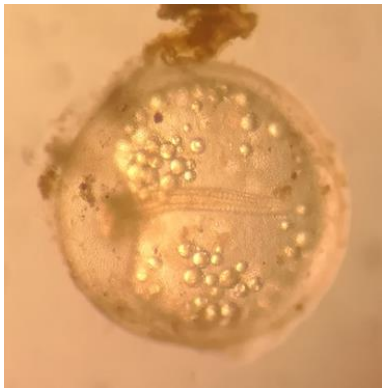
Photo from this experiment	Reference photos from (Mourabit et al., 2011)	Recognized stages
		**Stage 17. Optic vesicle and somite formation** The appearance of the optic vesicle and the brain development begins at this stage. Somite formation also occurs at this stage; however, it is difficult to observe clearly because of the oil droplets.
		**Stage 23. Increased vitelline circulation stage** Development of vitelline vessels and different developmental brain sections are visible at this stage. Oil droplets are found distributed all over the yolk globe.
		**Stage 25. Pectoral fin development** The development of pectoral fin appears at this stage along with dense aggregate otoliths.

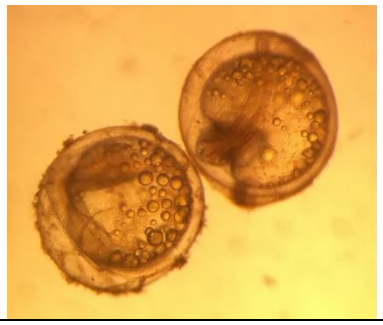
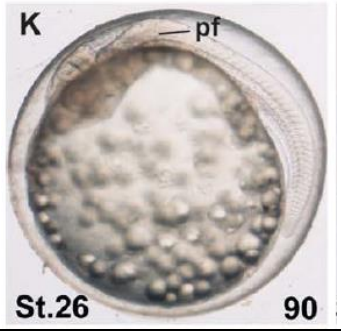
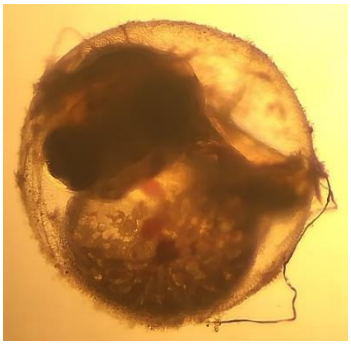
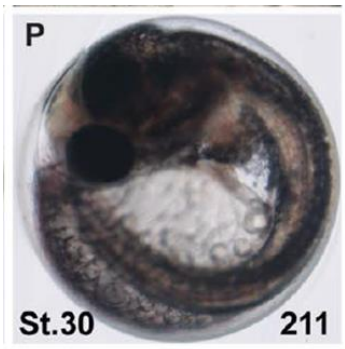
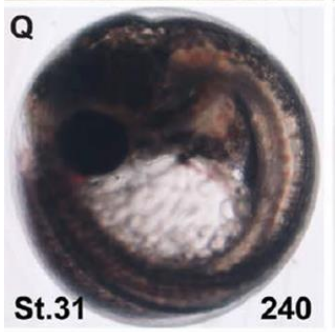
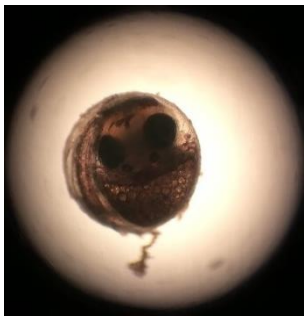
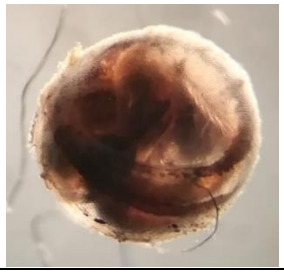
Photo from this experiment	Reference photos from (Mourabit et al., 2011)	Recognized stages
		**Stage 26. Liver formation stage** Liver bud starts to form behind the pectoral fin of the embryo, increased pigmentation seems to be dense at this stage.
	 	**Stage 30, 31. Jaw formation, pectoral fin movement, heartbeats stage** Pigmentation all over the body and brain is visible, the movement of pectoral fin and heartbeat is clearly possible to observe. The developing jaws are also the most remarkable to see at these stages.
 		**Stage 32. Ready to hatch** Well-defined fin rays and pigmentation are observed, most of the yolk sac is absorbed at this stage which makes the embryo ready to hatch.

Figure S1.5. C. Natural hatching and artificial dechorionization in the experimental killifish

(a) Naturally hatching of the embryo and newly hatched alevin

(b) Egg at embryonic diapause stage and alevin just after manual artificial dechorionizing

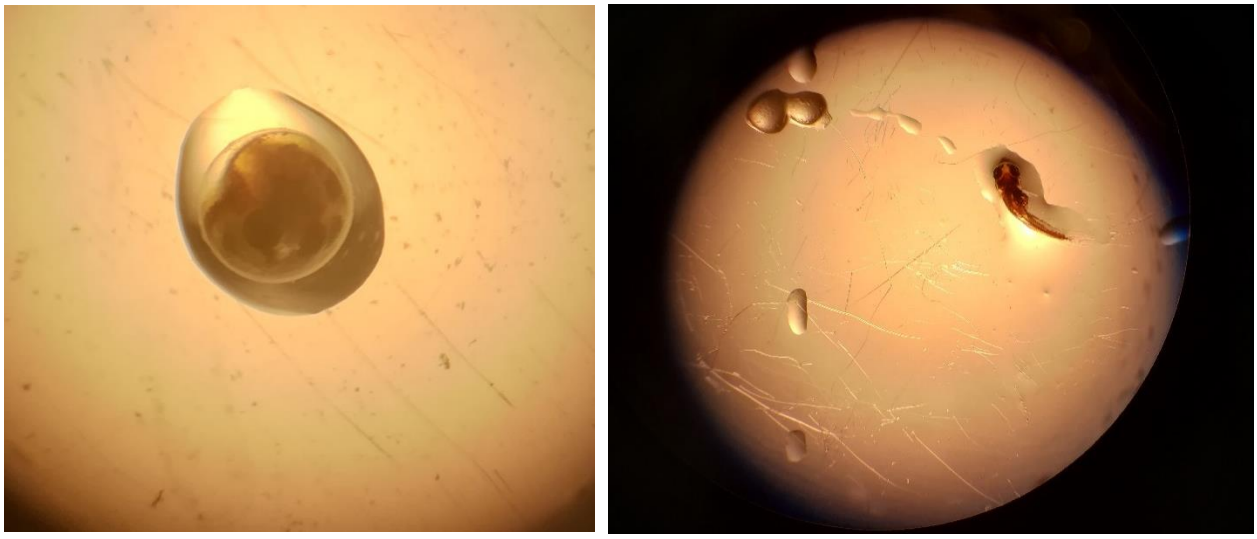


Figure S1.6. Behavioural test tank set up marked with acclimation zone (0) and five exploratory zones (1-5). A plastic Lego brick (orange colour) was used as a novel object placed at the middle of zone 3.

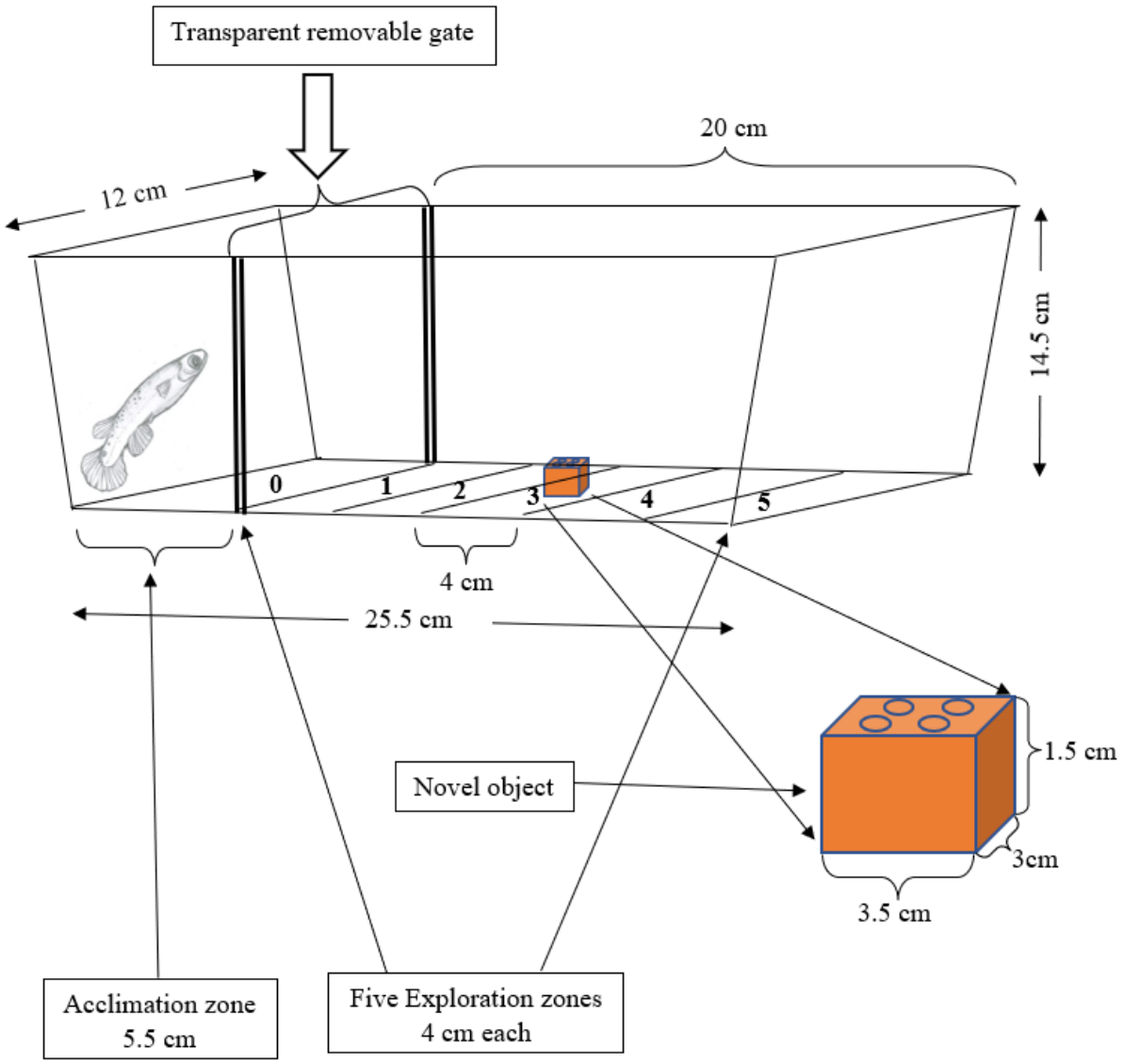


Figure S1.7. Respirometry measurement set up for experimental killifish (*K. marmoratus*)

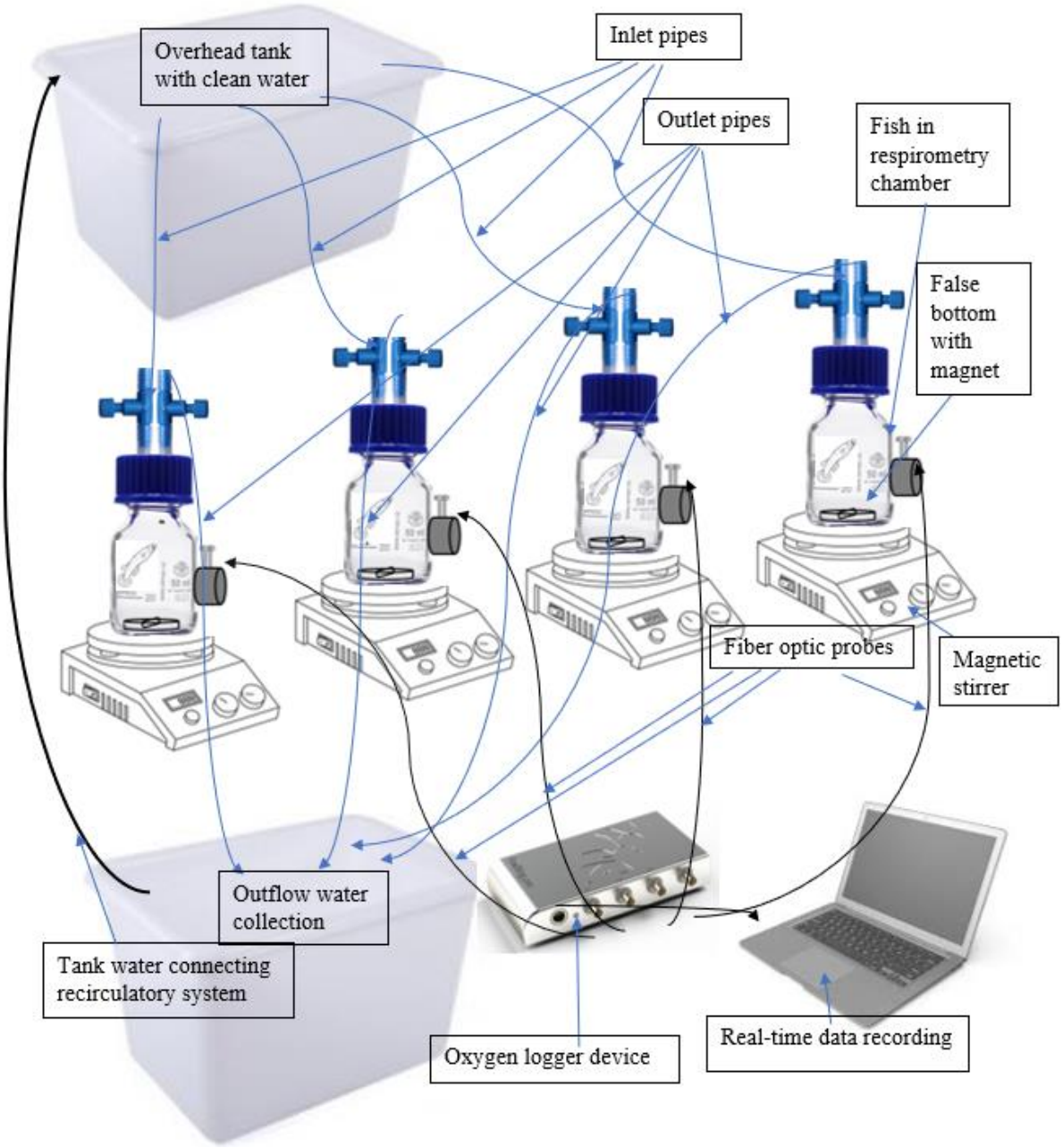


Table S1.8. Behavioural data from two strains of *K. marmoratus* from experimental diet and rearing environmental groups

Fish_ID	Strain	Diet	Environment	Latency	Inspections	Contacts	Activity	Exploration_total_s
DF1-02	DAN	Probiotic	Poor	278.411	14	2	59	921.589
DF1-03	DAN	EDS	Enriched	92.8	83	0	365	1107.2
DF1-04	DAN	EDS	Poor	161.634	2	0	14	1038.366
DF1-05	DAN	Probiotic	Enriched	383.605	0	0	4	816.395
DF1-06	DAN	Probiotic	Poor	0.652	37	0	205	1199.348
DF1-07	DAN	EDS	Enriched	153.506	27	0	162	1046.494
DF1-08	DAN	EDS	Poor	913.325	0	0	2	286.675
DF1-09	DAN	Probiotic	Enriched	73.404	3	0	31	1126.596
DF1-13	DAN	EDS	Enriched	597.177	8	0	52	602.823
DF1-14	DAN	Probiotic	Poor	335.529	16	0	212	864.471
DF1-15	DAN	Probiotic	Poor	310.746	24	0	99	889.254
DF1-16	DAN	Probiotic	Poor	202.08	0	0	6	997.92
DF1-17	DAN	EDS	Enriched	804.081	4	0	27	395.919
DF1-19	DAN	EDS	Poor	12.585	4	0	25	1187.415
DF1-20	DAN	Probiotic	Enriched	1200	0	0	0	0
DF1-21	DAN	Probiotic	Poor	477.849	29	0	179	722.151
DF1-22	DAN	EDS	Enriched	1200	0	0	0	0
DF1-23	DAN	EDS	Poor	0.347	13	3	52	1199.653
DF1-24	DAN	Probiotic	Enriched	157.984	4	0	21	1042.016
DF1-25	DAN	Probiotic	Poor	108.647	16	0	90	1091.353
DF1-26	DAN	EDS	Enriched	754.093	0	0	4	445.907
DF1-27	DAN	EDS	Poor	443.988	0	0	12	756.012
DF1-28	DAN	Probiotic	Enriched	152.61	14	0	65	1047.39
DF1-30	DAN	Probiotic	Enriched	42.671	0	0	2	1157.329
DF1-31	DAN	EDS	Poor	0.575	21	2	96	1199.425
DF1-32	DAN	EDS	Enriched	390.563	14	1	56	809.437
DF1-36	DAN	EDS	Enriched	504.713	7	0	30	695.287
DF1-37	DAN	Probiotic	Enriched	864.684	3	0	19	335.316
DF1-38	DAN	Probiotic	Poor	122.569	61	1	319	1077.431
DF1-39	DAN	Probiotic	Enriched	96.712	12	0	59	1103.288
DF1-40	DAN	EDS	Poor	940.016	2	0	12	259.984
DF1-41	DAN	EDS	Enriched	237.439	13	0	69	962.561
DF1-42	DAN	Probiotic	Poor	446.045	0	1	5	753.955
DF1-43	DAN	Probiotic	Poor	136.772	16	1	103	1063.228
DF1-45	DAN	EDS	Poor	177.808	10	1	57	1022.192

Table S1.8. Continued

Fish_ID	Strain	Diet	Environment	Latency	Inspections	Contacts	Activity	Exploration_total_s
DF1-46	DAN	EDS	Enriched	6.267	24	0	106	1193.733
DF1-47	DAN	Probiotic	Enriched	430.263	19	1	154	769.737
DF1-48	DAN	EDS	Poor	617.583	1	0	12	582.417
DF1-58	DAN	Probiotic	Enriched	260.926	4	0	36	939.074
DF1-63	DAN	EDS	Poor	617.641	1	0	20	582.359
HF1-02	HON9	EDS	Enriched	22.578	49	1	260	1177.422
HF1-04	HON9	EDS	Poor	0.52	10	0	56	1199.48
HF1-06	HON9	Probiotic	Enriched	1173.77	0	0	8	26.227
HF1-07	HON9	EDS	Poor	47.871	3	0	32	1152.129
HF1-08	HON9	EDS	Enriched	1200	0	0	0	0
HF1-10	HON9	EDS	Enriched	48.725	5	0	36	1151.275
HF1-11	HON9	Probiotic	Poor	2.123	22	0	253	1197.877
HF1-12	HON9	Probiotic	Enriched	39.428	8	1	36	1160.572
HF1-13	HON9	Probiotic	Poor	252.929	4	1	28	947.071
HF1-14	HON9	Probiotic	Enriched	751.089	13	0	57	448.911
HF1-15	HON9	EDS	Poor	753.534	11	0	51	446.466
HF1-16	HON9	EDS	Enriched	42.488	2	0	10	1157.512
HF1-17	HON9	Probiotic	Poor	759.18	0	0	10	440.82
HF1-18	HON9	Probiotic	Enriched	127.046	8	0	50	1072.954
HF1-19	HON9	EDS	Poor	294.281	1	0	6	905.719
HF1-20	HON9	EDS	Enriched	56.804	11	0	43	1143.196
HF1-22	HON9	Probiotic	Enriched	17.36	18	0	89	1182.64
HF1-23	HON9	Probiotic	Poor	91.452	0	0	4	1108.548
HF1-24	HON9	EDS	Enriched	179.175	0	0	4	1020.825
HF1-26	HON9	Probiotic	Enriched	116.824	5	0	27	1083.176
HF1-27	HON9	EDS	Poor	1200	0	0	0	0
HF1-28	HON9	EDS	Enriched	61.459	6	0	34	1138.541
HF1-29	HON9	EDS	Poor	898.987	3	0	10	301.013
HF1-30	HON9	Probiotic	Poor	213.446	0	0	6	986.554
HF1-31	HON9	Probiotic	Poor	102.233	8	0	40	1097.767
HF1-33	HON9	EDS	Poor	1200	0	0	0	0
HF1-35	HON9	Probiotic	Poor	1200	0	0	0	0
HF1-36	HON9	EDS	Enriched	859.15	0	0	4	340.85
HF1-37	HON9	EDS	Poor	1200	0	0	0	0
HF1-38	HON9	EDS	Enriched	193.248	14	1	63	1006.752
HF1-41	HON9	Probiotic	Poor	52.328	0	0	8	1147.672
HF1-43	HON9	EDS	Poor	624.571	1	0	8	575.429

Table S1.8. Continued

Fish_ID	Strain	Diet	Environment	Latency	Inspections	Contacts	Activity	Exploration_total_s
HF1-45	HON9	Probiotic	Poor	1.578	0	1	10	1198.422
HF1-50	HON9	Probiotic	Enriched	215.563	9	0	63	984.437
HF1-52	HON9	Probiotic	Enriched	497.263	7	1	30	702.737
HF1-56	HON9	EDS	Enriched	495.014	7	1	30	704.986
HF1-57	HON9	Probiotic	Poor	131.909	0	0	6	1068.091
HF1-60	HON9	Probiotic	Enriched	315.291	0	0	17	884.709
HF1-63	HON9	EDS	Poor	5.388	1	0	12	1194.612
HF1-66	HON9	Probiotic	Enriched	177.147	1	0	12	1022.853

Table S1.9. Respirometry data used for Basal metabolic rate (BMR) calculation using two strains of *K. marmoratus* from experimental diet and rearing environmental groups

FISH ID	Strain	Diet	Environment	Background as % of fish	O2_consumption_corrected (mg O2 h-1 g-1)	Weight_g	Length_cm	Hatching_type	Incubation_days
DF1-02	DAN	Probiotic	Poor	23.81	0.00346988	0.166	2.2	Natural	19
DF1-03	DAN	EDS	Enriched	16.95	0.009545455	0.0924	2.4	Natural	30
DF1-04	DAN	EDS	Poor	12.66	0.009857143	0.126	2.6	Natural	30
DF1-05	DAN	Probiotic	Enriched	13.35	0.01383	0.075	2.2	Natural	30
DF1-06	DAN	Probiotic	Poor	13.65	0.006646382	0.152	2.4	Natural	37
DF1-07	DAN	EDS	Enriched	12.66	0.010097561	0.123	2.2	Natural	38
DF1-08	DAN	EDS	Poor	22.22	0.004079137	0.139	2.4	Natural	38
DF1-09	DAN	Probiotic	Enriched	16.13	0.010516854	0.089	2.1	Natural	16
DF1-13	DAN	EDS	Enriched	22.99	0.005110169	0.118	2.4	Artificial_dechorionization	56
DF1-14	DAN	Probiotic	Poor	14.93	0.010058824	0.102	2.2	Artificial_dechorionization	56
DF1-15	DAN	Probiotic	Poor	9.34	0.016318421	0.095	2.3	Natural	53
DF1-16	DAN	Probiotic	Poor	14.60	0.011571429	0.091	2.2	Artificial_dechorionization	57
DF1-17	DAN	EDS	Enriched	15.52	0.008909091	0.099	2.4	Natural	57
DF1-19	DAN	EDS	Poor	21.05	0.008035714	0.084	2.2	Natural	64
DF1-20	DAN	Probiotic	Enriched	12.08	0.01179	0.1	2.3	Natural	52
DF1-21	DAN	Probiotic	Poor	21.98	0.007792683	0.082	2.1	Artificial_dechorionization	52
DF1-22	DAN	EDS	Enriched	21.51	0.006083333	0.108	2.4	Artificial_dechorionization	61
DF1-23	DAN	EDS	Poor	16.95	0.007229508	0.122	2.4	Artificial_dechorionization	49
DF1-24	DAN	Probiotic	Enriched	16.26	0.00927	0.1	2.2	Artificial_dechorionization	64
DF1-25	DAN	Probiotic	Poor	27.69	0.004272727	0.099	2.2	Natural	59
DF1-26	DAN	EDS	Enriched	16.51	0.0078	0.105	2.2	Natural	56
DF1-27	DAN	EDS	Poor	27.40	0.0045	0.106	2.4	Natural	57
DF1-28	DAN	Probiotic	Enriched	24.66	0.006111111	0.081	2.1	Artificial_dechorionization	73
DF1-30	DAN	Probiotic	Enriched	16.26	0.010905882	0.085	2.1	Artificial_dechorionization	79

Table S1.9. Continued

FISH ID	Strain	Diet	Environment	Background as % of fish	O2_consumption_corrected (mg O2 h-1 g-1)	Weight_g	Length_cm	Hatching_type	Incubation_days
DF1-31	DAN	EDS	Poor	23.81	0.004840336	0.119	2.4	Artificial_dechorionization	79
DF1-32	DAN	EDS	Enriched	11.69	0.012489796	0.098	2.4	Natural	79
DF1-36	DAN	EDS	Enriched	19.80	0.007923913	0.092	2.3	Artificial_dechorionization	63
DF1-37	DAN	Probiotic	Enriched	27.03	0.0050625	0.096	2.2	Artificial_dechorionization	87
DF1-38	DAN	Probiotic	Poor	23.81	0.0072	0.08	2.2	Artificial_dechorionization	64
DF1-39	DAN	Probiotic	Enriched	24.00	0.005896552	0.087	2.2	Artificial_dechorionization	95
DF1-40	DAN	EDS	Poor	26.67	0.00495	0.1	2.5	Artificial_dechorionization	77
DF1-41	DAN	EDS	Enriched	12.00	0.012	0.099	2.2	Artificial_dechorionization	80
DF1-42	DAN	Probiotic	Poor	24.69	0.0061	0.09	2.2	Artificial_dechorionization	80
DF1-43	DAN	Probiotic	Poor	22.99	0.00628125	0.096	2.3	Artificial_dechorionization	80
DF1-45	DAN	EDS	Poor	27.03	0.005855422	0.083	2.3	Artificial_dechorionization	80
DF1-46	DAN	EDS	Enriched	21.74	0.007363636	0.088	2.2	Artificial_dechorionization	17
DF1-47	DAN	Probiotic	Enriched	16.22	0.010084337	0.083	2.1	Artificial_dechorionization	14
DF1-48	DAN	EDS	Poor	16.95	0.008732673	0.101	2.2	Artificial_dechorionization	15
DF1-58	DAN	Probiotic	Enriched	17.65	0.010647887	0.071	1.9	Artificial_dechorionization	23
DF1-63	DAN	EDS	Poor	28.99	0.0049	0.09	2.1	Artificial_dechorionization	31
HF1-02	HON9	EDS	Enriched	13.79	0.011061947	0.1017	2.7	Natural	16
HF1-04	HON9	EDS	Poor	15.38	0.010113507	0.0881	2.4	Natural	16
HF1-06	HON9	Probiotic	Enriched	12.27	0.013268041	0.097	2	Natural	19
HF1-07	HON9	EDS	Poor	18.18	0.005813397	0.1254	2.4	Natural	11
HF1-08	HON9	EDS	Enriched	15.38	0.008291457	0.1194	2.3	Natural	12
HF1-10	HON9	EDS	Enriched	13.33	0.011842105	0.0988	2.3	Natural	14
HF1-11	HON9	Probiotic	Poor	19.05	0.004847909	0.1578	2.3	Natural	15
HF1-12	HON9	Probiotic	Enriched	27.78	0.004829721	0.0969	2.2	Natural	15
HF1-13	HON9	Probiotic	Poor	22.47	0.008808511	0.0705	2.4	Natural	15
HF1-14	HON9	Probiotic	Enriched	11.68	0.011730583	0.103	2.3	Natural	19
HF1-15	HON9	EDS	Poor	35.09	0.002466667	0.135	2.2	Natural	27

Table S1.9. Continued

FISH ID	Strain	Diet	Environment	Background as % of fish	O2_consumption_corrected (mg O2 h-1 g-1)	Weight_g	Length_cm	Hatching_type	Incubation_days
HF1-16	HON9	EDS	Enriched	17.86	0.007263158	0.114	2.3	Natural	38
HF1-17	HON9	Probiotic	Poor	19.78	0.006915789	0.095	2.3	Natural	17
HF1-18	HON9	Probiotic	Enriched	16.13	0.009852632	0.095	2.2	Natural	17
HF1-19	HON9	EDS	Poor	13.16	0.012122449	0.098	2.2	Natural	44
HF1-20	HON9	EDS	Enriched	26.47	0.004411765	0.102	2.3	Natural	42
HF1-22	HON9	Probiotic	Enriched	23.81	0.006545455	0.088	2.2	Natural	40
HF1-23	HON9	Probiotic	Poor	13.89	0.011747368	0.095	2.1	Natural	22
HF1-24	HON9	EDS	Enriched	11.63	0.012550459	0.109	2.2	Natural	23
HF1-26	HON9	Probiotic	Enriched	7.96	0.022023529	0.085	2.2	Natural	43
HF1-27	HON9	EDS	Poor	12.50	0.012115385	0.104	2.4	Natural	38
HF1-28	HON9	EDS	Enriched	10.11	0.0144	0.1	2.2	Natural	49
HF1-29	HON9	EDS	Poor	12.35	0.01278	0.1	2.2	Natural	46
HF1-30	HON9	Probiotic	Poor	11.17	0.0159	0.09	2.3	Natural	48
HF1-31	HON9	Probiotic	Poor	10.53	0.018	0.085	2.2	Artificial_dechorionization	44
HF1-33	HON9	EDS	Poor	23.38	0.010836735	0.049	1.9	Artificial_dechorionization	22
HF1-35	HON9	Probiotic	Poor	13.74	0.009778846	0.104	2.1	Natural	37
HF1-36	HON9	EDS	Enriched	14.93	0.008621849	0.119	2.4	Natural	36
HF1-37	HON9	EDS	Poor	20.41	0.0065	0.108	2.4	Natural	41
HF1-38	HON9	EDS	Enriched	16.81	0.006853846	0.13	2.2	Natural	22
HF1-41	HON9	Probiotic	Poor	17.70	0.008904255	0.094	2.1	Natural	44
HF1-43	HON9	EDS	Poor	16.81	0.00891	0.1	2.2	Natural	75
HF1-45	HON9	Probiotic	Poor	15.93	0.008465347	0.101	2.3	Natural	45
HF1-50	HON9	Probiotic	Enriched	22.99	0.006775281	0.089	2.4	Natural	43
HF1-52	HON9	Probiotic	Enriched	26.87	0.004642105	0.095	2.3	Natural	25
HF1-56	HON9	EDS	Enriched	24.10	0.006096774	0.093	2.2	Artificial_dechorionization	53
HF1-57	HON9	Probiotic	Poor	28.99	0.005582278	0.079	2.3	Natural	46
HF1-60	HON9	Probiotic	Enriched	41.67	0.003272727	0.077	2.3	Natural	59
HF1-63	HON9	EDS	Poor	35.71	0.004378378	0.074	2.1	Artificial_dechorionization	60
HF1-66	HON9	Probiotic	Enriched	40.91	0.002925	0.08	2.2	Natural	53

Table S1.10. Metadata for microbiome data analysis

Sample_ID	Fish_ID	Diet	Environment	Strain	Parent
IG-8	DF1-03	EDS	Enriched	DAN	DP_14
IG-9	DF1-04	EDS	Poor	DAN	DP_14
IG-14	DF1-05	Probiotic	Enriched	DAN	DP_14
IG-15	DF1-06	Probiotic	Poor	DAN	DP_15
IG-17	DF1-07	EDS	Enriched	DAN	DP_15
IG-18	DF1-08	EDS	Poor	DAN	DP_15
IG-40	DF1-13	EDS	Enriched	DAN	DP_15
IG-39	DF1-14	Probiotic	Poor	DAN	DP_15
IG-34	DF1-16	Probiotic	Poor	DAN	DP_4
IG-43	DF1-21	Probiotic	Poor	DAN	DP_14
IG-44	DF1-22	EDS	Enriched	DAN	DP_15
IG-37	DF1-23	EDS	Poor	DAN	DP_8
IG-45	DF1-24	Probiotic	Enriched	DAN	DP_15
IG-49	DF1-25	Probiotic	Poor	DAN	DP_14
IG-55	DF1-28	Probiotic	Enriched	DAN	DP_15
IG-56	DF1-30	Probiotic	Enriched	DAN	DP_14
IG-57	DF1-31	EDS	Poor	DAN	DP_14
IG-59	DF1-36	EDS	Enriched	DAN	DP_8
IG-60	DF1-37	Probiotic	Enriched	DAN	DP_15
IG-65	DF1-38	Probiotic	Poor	DAN	DP_8
IG-66	DF1-39	Probiotic	Enriched	DAN	DP_15
IG-71	DF1-40	EDS	Poor	DAN	DP_1
IG-72	DF1-41	EDS	Enriched	DAN	DP_1
IG-75	DF1-45	EDS	Poor	DAN	DP_1
IG-77	DF1-48	EDS	Poor	DAN	DP_1
IG-80	DF1-63	EDS	Poor	DAN	DP_22
IG-1	HF1-02	EDS	Enriched	HON9	HP_10
IG-2	HF1-04	EDS	Poor	HON9	HP_10
IG-3	HF1-06	Probiotic	Enriched	HON9	HP_10
IG-4	HF1-07	EDS	Poor	HON9	HP_2
IG-10	HF1-11	Probiotic	Poor	HON9	HP_18
IG-12	HF1-13	Probiotic	Poor	HON9	HP_18
IG-19	HF1-15	EDS	Poor	HON9	HP_2
IG-28	HF1-24	EDS	Enriched	HON9	HP_12
IG-31	HF1-28	EDS	Enriched	HON9	HP_15
IG-36	HF1-31	Probiotic	Poor	HON9	HP_14
IG-46	HF1-35	Probiotic	Poor	HON9	HP_2
IG-48	HF1-37	EDS	Poor	HON9	HP_2
IG-50	HF1-38	EDS	Enriched	HON9	HP_2
IG-51	HF1-41	Probiotic	Poor	HON9	HP_2
IG-62	HF1-50	Probiotic	Enriched	HON9	HP_12

Table S1.10. Continued

Sample_ID	Fish_ID	Diet	Environment	Strain	Parent
IG-63	HF1-52	Probiotic	Enriched	HON9	HP_2
IG-64	HF1-56	EDS	Enriched	HON9	HP_2
IG-67	HF1-57	Probiotic	Poor	HON9	HP_12
IG-68	HF1-60	Probiotic	Enriched	HON9	HP_12
IG-70	HF1-66	Probiotic	Enriched	HON9	HP_12

Table S1.11. Alpha diversity results for microbiome data analysis

Sample_ID	Fish_ID	Chao1	Simpson_evenness
IG-8	DF1-03	17	0.359
IG-9	DF1-04	22	0.195
IG-14	DF1-05	28	0.15
IG-15	DF1-06	47.6	0.09
IG-17	DF1-07	7	0.613
IG-18	DF1-08	5	0.228
IG-40	DF1-13	325.25	0.071
IG-39	DF1-14	305	0.166
IG-34	DF1-16	39.5	0.102
IG-43	DF1-21	210.769	0.042
IG-44	DF1-22	340.333	0.198
IG-37	DF1-23	309	0.198
IG-45	DF1-24	193.154	0.02
IG-49	DF1-25	297.429	0.213
IG-55	DF1-28	87.6	0.027
IG-56	DF1-30	274.243	0.022
IG-57	DF1-31	126.909	0.031
IG-59	DF1-36	278	0.217
IG-60	DF1-37	230.25	0.066
IG-65	DF1-38	211.75	0.016
IG-66	DF1-39	272.667	0.076
IG-71	DF1-40	274.833	0.017
IG-72	DF1-41	253.167	0.11
IG-75	DF1-45	383.875	0.182
IG-77	DF1-48	115.895	0.011
IG-80	DF1-63	534.055	0.083
IG-1	HF1-02	31	0.181
IG-2	HF1-04	38	0.12
IG-3	HF1-06	23.5	0.056

Table S1.11. Continued

Sample_ID	Fish_ID	Chao1	Simpson_evenness
IG-4	HF1-07	37	0.1
IG-10	HF1-11	20.5	0.345
IG-12	HF1-13	35	0.063
IG-19	HF1-15	54	0.153
IG-28	HF1-24	51	0.273
IG-31	HF1-28	55	0.202
IG-36	HF1-31	327.4	0.197
IG-46	HF1-35	94	0.074
IG-48	HF1-37	262	0.239
IG-50	HF1-38	377	0.179
IG-51	HF1-41	226	0.122
IG-62	HF1-50	325.5	0.084
IG-63	HF1-52	268.6	0.191
IG-64	HF1-56	399.565	0.173
IG-67	HF1-57	303.857	0.115
IG-68	HF1-60	715.356	0.09
IG-70	HF1-66	706.603	0.015

Figure S1.11.1 Alpha rarefaction curve for the samples (N=46) generated in Qiime2, DF1=DAN Fish individual, and HF1=HON9 Fish individual

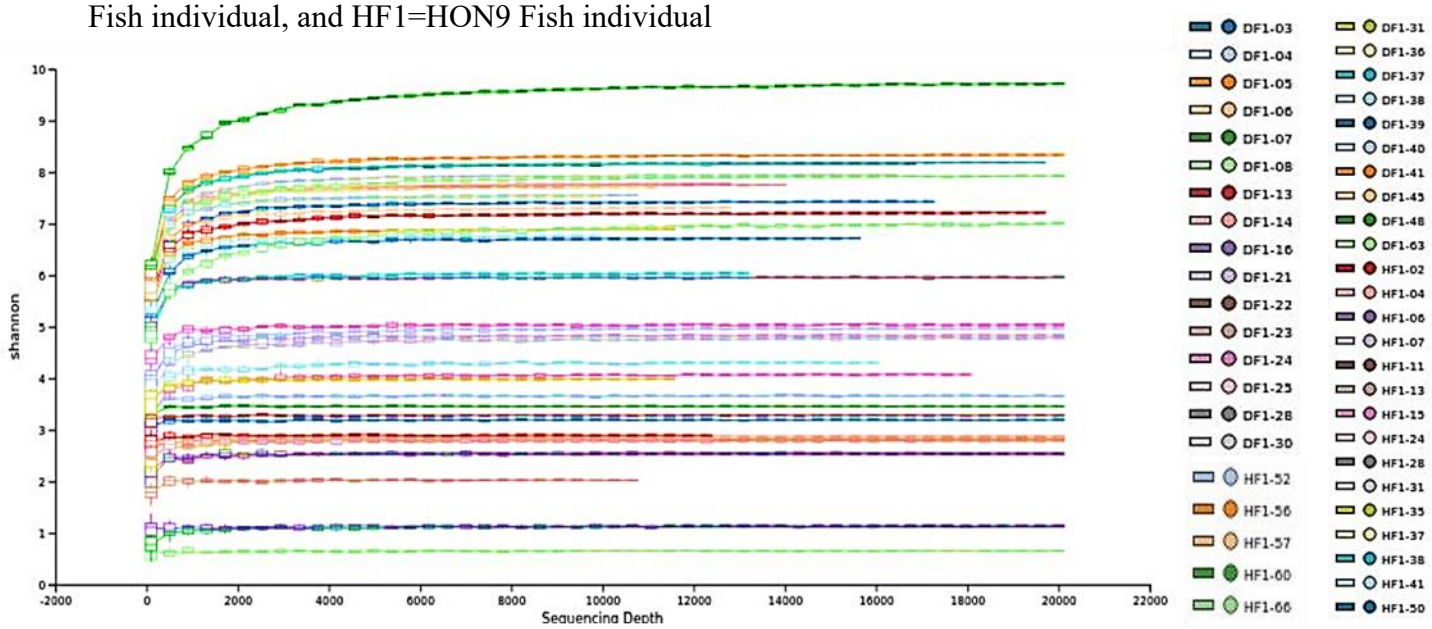


Table S1.12. Best model comparison using *glmulti* model method for inspections in experimental killifish. Best fitted linear models were chosen based on the Likelihood-Ratio chi-squared test (LR Chisq), and by using multi-model (generalized linear model) averaged approaches based on the corrected Akaike information criterion (AICC) and the weightings.

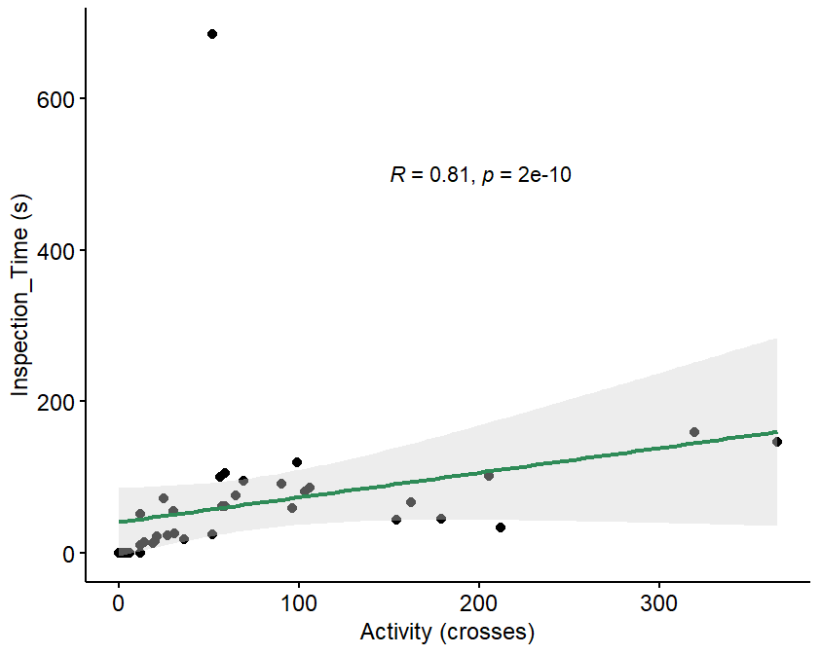
Parameter and models	**LR Chisq**	**Df**	***P*-value**	**AICC**	**weights**
Inspections					
Model 1				1316.917	0.570
Strain	106.726	1	**<0.001**		
Environment	6.446	1	**0.01**		
Hatching type	6.891	1	**0.11**		
Model 2				1318.594	0.246
Strain	106.952	1	**<0.001**		
Diet	0.600	1	**0.439**		
Environment	6. 471	1	**0.01**		
Hatching type	7.096	1	**0.01**		
Model 3				1321.145	0.069
Strain	108.011	1	**<0.001**		
Hatching type	7.333	1	0.11		

Table S1.13. Results of the best models for activity in *K. marmoratus* based on the LR Chisq, and by multi-model (generalized linear model) averaged approaches based on the corrected Akaike information criterion (AICC) and the weightings.

Parameter and models	LR Chisq	Df	*P*-value	AICC	weights
Activity					
Model 1				5754.552	0.737
Strain	419.97	1	**<0.001**		
Diet	84.27	1	**<0.001**		
Hatching type	9.67	1	**0.01**		
Model 2				5756.802	0.239
Strain	419.57	1	**<0.001**		
Diet	84.28	1	**<0.001**		
Environment	0.03	1	0.869		
Hatching type	9.64	1	**<0.01**		
Model 3				5762.005	0.177
Strain	488.79	1	**<0.001**		
Diet	82.09	1	**<0.001**		

Figure S1.14. Correlation between activity and inspection times in (a) DAN and (b) HON9

(a)



(b)

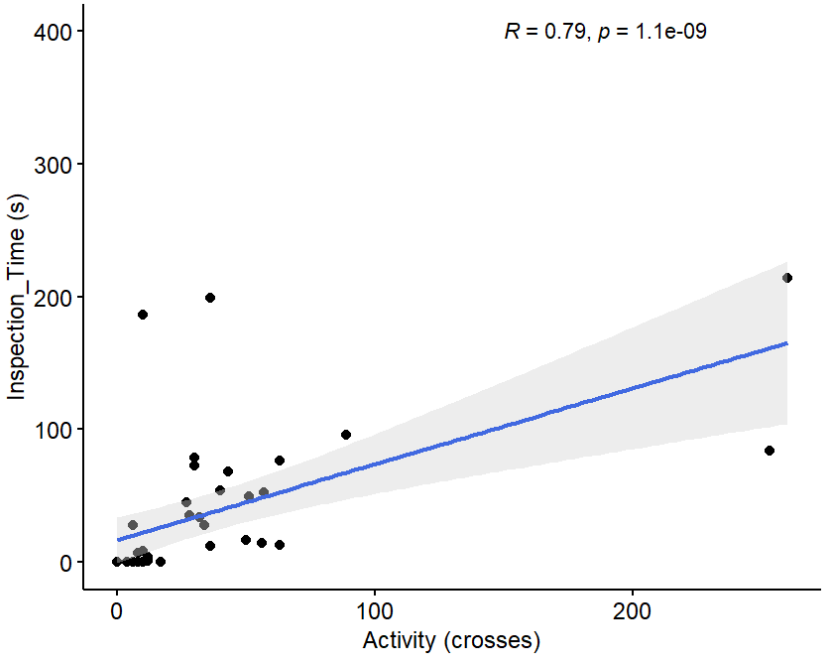


Table S1.15. Statistical result of BMR (O_2 consumption, mg O_2/h/g) with linear model (*lm*) run using the normally distributed data (Shapiro-Wilk normality test W = 0. 989, *p*-value = 0. 7265).

Parameter and Model	**Factors**	**Estimate**	**Std. Error**	**t- value**	**Pr(>|t|)**
O_2 consumption	Strain HON9	0.010	0.084	0.112	0.911
log10(O_2 consumption) ~ (Strain + Diet + Environment + Strain*Diet*Environment)	Diet Probiotic	0.027	0.084	0.321	0.750
	Environment Poor	-0.146	0.084	-1.729	0.088
	Strain HON9: Diet Probiotic	-0.111	0.119	-0.937	0.352
	Strain HON9: Environment Poor	0.098	0.119	0.820	0.415
	Diet Probiotic: Environment Poor	0.053	0.119	0.447	0.656
	Strain HON9: Diet Probiotic: Environment Poor	0.105	0.169	0.623	0.536

Table S1.16. Results showing Models' comparison for Chao1 richness in samples (N=46) from both hatching groups

Parameter and models	LR Chisq	Df	*P*-value	AICC	weights
Chao1					
Model 1				574.79	0.168
Strain	10.1331	1	**<0.01**		
Diet	3.3918	1	0.066		
Hatching type	13.3219	1	**<0.001**		
Diet:Strain	2.6913	1	0.101		
Diet:Hatching type	11.2265	1	**<0.001**		
Model 2				574.996	0.151
Strain	8.5452	1	**<0.01**		
Diet	2.8603	1	0.091		
Hatching type	12.1610	1	**<0.001**		
Diet:Hatching type	8.4439	1	**<0.01**		
Model 3				575.88	0.097
Strain	8.5359	1	**<0.01**		
Diet	3.1095	1	0.078		
Hatching type	12.1478	1	**<0.001**		
Strain:Hatching type	1.4543	1	0.228		
Diet:Hatching type	8.6513	1	**<0.01**		

Table S1.17. Results showing Models' comparison for simpson's evenness in samples (N=46) from both hatching groups

Parameter and models	LR Chisq	Df	*P*-value	aicc	weights
Simpson's evenness					
Model 1				-102.431	0.203
Diet	8.475	1	**<0.01**		
Environment	0.012	1	0.913		
Hatching type	10.648	1	**<0.01**		
Diet:Environment	9.904	1	**<0.01**		
Model 2				-101.176	0.108
Strain	0.301	1	0.583		
Diet	7.978	1	**<0.01**		
Environment	0.006	1	0.937		
Hatching type	9.767	1	**<0.01**		
Diet:Environment	6.732	1	**0.01**		
Strain:Hatching type	3.950	1	0.047		
Model 3				-100.058	0.062
Diet	8.782	1	**<0.01**		
Environment	0.012	1	0.912		
Hatching type	10.265	1	**<0.01**		
Diet:Environment	10.265	1	**<0.01**		
Environment: Hatching type	0.434	1	0.510		

Figure S1.18.1. Differentially abundant ASVs (DAA) across all samples (N=46) and sample groups by strain (DAN and HON9), Diet (EDS and Probiotic), and the rearing environment (Enriched and Poor).

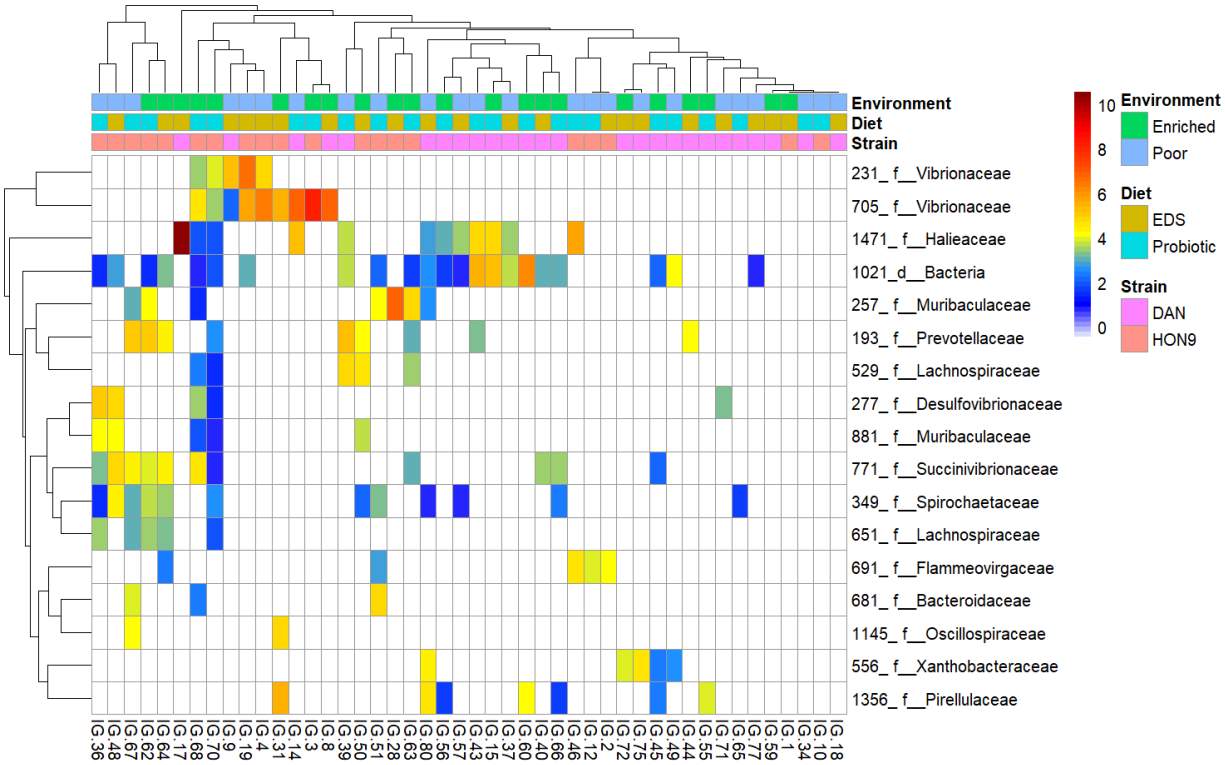


Table S1.18.2. Significance results for the 17 DAA in microbiome data across all sample groups

ASVs	baseMean	log2FoldChange	lfcSE	stat	p.value	p.adj
193_ f__Prevotellaceae	4.035721	-1.6991	0.526603	-3.22654	0.001253	0.048758
231_ f__Vibrionaceae	2.164865	-1.75058	0.498592	-3.51104	0.000446	0.035596
257_ f__Muribaculaceae	2.708627	-1.77427	0.495167	-3.58317	0.000339	0.032086
277_ f__Desulfovibrionaceae	2.797731	-1.65148	0.506334	-3.26165	0.001108	0.048758
349_ f__Spirochaetaceae	2.779185	-1.6377	0.444326	-3.68582	0.000228	0.032086
529_ f__Lachnospiraceae	1.987183	-1.49853	0.460984	-3.25072	0.001151	0.048758
556_ f__Xanthobacteraceae	2.512588	1.696301	0.518771	3.269848	0.001076	0.048758
651_ f__Lachnospiraceae	1.942643	-1.45054	0.433057	-3.34954	0.000809	0.048758
681_ f__Bacteroidaceae	2.11504	-1.62406	0.483591	-3.35833	0.000784	0.048758
691_ f__Flammeovirgaceae	2.481832	-1.9348	0.481052	-4.02203	5.77E-05	0.018329
705_ f__Vibrionaceae	5.845638	-3.36862	0.603895	-5.57816	2.43E-08	1.55E-05
771_ f__Succinivibrionaceae	4.124558	-1.78472	0.499411	-3.57365	0.000352	0.032086
881_ f__Muribaculaceae	2.181015	-1.68876	0.469831	-3.5944	0.000325	0.032086
1021_d__Bacteria	6.594273	1.718719	0.534402	3.216156	0.001299	0.048758
1145_ f__Oscillospiraceae	2.083609	-1.58777	0.490214	-3.23893	0.0012	0.048758
1356_ f__Pirellulaceae	2.426726	1.724487	0.520488	3.313213	0.000922	0.048758
1471_ f__Halieaceae	4.166785	2.12381	0.54089	3.926512	8.62E-05	0.018329

Table S1.19. Alpha diversity results from the naturally hatched fish samples (N=25) using *glmulti* model methods

Models	**LR Chisq**	***P*- value**	**Df**	**aicc**	**weight**
Chao1					
Model 1				299.72	0.303
Strain	5.82	**<0.05**	1		
Diet	6.64	**0.01**	1		
Model 2				300.10	0.250
Strain	7.56	**0.01**	1		
Diet	8.62	**<0.01**	1		
Strain:Diet	2.36	0.12	1		
Simpson evenness					
Model 1				-45.27	0.269
Strain	3.09	0.08	1		
Diet	4.72	**<0.05**	1		
Model 2				-44.24	0.162
Diet	6.33	**0.01**	1		

Table S1.20. Beta diversity results from naturally hatched fish samples (N=25) using *adonis2*

Diversity index and Experimental factors	Df	Sum of Sqs	R^2	F- value	*P*- value
Bray-curtis distance					
Strain	1	0.351	0.057	1.381	0.194
Diet	1	0.305	0.050	1.200	0.274
Environment	1	0.177	0.029	0.701	0.645
Weighted-Unifrac distance					
Strain	1	0.086	0.074	1.846	0.167
Diet	1	0.070	0.060	1.495	0.217
Environment	1	0.019	0.017	0.413	0.614

CHAPTER 2:

Intergenerational effects of early-rearing environment on inbred fish behaviour and basal metabolism

Table S2.1. List of Parents (F0) and offspring (F1) for the experiment

Strain	Parent_ID (F0)	F0_Environment	Offspring_ID (F1)	F1_Environment	F1_Environmental Code (Parental-own)
HON9	HF1-12	Enriched	HF2-72	Poor	E-P
	HF1-22	Enriched	HF2-37	Poor	E-P
	HF1-22	Enriched	HF2-70	Poor	E-P
	HF1-22	Enriched	HF2-86	Poor	E-P
	HF1-22	Enriched	HF2-87	Poor	E-P
	HF1-22	Enriched	HF2-88	Poor	E-P
	HF1-52	Enriched	HF2-89	Poor	E-P
	HF1-50	Enriched	HF2-94	Poor	E-P
	HF1-06	Enriched	HF2-95	Poor	E-P
	HF1-66	Enriched	HF2-96	Poor	E-P
	HF1-22	Enriched	HF1-62	Poor	E-P
			N=11		
HON9	HF1-22	Enriched	HF2-57	Enriched	E-E
	HF1-12	Enriched	HF2-71	Enriched	E-E
	HF1-12	Enriched	HF2-73	Enriched	E-E
	HF1-22	Enriched	HF2-36	Enriched	E-E
	HF1-52	Enriched	HF2-90	Enriched	E-E
	HF1-52	Enriched	HF2-91	Enriched	E-E
	HF1-52	Enriched	HF2-92	Enriched	E-E
	HF1-52	Enriched	HF2-93	Enriched	E-E
	HF1-60	Enriched	HF2-97	Enriched	E-E
	HF1-50	Enriched	HF2-105	Enriched	E-E
	HF1-22	Enriched	HF2-106	Enriched	E-E
	HF1-22	Enriched	HF1-63	Enriched	E-E
	HF1-22	Enriched	HF1-64	Enriched	E-E
			N=13		

Table S2.1. Continued

Strain	Parent_ID (F0)	F0_Environment	Offspring_ID (F1)	F1_Environment	F1_Environmental Code (Parental-own)
HON9	HF1-11	Poor	HF2-12	Poor	P-P
	HF1-11	Poor	HF2-18	Poor	P-P
	HF1-11	Poor	HF2-35	Poor	P-P
	HF1-17	Poor	HF2-31	Poor	P-P
	HF1-35	Poor	HF2-65	Poor	P-P
	HF1-41	Poor	HF2-79	Poor	P-P
	HF1-31	Poor	HF2-77	Poor	P-P
	HF1-41	Poor	HF2-59	Poor	P-P
	HF1-41	Poor	HF2-61	Poor	P-P
	HF1-13	Poor	HF2-74	Poor	P-P
	HF1-13	Poor	HF2-75	Poor	P-P
			N=11		
HON9	HF1-11	Poor	HF2-21	Enriched	P-E
	HF1-11	Poor	HF2-22	Enriched	P-E
	HF1-17	Poor	HF2-32	Enriched	P-E
	HF1-11	Poor	HF2-34	Enriched	P-E
	HF1-13	Poor	HF2-66	Enriched	P-E
	HF1-17	Poor	HF2-38	Enriched	P-E
	HF1-41	Poor	HF2-60	Enriched	P-E
	HF1-13	Poor	HF2-76	Enriched	P-E
	HF1-57	Poor	HF2-69	Enriched	P-E
			N=9		

Table S2.1. Continued

Strain	Parent_ID (F0)	F0_Environment	Offspring_ID (F1)	F1_Environment	F1_Environmental Code (Parental-own)
DAN	DF1-05	Enriched	DF2-03	Poor	E-P
	DF1-09	Enriched	DF2-35	Poor	E-P
	DF1-05	Enriched	DF2-21	Poor	E-P
	DF1-05	Enriched	DF2-112	Poor	E-P
	DF1-09	Enriched	DF2-114	Poor	E-P
	DF1-09	Enriched	DF2-116	Poor	E-P
	DF1-28	Enriched	DF2-86	Poor	E-P
	DF1-05	Enriched	DF2-63	Poor	E-P
	DF1-30	Enriched	DF2-80	Poor	E-P
	DF1-24	Enriched	DF2-87	Poor	E-P
	DF1-05	Enriched	DF2-84	Poor	E-P
	DF1-39	Enriched	DF2-128	Poor	E-P
	DF1-30	Enriched	DF2-79	Poor	E-P
			N=13		
DAN	DF1-05	Enriched	DF2-20	Enriched	E-E
	DF1-05	Enriched	DF2-22	Enriched	E-E
	DF1-28	Enriched	DF2-24	Enriched	E-E
	DF1-28	Enriched	DF2-115	Enriched	E-E
	DF1-09	Enriched	DF2-113	Enriched	E-E
	DF1-30	Enriched	DF2-88	Enriched	E-E
	DF1-30	Enriched	DF2-81	Enriched	E-E
	DF1-09	Enriched	DF2-83	Enriched	E-E
	DF1-24	Enriched	DF2-71	Enriched	E-E
	DF1-24	Enriched	DF2-89	Enriched	E-E
			N=10		

Table S2.1. Continued

Strain	Parent_ID (F0)	F0_Environment	Offspring_ID (F1)	F1_Environment	F1_Environmental Code (Parental-own)
DAN	DF1-02	Poor	DF2-05	Poor	P-P
	DF1-06	Poor	DF2-06	Poor	P-P
	DF1-14	Poor	DF2-39	Poor	P-P
	DF1-06	Poor	DF2-43	Poor	P-P
	DF1-02	Poor	DF2-36	Poor	P-P
	DF1-06	Poor	DF2-111	Poor	P-P
	DF1-38	Poor	DF2-72	Poor	P-P
	DF1-16	Poor	DF2-109	Poor	P-P
	DF1-38	Poor	DF2-56	Poor	P-P
	DF1-38	Poor	DF2-65	Poor	P-P
	DF1-38	Poor	DF2-66	Poor	P-P
	DF1-38	Poor	DF2-69	Poor	P-P
	DF1-25	Poor	DF2-59	Poor	P-P
			N=13		
DAN	DF1-06	Poor	DF2-07	Enriched	P-E
	DF1-14	Poor	DF2-40	Enriched	P-E
	DF1-14	Poor	DF2-44	Enriched	P-E
	DF1-21	Poor	DF2-23	Enriched	P-E
	DF1-21	Poor	DF2-52	Enriched	P-E
	DF1-06	Poor	DF2-58	Enriched	P-E
	DF1-02	Poor	DF2-108	Enriched	P-E
	DF1-14	Poor	DF2-77	Enriched	P-E
	DF1-14	Poor	DF2-78	Enriched	P-E
	DF1-38	Poor	DF2-67	Enriched	P-E
	DF1-38	Poor	DF2-68	Enriched	P-E
	DF1-38	Poor	DF2-70	Enriched	P-E
	DF1-25	Poor	DF2-60	Enriched	P-E
			N=13		

Table S2.1.1. Offspring's behavioural data as per their parental and own environmental groups from two strains of *K. marmoratus*

Fish_ID	Strain	Offspring's Own Environment	Parent _ID	Parental Environment	Latency	Inspections	Contacts	Activity	Exploration Total_s	Parental Activity
DF2-03	DAN	Poor	DF1-05	Enriched	374.317	27	0	130	825.683	4
DF2-05	DAN	Poor	DF1-02	Poor	126.564	2	0	13	1073.436	59
DF2-06	DAN	Poor	DF1-06	Poor	262.038	4	0	14	937.962	205
DF2-07	DAN	Enriched	DF1-06	Poor	955.75	22	0	86	244.25	205
DF2-108	DAN	Enriched	DF1-02	Poor	1200	0	0	0	0	59
DF2-109	DAN	Poor	DF1-16	Poor	190.497	0	0	12	1009.503	6
DF2-111	DAN	Poor	DF1-06	Poor	815.497	9	0	49	384.503	205
DF2-112	DAN	Poor	DF1-05	Enriched	52.046	19	0	83	1147.954	4
DF2-113	DAN	Enriched	DF1-09	Enriched	498.691	6	1	30	701.309	31
DF2-114	DAN	Poor	DF1-09	Enriched	868.578	0	0	2	331.422	31
DF2-115	DAN	Enriched	DF1-28	Enriched	501.318	4	0	31	698.682	65
DF2-116	DAN	Poor	DF1-09	Enriched	614.566	2	0	12	585.434	31
DF2-128	DAN	Poor	DF1-39	Enriched	479.767	4	0	18	720.233	59
DF2-20	DAN	Enriched	DF1-05	Enriched	334.975	10	0	48	865.025	4
DF2-21	DAN	Poor	DF1-05	Enriched	1200	0	0	0	0	4
DF2-22	DAN	Enriched	DF1-05	Enriched	66.004	15	0	56	1133.996	4
DF2-23	DAN	Enriched	DF1-21	Poor	382.452	10	0	47	817.548	179
DF2-24	DAN	Enriched	DF1-28	Enriched	77.951	12	2	66	1122.049	65
DF2-35	DAN	Poor	DF1-09	Enriched	518.955	9	1	45	681.045	31
DF2-36	DAN	Poor	DF1-02	Poor	131.031	6	0	23	1068.969	59

Table S2.1.1. Continued

Fish_ID	Strain	Offspring's Own Environment	Parent _ID	Parental Environment	Latency	Inspections	Contacts	Activity	Exploration Total_s	Parental Activity
DF2-39	DAN	Poor	DF1-14	Poor	238.044	8	0	49	961.956	212
DF2-40	DAN	Enriched	DF1-14	Poor	173.426	18	0	70	1026.574	212
DF2-43	DAN	Poor	DF1-06	Poor	79.12	12	2	65	1120.88	205
DF2-44	DAN	Enriched	DF1-14	Poor	194.353	10	0	54	1005.647	212
DF2-52	DAN	Enriched	DF1-21	Enriched	1200	0	0	0	0	179
DF2-56	DAN	Poor	DF1-38	Poor	419.092	5	1	18	780.908	319
DF2-58	DAN	Enriched	DF1-06	Poor	1081.74	1	0	6	118.26	205
DF2-59	DAN	Poor	DF1-25	Poor	166.961	9	1	49	1033.039	90
DF2-60	DAN	Enriched	DF1-25	Poor	187.772	0	0	2	1012.228	90
DF2-63	DAN	Poor	DF1-05	Enriched	137.46	13	0	61	1062.54	4
DF2-65	DAN	Poor	DF1-38	Poor	236.7	4	0	25	963.3	319
DF2-66	DAN	Poor	DF1-38	Poor	199.044	4	1	21	1000.956	319
DF2-67	DAN	Enriched	DF1-38	Poor	18.923	26	0	106	1181.077	319
DF2-68	DAN	Enriched	DF1-38	Poor	20.822	24	0	104	1179.178	319
DF2-69	DAN	Poor	DF1-38	Poor	1200	0	0	0	0	319
DF2-70	DAN	Enriched	DF1-38	Poor	1200	0	0	0	0	319
DF2-71	DAN	Enriched	DF1-24	Enriched	835.697	15	1	71	364.303	21
DF2-72	DAN	Poor	DF1-38	Poor	178.752	11	0	57	1021.248	319
DF2-77	DAN	Enriched	DF1-14	Poor	579.701	2	0	9	620.299	212
DF2-78	DAN	Enriched	DF1-14	Poor	54.7	2	0	12	1145.3	212
DF2-79	DAN	Poor	DF1-30	Enriched	174.525	14	0	54	1025.475	2

Table S2.1.1. Continued

Fish_ID	Strain	Offspring's Own Environment	Parent _ID	Parental Environment	Latency	Inspections	Contacts	Activity	Exploration Total_s	Parental Activity
DF2-80	DAN	Poor	DF1-30	Enriched	1200	0	0	0	0	2
DF2-81	DAN	Enriched	DF1-30	Enriched	77.555	1	0	7	1122.445	2
DF2-83	DAN	Enriched	DF1-09	Enriched	1200	0	0	0	0	31
DF2-84	DAN	Poor	DF1-05	Enriched	513.416	4	0	24	686.584	4
DF2-86	DAN	Poor	DF1-28	Enriched	0.959	14	1	74	1199.041	65
DF2-87	DAN	Poor	DF1-24	Enriched	149.737	2	1	16	1050.263	21
DF2-88	DAN	Enriched	DF1-30	Enriched	1200	0	0	0	0	2
DF2-89	DAN	Enriched	DF1-24	Enriched	689.573	1	0	6	510.427	21
HF2-105	HON9	Enriched	HF1-50	Enriched	174.223	0	0	5	1025.777	63
HF2-106	HON9	Enriched	HF1-22	Enriched	516.821	20	0	127	683.179	89
HF2-12	HON9	Poor	HF1-11	Poor	69.36	4	0	21	1130.64	253
HF2-18	HON9	Poor	HF1-11	Poor	303.944	1	0	3	896.056	253
HF2-21	HON9	Enriched	HF1-11	Poor	918.892	4	0	22	281.108	253
HF2-22	HON9	Enriched	HF1-11	Poor	111.683	0	0	2	1088.317	253
HF2-31	HON9	Poor	HF1-17	Poor	523.981	0	0	2	676.019	10
HF2-32	HON9	Enriched	HF1-17	Poor	276.605	7	1	43	923.395	10
HF2-34	HON9	Enriched	HF1-11	Poor	857.181	6	0	31	342.819	253
HF2-35	HON9	Poor	HF1-11	Poor	81.855	21	1	109	1118.145	253
HF2-36	HON9	Enriched	HF1-22	Enriched	446.153	0	0	17	753.847	89
HF2-37	HON9	Poor	HF1-22	Poor	285.549	8	0	29	914.451	89
HF2-38	HON9	Poor	HF1-17	Enriched	124.973	22	0	94	1075.027	10

Table S2.1.1. Continued

Fish_ID	Strain	Offspring's Own Environment	Parent _ID	Parental Environment	Latency	Inspections	Contacts	Activity	Exploration Total_s	Parental Activity
HF2-57	HON9	Enriched	HF1-22	Enriched	40.552	9	0	34	1159.448	89
HF2-59	HON9	Poor	HF1-41	Poor	1200	0	0	0	0	8
HF2-60	HON9	Enriched	HF1-41	Poor	45.854	5	0	50	1154.146	8
HF2-61	HON9	Poor	HF1-41	Poor	164.599	0	0	8	1035.401	8
HF2-62	HON9	Poor	HF1-22	Enriched	185.101	10	0	45	1014.899	89
HF2-63	HON9	Enriched	HF1-22	Enriched	43.606	38	1	241	1156.394	89
HF2-64	HON9	Enriched	HF1-22	Enriched	336.95	6	0	21	863.05	89
HF2-65	HON9	Poor	HF1-35	Poor	439.672	4	0	23	760.328	0
HF2-66	HON9	Enriched	HF1-13	Poor	1200	0	0	0	0	28
HF2-69	HON9	Enriched	HF1-57	Poor	916.061	0	0	7	283.939	6
HF2-70	HON9	Poor	HF1-22	Enriched	13.074	4	0	36	1186.926	89
HF2-71	HON9	Enriched	HF1-12	Enriched	336.95	2	0	16	863.05	36
HF2-72	HON9	Poor	HF1-12	Enriched	260.843	5	1	24	939.157	36
HF2-73	HON9	Enriched	HF1-12	Enriched	17.548	23	1	93	1182.452	36
HF2-74	HON9	Poor	HF1-13	Poor	78.725	6	1	34	1121.275	28
HF2-75	HON9	Poor	HF1-13	Poor	214.005	6	0	32	985.995	28
HF2-76	HON9	Enriched	HF1-13	Poor	775.65	7	0	34	424.35	28
HF2-77	HON9	Poor	HF1-31	Poor	583.416	0	0	6	616.584	40
HF2-79	HON9	Poor	HF1-41	Poor	589.9	7	0	28	610.1	8
HF2-86	HON9	Poor	HF1-22	Enriched	156.052	11	0	52	1043.948	89
HF2-87	HON9	Poor	HF1-22	Enriched	78.525	79	0	333	1121.475	89

Table S2.1.1. Continued

Fish_ID	Strain	Offspring's Own Environment	Parent _ID	Parental Environment	Latency	Inspections	Contacts	Activity	Exploration Total_s	Parental Activity
HF2-88	HON9	Poor	HF1-22	Enriched	289.171	6	0	29	910.829	89
HF2-89	HON9	Poor	HF1-52	Enriched	139.479	0	0	4	1060.521	30
HF2-90	HON9	Enriched	HF1-52	Enriched	965.776	0	0	4	234.224	30
HF2-91	HON9	Enriched	HF1-52	Enriched	21.8	1	0	9	1178.2	30
HF2-92	HON9	Enriched	HF2-52	Enriched	92.274	10	0	44	1107.726	30
HF2-93	HON9	Enriched	HF2-52	Enriched	5.801	31	0	252	1194.199	30
HF2-94	HON9	Poor	HF1-50	Enriched	223.715	2	0	13	976.285	63
HF2-95	HON9	Poor	HF1-06	Enriched	1200	0	0	0	0	8
HF2-96	HON9	Poor	HF1-66	Enriched	111.997	1	0	8	1088.003	12
HF2-97	HON9	Enriched	HF1-60	Enriched	122.699	3	0	21	1077.301	17

Table S2.1.2. Offspring's basal metabolism (BMR) data from environmental experimental groups of two *K. marmoratus* strains

Fish ID	Strain	Offspring's Environment	Parental Environment	Parent _ID	Background as % of fish	O2_consumption _parent (mg O2 h-1 g-1)	O2_consumption _offspring (mg O2 h-1 g-1)	Weight g	Length cm
DF2-03	DAN	Poor	Enriched	DF1-05	14.39	0.01383	0.01428	0.075	2.1
DF2-05	DAN	Poor	Poor	DF1-02	18.35	0.00346988	0.009423529	0.085	2
DF2-06	DAN	Poor	Poor	DF1-06	15.93	0.006646382	0.010301205	0.083	2.1
DF2-07	DAN	Enriched	Poor	DF1-06	22.47	0.006646382	0.008746479	0.071	2
DF2-108	DAN	Enriched	Poor	DF1-02	18.18	0.00346988	0.010125	0.072	2
DF2-109	DAN	Poor	Poor	DF1-16	19.80	0.011571429	0.009592105	0.076	1.9
DF2-111	DAN	Poor	Poor	DF1-06	18.35	0.006646382	0.01068	0.075	2
DF2-112	DAN	Poor	Enriched	DF1-05	10.05	0.01383	0.021197368	0.076	1.9
DF2-113	DAN	Enriched	Enriched	DF1-09	10.53	0.010516854	0.014648936	0.094	2.1
DF2-114	DAN	Poor	Enriched	DF1-09	12.35	0.010516854	0.011724771	0.109	2
DF2-115	DAN	Enriched	Enriched	DF1-28	10.05	0.006111111	0.020653846	0.078	2
DF2-116	DAN	Poor	Enriched	DF1-09	9.63	0.010516854	0.018777778	0.081	2.1

Table S2.1.2. Continued

Fish ID	Strain	Offspring's Environment	Parental Environment	Parent _ID	Background as % of fish	O2_consumption _parent (mg O2 h-1 g-1)	O2_consumption _offspring (mg O2 h-1 g-1)	Weight g	Length cm
DF2-128	DAN	Poor	Enriched	DF1-39	12.66	0.005896552	0.0135	0.092	2.1
DF2-20	DAN	Enriched	Enriched	DF1-05	23.68	0.01383	0.007352113	0.071	1.9
DF2-21	DAN	Poor	Enriched	DF1-05	74.07	0.01383	0.000828947	0.076	2
DF2-22	DAN	Enriched	Enriched	DF1-05	19.05	0.01383	0.011769231	0.065	2.1
DF2-23	DAN	Enriched	Poor	DF1-21	29.85	0.007792683	0.006409091	0.066	2
DF2-24	DAN	Enriched	Enriched	DF1-28	15.25	0.006111111	0.011392405	0.079	2
DF2-35	DAN	Poor	Enriched	DF1-09	16.39	0.010516854	0.013909091	0.066	1.9
DF2-36	DAN	Poor	Poor	DF1-02	35.71	0.00346988	0.004695652	0.069	2
DF2-39	DAN	Poor	Poor	DF1-14	17.54	0.010058824	0.012441176	0.068	1.9
DF2-40	DAN	Enriched	Poor	DF1-14	22.99	0.010058824	0.009885246	0.061	1.9
DF2-43	DAN	Poor	Poor	DF1-06	51.43	0.006646382	0.002637931	0.058	2
DF2-44	DAN	Enriched	Poor	DF1-14	19.35	0.010058824	0.010227273	0.066	1.8
DF2-52	DAN	Enriched	Poor	DF1-21	18.37	0.007792683	0.007058824	0.102	1.8
DF2-56	DAN	Poor	Poor	DF1-38	16.13	0.0072	0.011848101	0.079	2
DF2-58	DAN	Enriched	Poor	DF1-06	23.26	0.006646382	0.008735294	0.068	1.8
DF2-59	DAN	Poor	Poor	DF1-25	15.63	0.004272727	0.012461538	0.078	2.1
DF2-60	DAN	Enriched	Poor	DF1-25	15.75	0.004272727	0.013956522	0.069	1.9
DF2-63	DAN	Poor	Enriched	DF1-05	11.70	0.01383	0.017202532	0.079	2
DF2-65	DAN	Poor	Poor	DF1-38	16.39	0.0072	0.012405405	0.074	1.9
DF2-66	DAN	Poor	Poor	DF1-38	14.52	0.0072	0.014029412	0.068	1.8

Table S2.1.2. Continued

Fish ID	Strain	Offspring's Environment	Parental Environment	Parent _ID	Background as % of fish	O2_consumption _parent (mg O2 h-1 g-1)	O2_consumption _offspring (mg O2 h-1 g-1)	Weight g	Length cm
DF2-67	DAN	Enriched	Poor	DF1-38	28.17	0.0072	0.00612	0.075	2.1
DF2-68	DAN	Enriched	Poor	DF1-38	21.69	0.0072	0.007597403	0.077	2.1
DF2-69	DAN	Poor	Poor	DF1-38	30.30	0.0072	0.006179104	0.067	2
DF2-70	DAN	Enriched	Poor	DF1-38	19.61	0.0072	0.010394366	0.071	2
DF2-71	DAN	Enriched	Enriched	DF1-24	14.60	0.00927	0.011965909	0.088	2
DF2-72	DAN	Poor	Poor	DF1-38	15.79	0.0072	0.011076923	0.078	2.1
DF2-77	DAN	Enriched	Poor	DF1-14	17.54	0.010058824	0.010575	0.08	1.9
DF2-78	DAN	Enriched	Poor	DF1-14	19.42	0.010058824	0.010521127	0.071	1.9
DF2-79	DAN	Poor	Enriched	DF1-30	13.42	0.010905882	0.018725806	0.062	1.8
DF2-80	DAN	Poor	Enriched	DF1-30	11.76	0.010905882	0.015517241	0.087	2
DF2-81	DAN	Enriched	Enriched	DF1-30	9.30	0.010905882	0.020647059	0.085	2.1
DF2-83	DAN	Enriched	Enriched	DF1-09	23.81	0.010516854	0.006063158	0.095	2
DF2-84	DAN	Poor	Enriched	DF1-05	18.52	0.01383	0.01056	0.075	1.9
DF2-86	DAN	Poor	Enriched	DF1-28	12.05	0.006111111	0.016425	0.08	1.9
DF2-87	DAN	Poor	Enriched	DF1-24	19.78	0.00927	0.0082125	0.08	2.1
DF2-88	DAN	Enriched	Enriched	DF1-30	11.05	0.010905882	0.017888889	0.081	2.1
DF2-89	DAN	Enriched	Enriched	DF1-24	25.35	0.00927	0.005678571	0.084	1.9
HF2-105	HON9	Enriched	Enriched	HF1-50	14.75	0.006775281	0.008357143	0.112	2.1
HF2-106	HON9	Enriched	Enriched	HF1-22	11.49	0.006545455	0.012953271	0.107	2.2

Table S2.1.2. Continued

Fish ID	Strain	Offspring's Environment	Parental Environment	Parent _ID	Background as % of fish	O2_consumption _parent (mg O2 h-1 g-1)	O2_consumption _offspring (mg O2 h-1 g-1)	Weight g	Length cm
HF2-12	HON9	Poor	Poor	HF1-11	22.73	0.004847909	0.00816	0.075	2
HF2-18	HON9	Poor	Poor	HF1-11	30.51	0.004847909	0.0046125	0.080	2
HF2-21	HON9	Enriched	Poor	HF1-11	17.09	0.004847909	0.011486842	0.076	2
HF2-22	HON9	Enriched	Poor	HF1-11	18.87	0.004847909	0.01075	0.072	2.1
HF2-31	HON9	Poor	Poor	HF1-17	16.67	0.006915789	0.010945946	0.074	2
HF2-32	HON9	Enriched	Poor	HF1-17	29.41	0.006915789	0.008150943	0.053	1.8
HF2-34	HON9	Enriched	Poor	HF1-11	30.77	0.004847909	0.005192308	0.078	2
HF2-35	HON9	Poor	Poor	HF1-11	35.71	0.004847909	0.004438356	0.073	2.1
HF2-36	HON9	Enriched	Enriched	HF1-22	16.39	0.006545455	0.013701493	0.067	1.8
HF2-37	HON9	Poor	Enriched	HF1-22	13.07	0.006545455	0.015151899	0.079	2
HF2-38	HON9	Enriched	Poor	HF1-17	37.04	0.006915789	0.005016393	0.061	2
HF2-57	HON9	Enriched	Enriched	HF1-22	11.17	0.006545455	0.018828947	0.076	2.1
HF2-59	HON9	Poor	Poor	HF1-41	11.49	0.008904255	0.01925	0.072	1.9
HF2-60	HON9	Enriched	Poor	HF1-41	18.35	0.008904255	0.011608696	0.069	2
HF2-61	HON9	Poor	Poor	HF1-41	13.07	0.008904255	0.013758621	0.087	2
HF2-62	HON9	Poor	Enriched	HF1-22	15.27	0.006545455	0.015609375	0.064	1.9
HF2-63	HON9	Enriched	Enriched	HF1-22	7.43	0.006545455	0.02605814	0.086	2.1
HF2-64	HON9	Enriched	Enriched	HF1-22	7.17	0.006545455	0.027423529	0.085	2.1
HF2-65	HON9	Poor	Poor	HF1-35	14.39	0.009778846	0.014092105	0.076	2
HF2-66	HON9	Enriched	Poor	HF1-13	9.62	0.008808511	0.020385542	0.083	2
HF2-69	HON9	Enriched	Poor	HF1-57	11.90	0.005582278	0.015488372	0.086	2.1

Table S2.1.2. Continued

Fish ID	Strain	Offspring's Environment	Parental Environment	Parent _ID	Background as % of fish	O2_consumption _parent (mg O2 h-1 g-1)	O2_consumption _offspring (mg O2 h-1 g-1)	Weight g	Length cm
HF2-70	HON9	Poor	Enriched	HF1-22	16.26	0.006545455	0.012197368	0.076	2.1
HF2-71	HON9	Enriched	Enriched	HF1-12	6.85	0.004829721	0.029493976	0.083	2
HF2-72	HON9	Poor	Enriched	HF1-12	14.17	0.004829721	0.014217391	0.069	2
HF2-73	HON9	Enriched	Enriched	HF1-12	7.96	0.004829721	0.024631579	0.076	1.9
HF2-74	HON9	Poor	Poor	HF1-13	9.68	0.008808511	0.0216	0.07	1.9
HF2-75	HON9	Poor	Poor	HF1-13	9.85	0.008808511	0.024220588	0.068	1.9
HF2-76	HON9	Enriched	Poor	HF1-13	10.17	0.008808511	0.018346154	0.078	2
HF2-77	HON9	Poor	Poor	HF1-31	16.81	0.018	0.012728571	0.07	1.9
HF2-79	HON9	Poor	Poor	HF1-41	12.24	0.008904255	0.015689189	0.074	2
HF2-86	HON9	Poor	Enriched	HF1-22	19.23	0.006545455	0.007339806	0.103	2
HF2-87	HON9	Poor	Enriched	HF1-22	18.00	0.006545455	0.008682353	0.085	1.9
HF2-88	HON9	Poor	Enriched	HF1-22	25.32	0.006545455	0.005418367	0.098	2.2
HF2-89	HON9	Poor	Enriched	HF1-52	21.98	0.004642105	0.006144231	0.104	2.2
HF2-90	HON9	Enriched	Enriched	HF1-52	11.49	0.004642105	0.0126	0.11	2.2
HF2-91	HON9	Enriched	Enriched	HF1-52	15.25	0.004642105	0.008571429	0.105	2.1
HF2-92	HON9	Enriched	Enriched	HF2-52	11.43	0.004642105	0.012130435	0.115	2.1
HF2-93	HON9	Enriched	Enriched	HF2-52	13.33	0.004642105	0.010833333	0.108	2.1
HF2-94	HON9	Poor	Enriched	HF1-50	12.74	0.006775281	0.012978947	0.095	2.2
HF2-95	HON9	Poor	Enriched	HF1-06	15.52	0.013268041	0.009910112	0.089	2.2
HF2-96	HON9	Poor	Enriched	HF1-66	18.87	0.002925	0.007663366	0.101	2.2
HF2-97	HON9	Enriched	Enriched	HF1-60	15.38	0.003272727	0.009519231	0.104	2.2

Table S2.2.1. Models' comparison (model run with "gaussian" family) and *anova* (Analysis of variance) outcomes for F1 exploration.

Parameter and models	AIC	BIC	logLik	deviance	Chisq	*P*-value
Total Exploration	1381.7	1399.4	-683.83	1367.7	1	1
Model 1 Total Exploration ~ Strain + Length + Offspring's own Environment + Parental Environment + (1 | Parent ID)						
Model 2 Total Exploration ~ Strain + Length + Offspring's own Environment + Parental Environment	**1379.7**	**1394.9**	-683.83	1367.7		

Table S2.2.2. Results of the best model, model 2 (using *glm2*) including only the fixed factors for F1 exploration.

Model and fixed factors	Estimate	Std. Error	t-value	*P*-value
Model 2 Total Exploration ~ Strain + Length + Offspring's own Environment + Parental Environment				
Strain (HON9)	116.23	83.74	1.388	0.169
Length	63.40	412.40	0.154	0.878
Offspring's own Environment (Poor)	115.83	80.77	1.434	0.155
Parental Environment (Poor)	-45.94	84.14	-0.546	0.586

Table S2.3.1. Model comparison (run with "Poisson" family) and *anova* (Analysis of variance) results for inspections in offspring, best model was chosen based on the lowest Akaike information criterion (AIC), Bayesian Information Criterion (BIC).

Parameter and models	AIC	BIC	logLik	deviance	Chisq	*P*-value
Inspections						
Model 1						
Inspections ~ Strain + Length + Offspring's Environment + Parental Environment + (1 | Parent_ID) + Offspring's Environment * Parental Environment	**1023.6**	**1041.3**	-504.78	1009.6	225.11	**< 0.001**
Model 2						
Inspections ~ Strain + Length + Offspring_Environment + Parent_Environment + Offspring_Environment * Parent_Environment	1246.7	1261.9	-617.33	1234.7		

Table S2.3.2. For F1 inspections, statistical results of fixed factors from the best model (model 1, a mixed linear model) using *lme4*

Model and fixed factors	**Estimate**	**Std. Error**	**Z-value**	***P*-value**
Model 1 Inspections ~ Strain + Length + Offspring's Environment + Parental Environment + (1 | Parent_ID) + Offspring's Environment * Parental Environment				
Strain HON9	-0.500	0.411	-1.22	0.22
Length	0.170	0.412	0.41	0.68
Offspring's own Environment (Poor)	0.299	0.105	2.84	**0.004**
Parental Environment (Poor)	-0.290	0.234	-1.241	0.214
Offspring's own Environment (Poor): Parental Environment (Poor)	-0.597	0.164	-3.63	**<0.001**

Table S2.4.1. Model (run with "Poisson" family) comparison between models with and without parent ID as random factor for activity in F1 individuals, and the best model selection was done checking the lowest AIC and BIC as follows.

Parameter and models	AIC	BIC	logLik	deviance	Chisq	*P*-value
Activity						
Model 1						
Activity ~ Strain + Length + Offspring's own Environment + Parental Environment + (1 | Parent_ID) + Offspring's own Environment * Parental Environment	**3415.7**	**3433.5**	-1700.9	3401.7	1375.5	**<0.001**
Model 2						
Activity ~ Strain + Length + Offspring's own Environment + Parental Environment + Offspring's own Environment * Parental Environment	4789.3	4804.5	-2388.7	4777.3		

Table S2.4.2. Statistical results (using *lme4*) of fixed factor's effects in model 1 for F1 activity

Model and fixed factors	Estimate	Std. Error	Z-value	*P*- value
Model 1 Activity ~ Strain + Length + Offspring's own Environment + Parental Environment + (1 | Parent_ID) + Offspring's own Environment * Parental Environment				
Strain HON9	-0.428	0.377	-1.138	0.255
Length	0.561	0.189	2.972	**<0.01**
Offspring's own Environment (Poor)	0.180	0.047	3.822	**<0.001**
Parental Environment (Poor)	-0.485	0.116	-4.180	**<0.001**
Offspring's own Environment (Poor): Parental Environment (Poor)	-0.385	0.0744	-5.185	**<0.001**

Table S2.5. Model (run with "Poisson" family using *glm*) results to check the effect of parental activity on offspring's activity

Model and Predictors	Estimate	Std. Error	Z-value	*P*-value
Model Offspring's activity ~ Parental activity + Length				
Parental activity	0.001	0.0002	3.265	**<0.001**
Length	0.395	0.157	2.522	**0.012**

Table S2.6.1. Model comparison for offspring's BMR analyses with and without parent ID as a random factor using *lm*

Parameter and models	AIC	BIC	logLik	deviance	Chisq	*P*-value
O_2 consumption/h/g						
Model 1						
O2 consumption ~ Strain + Length + Offspring's own Environment + Parental Environment + (1 | Parent_ID)	**-707.99**	**-690.27**	361	-721.99	6.256	**0.012**
Model 2						
O2 consumption ~ Strain + Length + Offspring's own Environment + Parental Environment	-703.74	-688.54	357.87	-715.74		

Table S2.6.2. Best model results of fixed factors from the mixed linear model (with parent ID as random factor) as follows.

Model and fixed factors	Estimate	Std. Error	t-value	*P*- value
Model 1				
O2 consumption ~ Strain + Length + Offspring's own Environment + Parental Environment + (1 | Parent_ID)				
Strain HON9	0.002	0.002	1.165	0.256
Length	-0.007	0.005	-1.209	0.230
Offspring's own Environment (Poor)	-0.001	0.001	-0.868	0.388
Parental Environment (Poor)	-0.002	0.002	-1.441	0.163

Table S2.7. Model (using "Gamma" family in *glm*) results to check the effect of parental BMR on offspring's BMR

Model and Predictors	Estimate	Std. Error	t-value	*P*- value
Model				
Offspring's BMR ~ Parental BMR + Length				
Parental BMR	-1096.69	1288.21	-0.851	0.397
Length	17.33	37.68	0.460	0.647

CHAPTER 3: Intergenerational effects of the rearing environment on the microbiome of fish with low genetic diversity

Figure S3.1. Alpha rarefaction curve for the samples (N=115, F0 n=46, F1 n=69) generated in Qiime2, DF1=Parental DAN Fish individual, and HF1= Parental HON9 Fish individual,

DF2=Offspring DAN Fish individual, and HF2= Offspring HON9 Fish individual, 5 samples were excluded due to features bellow sampling depth (2,116) at this stage and further analysis was proceed with the parents (F0 n= 22) and respective offspring (F1 n= 64) in this study.

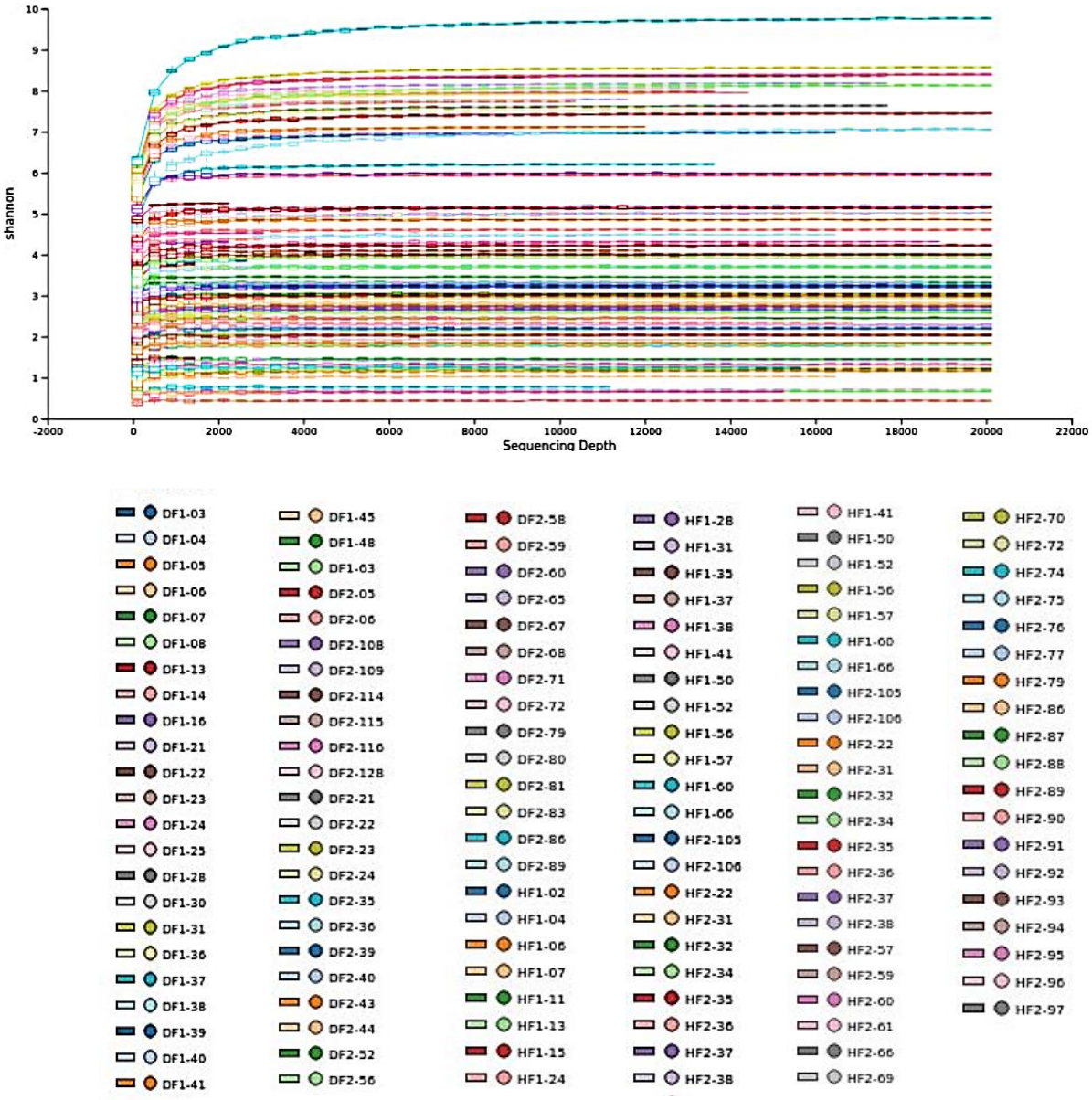


Table S3.1.1. Alpha diversity results for offspring's (F1 n=64) microbiome

Sample_ID	F1_Fish_ID	Chao1	Simpson's evenness
IG-100	HF2-32	25.5	0.126668
IG-101	HF2-34	53.42857	0.081218
IG-102	DF2-35	42	0.091724
IG-103	DF2-24	25	0.116489
IG-104	HF2-36	21	0.288521
IG-105	HF2-38	25	0.077032
IG-106	HF2-37	46.5	0.071682
IG-107	DF2-52	15.5	0.110894
IG-109	DF2-72	26.14286	0.132787
IG-110	DF2-109	91	0.123052
IG-111	DF2-56	43.25	0.086287
IG-112	DF2-65	19	0.095354
IG-115	DF2-59	68	0.174878
IG-116	DF2-58	42	0.156387
IG-117	DF2-108	29	0.137105
IG-120	DF2-67	47.2	0.177011
IG-121	DF2-68	6	0.183622
IG-123	DF2-60	54	0.118566
IG-125	HF2-79	26	0.116037
IG-127	HF2-59	13	0.329187
IG-128	HF2-61	46.2	0.044016
IG-129	HF2-74	24	0.067499
IG-130	HF2-75	16	0.136234
IG-131	HF2-66	24.5	0.086258
IG-132	HF2-60	32.5	0.036995
IG-133	HF2-76	29.5	0.095132
IG-134	HF2-69	23	0.133695
IG-136	DF2-114	69.75	0.026559
IG-137	DF2-116	17	0.227513
IG-138	DF2-86	22.25	0.055035
IG-140	DF2-80	15.2	0.079946
IG-143	DF2-79	21	0.081313
IG-144	DF2-115	23	0.113206
IG-147	DF2-81	14.5	0.257687
IG-148	DF2-83	20	0.110269
IG-149	HF2-72	17.6	0.090343
IG-156	DF2-71	12	0.135567
IG-158	HF2-86	11	0.103127
IG-159	HF2-87	50	0.096972

Table S3.1.1. Continued

Sample_ID	F1_Fish_ID	Chao1	Simpson's evenness
IG-160	HF2-88	109.1429	0.118487
IG-161	HF2-89	82.27273	0.059785
IG-163	HF2-91	57	0.098406
IG-164	HF2-92	55.5	0.109922
IG-165	HF2-93	33.33333	0.078037
IG-167	DF2-128	24	0.162712
IG-168	HF2-94	55.375	0.089729
IG-169	HF2-95	26	0.406291
IG-170	HF2-96	34.2	0.089511
IG-171	HF2-97	23.5	0.11261
IG-172	HF2-105	26.5	0.276996
IG-173	HF2-106	38	0.103751
IG-174	HF2-90	8	0.328834
IG-86	DF2-06	14	0.163786
IG-88	HF2-22	18	0.14224
IG-89	DF2-21	31.5	0.094592
IG-91	DF2-22	35	0.470831
IG-92	DF2-23	51	0.111753
IG-93	DF2-39	23	0.247417
IG-94	DF2-43	37	0.199664
IG-95	DF2-36	31.5	0.06589
IG-96	DF2-40	20	0.137789
IG-97	DF2-44	24.85714	0.053834
IG-98	HF2-35	20	0.760315
IG-99	HF2-31	29	0.164844

Figure S3.1.2. Differentially abundant 37 ASVs were detected across all offspring gut samples grouped by strain (DAN and HON9) and offspring's parental-own environments as follows: E-E, E-P, P-E, and P-P (E=Enriched, P=Poor).

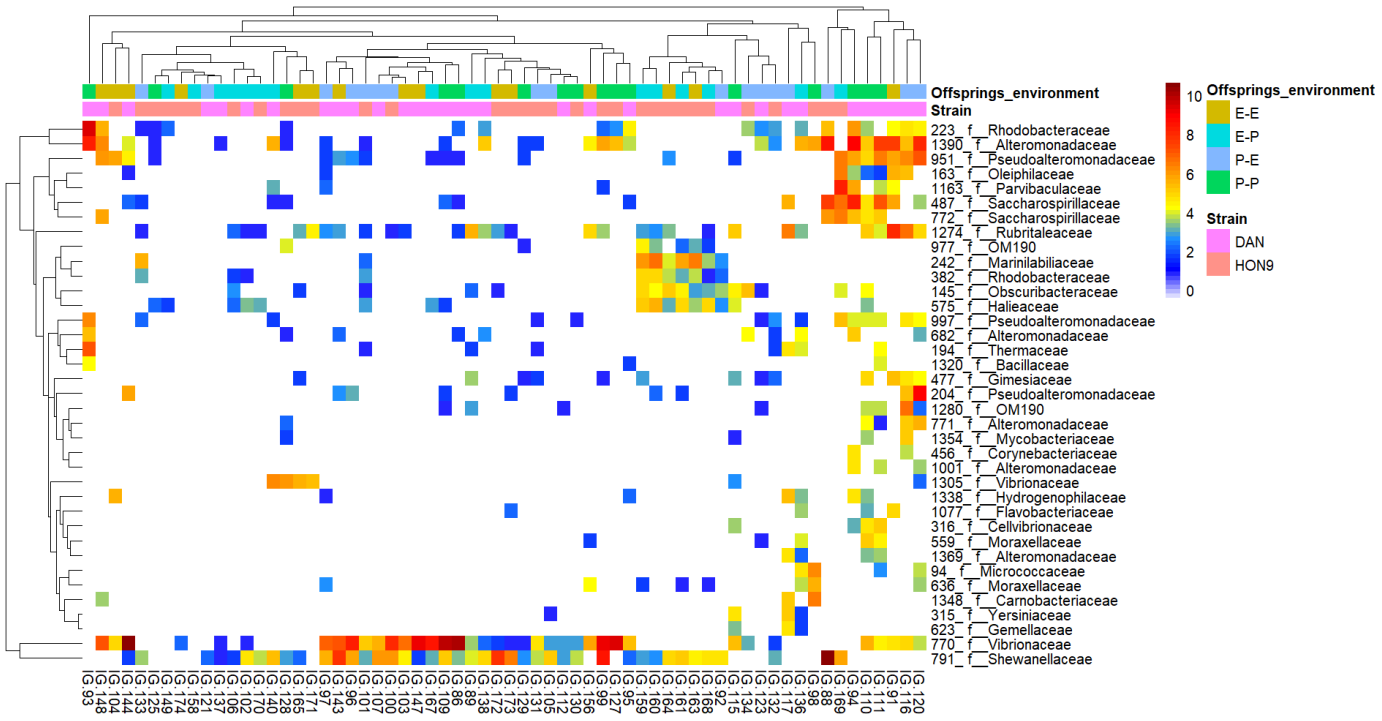


Table S3.1.3. Significance results for 37 differentially abundant ASVs from the offspring microbiome data

ASVs	baseMean	log2FoldChange	lfcSE	stat	P value	P adj
94_ f__Micrococcaceae	1.5625	1.087463	0.383432	2.836132	0.004566	0.027616
145_ f__Obscuribacteraceae	4.03125	-1.25987	0.453482	-2.77821	0.005466	0.028405
163_ f__Oleiphilaceae	1.75	1.321928	0.408971	3.232327	0.001228	0.012995
194_ f__Thermaceae	1.828125	1.169925	0.406064	2.881131	0.003963	0.025162
204_ f__Pseudoalteromonadaceae	2.59375	1.703282	0.451369	3.773594	0.000161	0.002919

Table S3.1.3. Continued

ASVs	baseMean	log2FoldChange	lfcSE	stat	P value	P adj
223_ f__Rhodobacteraceae	4.8125	1.299053	0.466053	2.787352	0.005314	0.028405
242_ f__Marinilabiliaceae	6.421875	-3.38082	0.525308	-6.43589	1.23E-10	1.56E-08
315_ f__Yersiniaceae	1.78125	1.295456	0.420632	3.079787	0.002071	0.017539
316_ f__Cellvibrionaceae	1.96875	1.554588	0.428913	3.624487	0.00029	0.004086
382_ f__Rhodobacteraceae	2.46875	-1.76081	0.419074	-4.20167	2.65E-05	0.000841
456_ f__Corynebacteriaceae	1.5	1	0.395624	2.527651	0.011483	0.045493
477_ f__Gimesiaceae	2.921875	1.615989	0.436217	3.704553	0.000212	0.003362
487_ f__Saccharospirillaceae	2.921875	2.118644	0.454433	4.662172	3.13E-06	0.000132
559_ f__Moraxellaceae	1.953125	1.539158	0.427981	3.596322	0.000323	0.004099
575_ f__Halieaceae	3.9375	-1.37851	0.445693	-3.09296	0.001982	0.017539
623_ f__Gemellaceae	1.46875	0.954196	0.386182	2.470845	0.013479	0.048911
636_ f__Moraxellaceae	1.65625	0.959358	0.376707	2.546696	0.010875	0.045493
682_ f__Alteromonadaceae	2.671875	1.316027	0.437863	3.005571	0.002651	0.021041
770_ f__Vibrionaceae	101.7969	1.680745	0.68343	2.459279	0.013922	0.049112
771_ f__Alteromonadaceae	1.71875	1.099535	0.409022	2.688209	0.007184	0.035089
772_ f__Saccharospirillaceae	2.1875	1.754887	0.449415	3.904829	9.43E-05	0.002395
791_ f__Shewanellaceae	20.98438	-1.43281	0.517108	-2.77082	0.005592	0.028405
951_ f__Pseudoalteromonadaceae	10.9375	1.685655	0.566155	2.977372	0.002907	0.02127
977_ f__OM190	1.765625	-1.33985	0.394378	-3.39738	0.00068	0.007855

Table S3.1.3. Continued

ASVs	baseMean	log2FoldChange	lfcSE	stat	P value	P adj
997_ f__Pseudoalteromonadaceae	3.859375	1.197446	0.475659	2.517445	0.011821	0.045493
1001_ f__Alteromonadaceae	1.609375	1.149747	0.389801	2.94957	0.003182	0.02127
1077_ f__Flavobacteriaceae	1.609375	0.95818	0.383235	2.500239	0.012411	0.046359
1163_ f__Parvibaculaceae	1.578125	0.978626	0.370456	2.641679	0.00825	0.037951
1274_ f__Rubritaleaceae	11.70313	2.78329	0.510704	5.449911	5.04E-08	3.20E-06
1280_ f__OM190	1.484375	0.97728	0.348556	2.803793	0.005051	0.028405
1305_ f__Vibrionaceae	2.25	-1.42884	0.450814	-3.16947	0.001527	0.014919
1320_ f__Bacillaceae	1.453125	0.930737	0.38034	2.447118	0.0144	0.049428
1338_ f__Hydrogenophilaceae	2.0625	1.643856	0.427039	3.849432	0.000118	0.002506
1348_ f__Carnobacteriaceae	1.546875	1.066089	0.404299	2.636881	0.008367	0.037951
1354_ f__Mycobacteriaceae	1.578125	0.978626	0.386455	2.532313	0.011331	0.045493
1369_ f__Alteromonadaceae	1.59375	1.129283	0.381993	2.956289	0.003114	0.02127
1390_ f__Alteromonadaceae	36.17188	1.702884	0.650788	2.616648	0.00888	0.038887

Table S3.2.1. Parental (F0) analyses results including the fixed factors (using *lm*) for Chao1 richness.

Model and factors	Estimate	Std. Error	t-value	*P*-value
Model				
Chao 1 ~ Strain + Environment + Strain : Environment				
Strain (HON9)	-24.499	99.959	-0.245	0.810
Environment (Enriched)	9.301	93.769	0.099	0.922
Strain (HON9) : Environment (Enriched)	168.782	139.942	1.206	0.245

Table S3.2.2. Parental (F0) results including the fixed factors (using *lm*) for Simpson's evenness.

Model and factors	Estimate	Std. Error	t-value	*P*-value
Model				
Simpson's evenness ~ Strain + Environment + Strain : Environment				
Strain (HON9)	0.031	0.052	0.606	0.553
Environment (Enriched)	-0.071	0.049	-1.451	0.166
Strain (HON9) : Environment (Enriched)	0.015	0.073	0.210	0.837

Table S3.3.1. Model comparison and *anova* (Analysis of variance) outcomes in offspring's (F1) Chao1 richness.

Parameter and models	AIC	BIC	logLik	deviance	Chisq	*P*-value
Chao1						
Model 1						
Chao1 ~ Strain + Offspring's own Environment + Parental Environment + Offspring's own Environment: Parental Environment + (1 | Parent ID)	**542.75**	**557.86**	-264.38	528.75	5.371	0.023
Model 2						
Chao1 ~ Strain + Offspring's own Environment + Parental Environment + Offspring's own Environment: Parental Environment	545.12	559.07	-267.06	534.12		

Table S3.3.2. Results of the best model, model 2 (using *lme4*) including the fixed factors for Chao1 richness in offspring (F1).

Model and fixed factors	**Estimate**	**Std. Error**	**t-value**	***P*-value**
Model 1				
Chao1 ~ Strain + Offspring's own Environment + Parental Environment + Offspring's own Environment: Parental Environment + (1 | Parent ID)				
Strain (HON9)	3.438e-04	6.121e-03	0.056	0.955
Offspring's own Environment (Enriched)	-9.693e-06	5.254e-03	-0.002	0.999
Parental Environment (Enriched)	-1.852e-03	6.845e-03	-0.270	0.787
Offspring's own Environment Enriched: Parental Environment Enriched	1.299e-02	7.412e-03	1.753	0.080

Table S3.4.1. Model comparison and *anova* outcomes for F1 Simpson's evenness.

Parameter and models	AIC	BIC	logLik	deviance	Chisq	*P*-value
Simpson's evenness						
Model 1						
Simpson's evenness ~ Strain + Offspring's own Environment + Parental Environment + Offspring's own Environment: Parental Environment+ (1 | Parent ID)	14.06	29.17	-0.03	0.06	1	1
Model 2						
Simpson's evenness ~ Strain + Offspring's own Environment + Parental Environment + Offspring's own Environment: Parental Environment	**12.06**	**25.01**	-0.03	0.059		

Table S3.4.2. Results of the best model, model 2 (using *glm2*) including the fixed factors for Simpson's evenness.

Model and fixed factors	Estimate	Std. Error	t-value	*P*-value
Model 2				
Simpson's evenness ~ Strain + Offspring's own Environment + Parental Environment + Offspring's own Environment: Parental Environment				
Strain (HON9)	-0.004	0.06	-0.062	0.95
Offspring's own Environment (Enriched)	-0.12	0.09	-1.33	0.19
Parental Environment (Enriched)	-0.164	0.089	-1.84	0.07
Offspring's own Environment Enriched: Parental Environment Enriched	0.313	0.126	2.478	**0.02**

Table S3.5. Parental (F0) Beta diversity results from Bray-Curtis distance using *adonis2*

Model and Experimental factors	**Df**	**Sum of Sqs**	**R^2**	**F- value**	***P*- value**
Model					
Bray-Curtis distance ~ Strain + Environment					
Strain	1	0.32	0.067	1.28	0.22
Environment	1	0.21	0.04	0.81	0.59

Table S3.6. Parental (F0) Weighted UniFrac distance results under beta diversity index using *adonis2*

Model and Experimental factors	**Df**	**Sum of Sqs**	**R^2**	**F- value**	***P*- value**
Model					
Weighted UniFrac distance ~ Strain + Environment					
Strain	1	0.04	0.059	1.09	0.30
Environment	1	0.009	0.013	0.23	0.83

Table S3.7. Offspring's (F1) Beta diversity results from Bray-Curtis distance using *adonis2*

Model and Experimental factors	**Df**	**Sum of Sqs**	**R^2**	**F- value**	***P*- value**
Model					
Bray-Curtis distance ~ Strain + Offspring's own Environment + Parental Environment					
Strain	1	0.2749	0.01746	1.0998	0.3231
Offspring's own Environment	1	0.1618	0.01027	0.6472	0.7292
Parental Environment	1	0.3117	0.01979	1.2469	0.2408

Table S3.8. Offspring's (F1) Weighted Unifrac distance results under beta diversity index using *adonis2*

Model and Experimental factors	**Df**	**Sum of Sqs**	**R^2**	**F- value**	***P*- value**
Model					
Weighted Unifrac distance ~ Strain + Offspring's own Environment + Parental Environment					
Strain	1	0.02076	0.01227	0.7655	0.4696
Offspring's own Environment	1	0.01034	0.00612	0.3814	0.8242
Parental Environment	1	0.03314	0.01960	1.2221	0.2588

CHAPTER 4: Microbiome and epigenetic variation in wild fish with low genetic diversity

Table S4.1. Sampling location, standard length (mm), alpha diversity metrics (chao1, shannon, simpson_eveness and faith_phylogenetic diversity), individual heterozygosity (H-indiv) based on SNPs, and coefficient of variation for DNA methylation (CV) measurements for *K. ocellatus* (Koce) and *K. hermaphroditus* (Kher).

Sample	Species	Location	Location type	SL	chao1	shannon	simpson_e	faith_pd	H-indiv	CV
IA-11	Koce	FLO	ALLOPATRIC	29.483	263	7.118	0.356	25.304	0.064	NA
IA-12	Koce	FLO	ALLOPATRIC	24.971	482.886	7.647	0.147	33.745	0.081	NA
IA-13	Koce	FLO	ALLOPATRIC	22.817	365.3	7.595	0.343	28.748	0.068	NA
IA-17	Koce	FLO	ALLOPATRIC	21.032	253	7.301	0.433	23.11	0.068	NA
IA-18	Koce	FLO	ALLOPATRIC	19.424	319	7.221	0.17	26.325	0.066	NA
IA-19	Koce	FLO	ALLOPATRIC	22.299	160	6.614	0.467	16.72	0.063	NA
IA-23	Koce	FLO	ALLOPATRIC	22.484	237.333	7.2	0.462	22.464	0.072	NA
IA-14	Kher	PIC	ALLOPATRIC	27.181	248.5	6.538	0.167	19.578	NA	NA
IA-15	Kher	PIC	ALLOPATRIC	27.791	277.955	6.572	0.17	18.344	NA	NA
IA-16	Kher	PIC	ALLOPATRIC	24.527	103	5.417	0.212	11.781	NA	NA
IA-20	Kher	PIC	ALLOPATRIC	25.471	60.5	4.735	0.256	7.133	NA	NA
IA-21	Kher	PIC	ALLOPATRIC	24.842	88	5.464	0.32	9.025	NA	NA
IA-10	Koce	SFR	ALLOPATRIC	26.673	246	6.813	0.181	21.662	NA	NA
IA-24	Koce	SFR	ALLOPATRIC	34.476	332.105	7.449	0.337	26.225	0.072	NA
IA-25	Koce	SFR	ALLOPATRIC	43.778	397.789	7.523	0.112	35.637	0.072	NA
IA-26	Koce	SFR	ALLOPATRIC	36.492	1002.067	9.161	0.199	55.505	0.068	NA
IA-9	Koce	SFR	ALLOPATRIC	27.838	144.25	6.22	0.302	17.517	NA	NA
IA-22	Kher	ARA	ALLOPATRIC	38.221	254.214	7.145	0.365	22.288	NA	NA
IA-28	Kher	ARA	ALLOPATRIC	38.15	31.5	3.777	0.305	4.967	NA	NA
IA-29	Kher	ARA	ALLOPATRIC	37.25	76	5.103	0.322	8.082	NA	NA
IA-31	Koce	GUA	SYMPATRIC	40.08	400.844	3.722	0.015	33.529	NA	NA
IA-32	Koce	GUA	SYMPATRIC	34.032	261.019	2.852	0.013	25.495	0.089	0.887

Table S4.1. Continued

Sample	Species	Location	Location type	SL	chao1	shannon	simpson_e	faith_pd	H-indiv	CV
IA-33	Koce	GUA	SYMPATRIC	33.579	528.777	3.229	0.008	37.928	0.086	0.643
IA-34	Koce	GUA	SYMPATRIC	30.963	700.614	6.415	0.018	48.123	0.093	0.746
IA-35	Koce	GUA	SYMPATRIC	19.229	679.692	7.751	0.067	42.445	0.091	0.612
IA-39	Kher	GUA	SYMPATRIC	22.965	705.581	6.911	0.03	46.201	0.045	0.597
IA-40	Kher	GUA	SYMPATRIC	24.315	402.758	7.066	0.135	30.323	0.037	0.803
IA-41	Kher	GUA	SYMPATRIC	33.9	327.774	4.572	0.014	29.237	0.035	0.538
IA-42	Kher	GUA	SYMPATRIC	25.859	318.1	6.848	0.133	24.039	0.036	0.69
IA-43	Kher	GUA	SYMPATRIC	34.68	285	3.434	0.009	24.942	0.036	0.711
IA-44	Kher	GUA	SYMPATRIC	23.631	266.615	6.969	0.273	21.306	0.04	0.928
IA-45	Kher	GUA	SYMPATRIC	26.432	486.026	7.004	0.036	31.093	0.065	0.87
IA-46	Kher	GUA	SYMPATRIC	23.316	282.333	7.24	0.36	21.919	0.037	1.208
IA-47	Kher	GUA	SYMPATRIC	25.119	64	2.729	0.038	8.69	0.039	0.649
IA-48	Kher	GUA	SYMPATRIC	29.409	197.333	5.958	0.111	18.866	0.036	0.83
IA-36	Koce	FUN	SYMPATRIC	36.945	158	6.319	0.308	15.278	NA	NA
IA-38	Koce	FUN	SYMPATRIC	23.945	224.875	6.771	0.305	18.593	NA	NA
IA-49	Kher	FUN	SYMPATRIC	25.776	205.5	4.604	0.031	15.224	0.036	0.65
IA-50	Kher	FUN	SYMPATRIC	29.086	84	5.428	0.384	9.435	0.038	1.01
IA-51	Kher	FUN	SYMPATRIC	28.938	190.143	6.287	0.244	16.774	0.037	0.591
IA-52	Kher	FUN	SYMPATRIC	27.172	51	4.748	0.362	6.441	0.036	1.09
IA-59	Koce	FUN	SYMPATRIC	25.47	992.603	9.167	0.273	59.339	NA	NA

Table S4.2.a. Analysis of indicator ASVs associated with the locations (GUA and FUN) where *Kryptolebias hermaphroditus* (KH) and *K. ocellatus* (KO) were sampled in sympatry. The association statistic indicates the strength of association for the respective ASV with the respective group.

ASV	Taxa	Representative location	A (specificity)	B (sensitivity)	Association statistic	p. value	IA-40	IA-41	IA-42	IA-43	IA-44	IA-45	IA-46	IA-47	IA-48	IA-39	IA-49	IA-50	IA-51	IA-52
1637_	g__Phycisphaera	GUA	0.9934	0.9286	0.96	0.001	125	5970	1572	7958	542	2967	219	8111	1664	140	3	5	1	3
6379_	g__Exiguobacterium	GUA	0.8228	0.9286	0.874	0.008	12	47	1	10	20	172	118	0	34	31	2	0	0	0
1461_	g__Sulfurovum	GUA	0.9658	0.7857	0.871	0.02	2	250	45	30	109	50	0	0	0	17	0	0	0	0
8758_	f__Flavobacteriaceae	GUA	0.8414	0.8571	0.849	0.013	291	33	140	63	20	28	65	0	158	102	0	0	0	0
2984_	f__Rhodobacteraceae	GUA	0.996	0.7143	0.843	0.006	31	20	9	11	92	74	0	0	0	1	0	0	0	0
5476_	g__Sulfurimonas	GUA	0.9517	0.7143	0.824	0.013	402	118	196	128	0	35	9	0	209	34	0	0	0	0
362_	g__Sulfurovum	GUA	0.775	0.8571	0.815	0.029	517	451	240	251	127	83	196	47	62	334	0	0	0	0
6769_	g__Sulfurimonas	GUA	0.9838	0.6429	0.795	0.023	107	34	21	23	0	48	0	0	0	16	0	0	0	0
7805_	g__Hydrogenophaga	GUA	0.925	0.6429	0.771	0.042	3	0	140	6	0	24	4	0	49	14	0	0	0	0
6323_	g__Aridibacter	GUA	1	0.5714	0.756	0.022	0	2	73	0	41	99	0	0	81	0	0	0	0	0
5583_	f__Rhodobacteraceae	GUA	0.9749	0.5714	0.746	0.032	11	2	0	4	0	7	0	10	29	2	0	0	0	0
4871_	g__Massilia	GUA	1	0.5	0.707	0.043	0	0	11	4	0	41	30	0	64	0	0	0	0	0
8690_	g__Rhizobacter	GUA	1	0.5	0.707	0.036	10	15	0	7	0	0	79	0	0	0	0	0	0	0
5588_	g__Arcobacter	FUN	1	0.875	0.935	0.001	0	0	0	0	0	0	0	0	0	0	73	27	628	168
5793_	g__Staphylococcus	FUN	0.8273	0.875	0.851	0.029	8	9	0	8	0	24	1	0	0	31	0	289	29	174

Table S4.2.a. Continued

ASV	Taxa	Representative location	A (specificity)	B (sensitivity)	Association statistic	p. value	IA-40	IA-41	IA-42	IA-43	IA-44	IA-45	IA-46	IA-47	IA-48	IA-39	IA-49	IA-50	IA-51	IA-52
7236_	g__Vibrio	FUN	0.96	0.75	0.849	0.015	56	14	36	10	0	0	70	0	0	16	0	480	907	1085
5244_	g__Vibrio	FUN	0.9322	0.75	0.836	0.018	18	0	16	0	0	8	38	0	0	6	0	162	314	0
3672_	g__Defluviicoccus	FUN	1	0.625	0.791	0.002	0	0	0	0	0	0	0	0	0	0	6	41	44	0
8863_	c__Actinobacteria	FUN	1	0.625	0.791	0.001	0	0	0	0	0	0	0	0	0	0	4	14	10	0
889_	g__Thermus	FUN	0.826	0.75	0.787	0.045	69	0	11	0	0	1	0	0	0	7	6	0	57	126
7029_	f__Intrasporangiaceae	FUN	0.9871	0.625	0.785	0.003	0	0	0	0	0	0	0	0	0	0	15	0	0	196
1831_	f__Rhodobacteraceae	FUN	0.9568	0.625	0.773	0.004	0	0	0	0	0	0	0	0	0	17	40	0	57	10
8419_	f__Intrasporangiaceae	FUN	1	0.5	0.707	0.009	0	0	0	0	0	0	0	0	0	6	137	0	45	626
3362_	g__Limnothrix	FUN	1	0.5	0.707	0.007	0	0	0	0	0	0	0	0	0	0	85	0	96	0
5509_	g__Arcobacter	FUN	1	0.5	0.707	0.005	0	0	0	0	0	0	0	0	0	0	7	82	11	0
1403_	o__Micrococcales	FUN	1	0.5	0.707	0.009	0	0	0	0	0	0	0	0	0	0	27	0	4	0
5754_	g__Sulfurimonas	FUN	1	0.375	0.612	0.038	0	0	0	0	0	0	0	0	0	0	1	0	0	698
9357_	f__Rhodobacteraceae	FUN	1	0.375	0.612	0.027	0	0	0	0	0	0	0	0	0	0	328	0	28	0
6622_	g__Halioglobus	FUN	1	0.375	0.612	0.038	0	0	0	0	0	0	0	0	0	0	0	0	14	0
2356_	f__Arcobacteraceae	FUN	1	0.375	0.612	0.035	0	0	0	0	0	0	0	0	0	0	31	0	33	0
8051_	g__Sulfurimonas	FUN	1	0.375	0.612	0.037	0	0	0	0	0	0	0	0	0	0	69	171	0	0

Table S4.2.a. Continued

ASV	Taxa	Representative location	A (specificity)	B (sensitivity)	Association statistic	p. value	IA-40	IA-41	IA-42	IA-43	IA-44	IA-45	IA-46	IA-47	IA-48	IA-39	IA-49	IA-50	IA-51	IA-52
6302_	g__Defluviicoccus	FUN	1	0.375	0.612	0.04	0	0	0	0	0	0	0	0	0	0	56	0	0	0
9324_	f__Rhodobacteraceae	FUN	1	0.375	0.612	0.04	0	0	0	0	0	0	0	0	0	0	143	0	0	0
2716_	g__Romboutsia	FUN	1	0.375	0.612	0.032	0	0	0	0	0	0	0	0	0	0	5	19	0	141
8728_	g__Pseudarcobacter	FUN	1	0.375	0.612	0.035	0	0	0	0	0	0	0	0	0	0	14	0	5	0
508_	f__Arcobacteraceae	FUN	1	0.375	0.612	0.04	0	0	0	0	0	0	0	0	0	0	12	0	0	0
3358_	g__Thiothrix	FUN	1	0.375	0.612	0.041	0	0	0	0	0	0	0	0	0	0	0	0	50	0
9220_	g__Desulfobacter	FUN	1	0.375	0.612	0.036	0	0	0	0	0	0	0	0	0	1	0	0	4	0
8550_	g__Dermacoccus	FUN	0.9771	0.375	0.605	0.038	0	5	0	0	0	0	0	0	0	0	0	0	41	0

Table S4.2.b. Analysis of indicator ASVs associated with *Kryptolebias hermaphroditus* (KH) and *K. ocellatus* (KO) sampled in sympatry in GUA and FUN locations. The association statistic indicates the strength of association for the respective ASV with the respective group.

ASV	Taxa	Representative species	A (specificity)	B (sensitivity)	Association statistic	p.value	IA-40	IA-41	IA-42	IA-43	IA-44	IA-45	IA-46	IA-47	IA-48	IA-39	IA-49	IA-50	IA-51	IA-52	IA-36	IA-38	IA-59	IA-10	IA-32	IA-33	IA-34	IA-35
1888_	f__Xanthobacteraceae	*K. hermaphroditus*	0.9744	0.8571	0.914	0.019	6	2	127	18	13	58	83	143	33	0	0	371	143	1935	0	0	33	0	7	0	4	0
362_	g__Sulfurovum	*K. hermaphroditus*	0.9141	0.7143	0.808	0.04	517	451	240	251	127	83	196	47	62	334	0	0	0	0	0	12	0	0	0	2	89	21
2273_	g__Sulfurimonas	*K. hermaphroditus*	0.9961	0.5	0.706	0.038	60	3	102	30	111	52	0	0	0	87	0	0	0	0	0	0	0	0	0	1	0	0
1461_	g__Sulfurovum	*K. ocelatus*	0.9676	0.75	0.852	0.028	2	250	45	30	109	50	0	0	0	17	0	0	0	0	0	163	0	53	3494	3365	869	631
3700_	c__Gammaproteobacteria	*K. ocelatus*	0.9958	0.5	0.706	0.01	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	37	79	0	0	4	688
8682_	f__Desulfosarcinaceae	*K. ocelatus*	0.9819	0.5	0.701	0.022	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	40	0	11	1	0	10
7051_	g__Methylomonas	*K. ocelatus*	0.8537	0.5	0.653	0.035	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	6	1	2	11
2428_	g__Nocardioides	*K. ocelatus*	0.801	0.5	0.633	0.031	0	0	0	0	30	0	0	0	0	0	0	0	0	0	0	9	0	0	0	4	43	13
2495_	f__Rhodobacteraceae	*K. ocelatus*	1	0.375	0.612	0.034	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	1	9	0
1878_	g__Woeseia	*K. ocelatus*	1	0.375	0.612	0.034	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	112	5	0	0	0
6978_	g__Marinobacter	*K. ocelatus*	1	0.375	0.612	0.034	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	101	0	0	4	3	0	0
4405_	o__Vicinamibacterales	*K. ocelatus*	1	0.375	0.612	0.034	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	23	65
7642_	o__Vicinamibacterales	*K. ocelatus*	1	0.375	0.612	0.034	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	30	11
5854_	p__Chloroflexi	*K. ocelatus*	1	0.375	0.612	0.034	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	16	15
552_	g__Alkanindiges	*K. ocelatus*	1	0.375	0.612	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	2	8	0	0
1914_	o__Vicinamibacterales	*K. ocelatus*	1	0.375	0.612	0.035	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	2	13	0
1792_	g__Vicinamibacteraceae	*K. ocelatus*	1	0.375	0.612	0.046	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0	0	0	6	0	5
5785_	o__Sphingobacteriales	*K. ocelatus*	1	0.375	0.612	0.034	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	5	14
7406_	f__Oxalobacteraceae	*K. ocelatus*	1	0.375	0.612	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	4
7006_	f__Vicinamibacteraceae	*K. ocelatus*	1	0.375	0.612	0.035	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	9	0
33_	f__Roseiflexaceae	*K. ocelatus*	1	0.375	0.612	0.034	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	6
3431_	g__Hyphomonas	*K. ocelatus*	0.9952	0.375	0.611	0.04	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	33	50	0	0	0	0	36

Table S4.3. Snapshot of water parameters at the time of sampling each one of the locations.

Location code	Sampling site	Distance from the sea (m)	Temperature ºC	Dissolved O2 (ppm)	Conductivity	Salinity (ppm)	pH
FLO	Poço das Pedras, estuário do rio Ratones, Florianópolis, SC	3157	20.8	70	31.57	21.76	7.3
SFR	Manguezal no canal do Linguado, São Francisco do Sul, SC	8600	18.2	80.4	31.98	23.31	7.5
PIC	Manguezal do rio da Fazenda, P. E. S. M. Picinguaba, Picinguaba, SP	1048	24.1	56.2	36.85	23.87	8.52
ARA	Alagado na praia de Coqueiral, Aracruz, ES	33	22.8	48.3	10.836	6.53	12.14
GUA	Manguezal do rio Piracao, Guaratiba, RJ	11640	22	6.7	24.88	16.14	6.75
FUN	Manguezal da Ilha do Fundao, Rio de Janeiro, RJ	1790	22	8.5	41.45	9.48	7.75

Table S4.4. Individual, sampling location and pairwise distances Bray-Curtis and Unifrac for microbiome, Euclidean for genetic distance based on SNPs for fish (14 *Kryptolebias hermaphroditus* and 14 *K. ocelatus*) living in sympatry (FUN, GUA) and allopatry (FLO, SFR).

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-11	IA-12	Koce	Koce	FLO	FLO	0.83639412	0.21386238	38.05132462
IA-11	IA-13	Koce	Koce	FLO	FLO	0.858356058	0.222601631	31.16703655
IA-11	IA-17	Koce	Koce	FLO	FLO	0.887915255	0.239954272	29.34617637
IA-11	IA-18	Koce	Koce	FLO	FLO	0.911038405	0.257869263	30.61583944
IA-11	IA-19	Koce	Koce	FLO	FLO	0.896109965	0.245510066	29.37172812
IA-11	IA-23	Koce	Koce	FLO	FLO	0.885596355	0.212732864	29.34601153
IA-11	IA-24	Koce	Koce	FLO	SFR	0.978570872	0.248592154	29.58179244
IA-11	IA-25	Koce	Koce	FLO	SFR	0.961196821	0.339435956	33.53611515
IA-11	IA-26	Koce	Koce	FLO	SFR	0.966893105	0.296655141	33.6181716
IA-11	IA-32	Koce	Koce	FLO	GUA	0.99563489	0.567870739	39.35908694
IA-11	IA-33	Koce	Koce	FLO	GUA	0.99639682	0.6101349	39.47686587
IA-11	IA-34	Koce	Koce	FLO	GUA	0.988143063	0.428420126	44.5916431
IA-11	IA-35	Koce	Koce	FLO	GUA	0.982609374	0.332726965	46.51286491
IA-11	IA-39	Koce	Kher	FLO	GUA	0.970737417	0.309245323	19.03505538
IA-11	IA-40	Koce	Kher	FLO	GUA	0.953240073	0.303601353	19.08815187
IA-11	IA-41	Koce	Kher	FLO	GUA	0.982156076	0.554823841	19.36046451
IA-11	IA-42	Koce	Kher	FLO	GUA	0.976940755	0.329485683	18.98239952
IA-11	IA-43	Koce	Kher	FLO	GUA	0.981923721	0.609849446	18.98239952
IA-11	IA-44	Koce	Kher	FLO	GUA	0.98558741	0.30593233	21.79888201
IA-11	IA-45	Koce	Kher	FLO	GUA	0.952069207	0.346912096	20.44692943
IA-11	IA-46	Koce	Kher	FLO	GUA	0.956145817	0.272623252	17.82796279
IA-11	IA-47	Koce	Kher	FLO	GUA	0.995546701	0.576858639	20.44692943
IA-11	IA-48	Koce	Kher	FLO	GUA	0.957009346	0.385173335	17.37654869

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-11	IA-49	Koce	Kher	FLO	FUN	0.990263281	0.376144721	17.2808094
IA-11	IA-50	Koce	Kher	FLO	FUN	0.950471147	0.30569979	17.37654869
IA-11	IA-51	Koce	Kher	FLO	FUN	0.928738318	0.270696126	17.32848069
IA-11	IA-52	Koce	Kher	FLO	FUN	0.979440854	0.327746114	17.67360523
IA-12	IA-13	Koce	Koce	FLO	FLO	0.81966382	0.188840379	38.1957072
IA-12	IA-17	Koce	Koce	FLO	FLO	0.872036806	0.218495467	37.1573051
IA-12	IA-18	Koce	Koce	FLO	FLO	0.926769351	0.214852174	37.43742558
IA-12	IA-19	Koce	Koce	FLO	FLO	0.87759239	0.214062806	37.33081493
IA-12	IA-23	Koce	Koce	FLO	FLO	0.875565258	0.194922009	37.13902232
IA-12	IA-24	Koce	Koce	FLO	SFR	0.98299333	0.251769052	39.81612505
IA-12	IA-25	Koce	Koce	FLO	SFR	0.966145325	0.292573467	38.93859202
IA-12	IA-26	Koce	Koce	FLO	SFR	0.946765683	0.244506582	42.16525706
IA-12	IA-32	Koce	Koce	FLO	GUA	0.993210816	0.530787657	42.93916804
IA-12	IA-33	Koce	Koce	FLO	GUA	0.992628607	0.573742619	44.37235243
IA-12	IA-34	Koce	Koce	FLO	GUA	0.979695431	0.414007329	48.75390994
IA-12	IA-35	Koce	Koce	FLO	GUA	0.967677714	0.312658457	49.13252624
IA-12	IA-39	Koce	Kher	FLO	GUA	0.971016757	0.297345719	40.54326205
IA-12	IA-40	Koce	Kher	FLO	GUA	0.963407974	0.274058375	39.88397808
IA-12	IA-41	Koce	Kher	FLO	GUA	0.965129885	0.515987525	40.68574627
IA-12	IA-42	Koce	Kher	FLO	GUA	0.980424271	0.292754391	39.69083419
IA-12	IA-43	Koce	Kher	FLO	GUA	0.974804992	0.570053253	39.69083419
IA-12	IA-44	Koce	Kher	FLO	GUA	0.994618414	0.299725959	40.79590935
IA-12	IA-45	Koce	Kher	FLO	GUA	0.957944837	0.341197212	39.02371384
IA-12	IA-46	Koce	Kher	FLO	GUA	0.963270558	0.239555474	39.60891288
IA-12	IA-47	Koce	Kher	FLO	GUA	0.990848651	0.585365283	40.34692549
IA-12	IA-48	Koce	Kher	FLO	GUA	0.975361584	0.363443592	39.11831692

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-12	IA-49	Koce	Kher	FLO	FUN	0.984715561	0.37121379	39.02371384
IA-12	IA-50	Koce	Kher	FLO	FUN	0.962421549	0.298635295	39.21361138
IA-12	IA-51	Koce	Kher	FLO	FUN	0.975049706	0.280847353	39.11831692
IA-12	IA-52	Koce	Kher	FLO	FUN	0.98284801	0.311250767	39.8003813
IA-13	IA-17	Koce	Koce	FLO	FLO	0.890226325	0.204700093	33.05241764
IA-13	IA-18	Koce	Koce	FLO	FLO	0.93392551	0.244688842	31.01676066
IA-13	IA-19	Koce	Koce	FLO	FLO	0.914079844	0.236033699	32.05849694
IA-13	IA-23	Koce	Koce	FLO	FLO	0.870223953	0.22020256	31.27695582
IA-13	IA-24	Koce	Koce	FLO	SFR	0.983476228	0.231393498	34.25861754
IA-13	IA-25	Koce	Koce	FLO	SFR	0.970072481	0.298059334	32.67553672
IA-13	IA-26	Koce	Koce	FLO	SFR	0.954233052	0.238547887	34.56516887
IA-13	IA-32	Koce	Koce	FLO	GUA	0.996413535	0.560612201	37.6382997
IA-13	IA-33	Koce	Koce	FLO	GUA	0.993340385	0.601973108	39.47994219
IA-13	IA-34	Koce	Koce	FLO	GUA	0.992665704	0.435147895	43.58299027
IA-13	IA-35	Koce	Koce	FLO	GUA	0.984321373	0.3361481	46.35777569
IA-13	IA-39	Koce	Kher	FLO	GUA	0.974477053	0.327893166	25.42290944
IA-13	IA-40	Koce	Kher	FLO	GUA	0.974042172	0.311678589	24.30557281
IA-13	IA-41	Koce	Kher	FLO	GUA	0.978566697	0.544269522	24.85183366
IA-13	IA-42	Koce	Kher	FLO	GUA	0.989402735	0.319692745	24.10982004
IA-13	IA-43	Koce	Kher	FLO	GUA	0.989245217	0.598426689	24.17454451
IA-13	IA-44	Koce	Kher	FLO	GUA	0.988856853	0.293267337	25.35447618
IA-13	IA-45	Koce	Kher	FLO	GUA	0.972949797	0.351051207	25.28659259
IA-13	IA-46	Koce	Kher	FLO	GUA	0.967666537	0.246240722	24.85183366
IA-13	IA-47	Koce	Kher	FLO	GUA	0.994764193	0.580993833	24.17454451
IA-13	IA-48	Koce	Kher	FLO	GUA	0.981927242	0.385912603	22.99588682
IA-13	IA-49	Koce	Kher	FLO	FUN	0.99524737	0.392581418	22.87258359

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-13	IA-50	Koce	Kher	FLO	FUN	0.969777224	0.33359289	23.05829097
IA-13	IA-51	Koce	Kher	FLO	FUN	0.986601231	0.302911427	22.93398661
IA-13	IA-52	Koce	Kher	FLO	FUN	0.983097056	0.347025858	23.37811611
IA-17	IA-18	Koce	Koce	FLO	FLO	0.945550146	0.278493006	28.48419355
IA-17	IA-19	Koce	Koce	FLO	FLO	0.935596916	0.258916164	29.9578788
IA-17	IA-23	Koce	Koce	FLO	FLO	0.868000935	0.230024515	29.87356012
IA-17	IA-24	Koce	Koce	FLO	SFR	0.952390229	0.250063118	33.20892572
IA-17	IA-25	Koce	Koce	FLO	SFR	0.977404652	0.323191782	31.59900107
IA-17	IA-26	Koce	Koce	FLO	SFR	0.962678118	0.270506394	34.7088991
IA-17	IA-32	Koce	Koce	FLO	GUA	0.991893683	0.575447047	37.6382997
IA-17	IA-33	Koce	Koce	FLO	GUA	0.993498864	0.617940533	39.77220585
IA-17	IA-34	Koce	Koce	FLO	GUA	0.980889236	0.452661616	43.20376901
IA-17	IA-35	Koce	Koce	FLO	GUA	0.972628378	0.360607943	45.03086928
IA-17	IA-39	Koce	Kher	FLO	GUA	0.971830986	0.347939611	24.50617241
IA-17	IA-40	Koce	Kher	FLO	GUA	0.973581671	0.323093916	24.43875503
IA-17	IA-41	Koce	Kher	FLO	GUA	0.978027115	0.561471595	24.99425221
IA-17	IA-42	Koce	Kher	FLO	GUA	0.966113578	0.323401947	24.30557281
IA-17	IA-43	Koce	Kher	FLO	GUA	0.976392676	0.615016335	24.30557281
IA-17	IA-44	Koce	Kher	FLO	GUA	0.979589452	0.326630344	26.5533629
IA-17	IA-45	Koce	Kher	FLO	GUA	0.947005416	0.372657198	25.42290944
IA-17	IA-46	Koce	Kher	FLO	GUA	0.947579546	0.263360039	24.99425221
IA-17	IA-47	Koce	Kher	FLO	GUA	0.993828125	0.588165421	25.49189982
IA-17	IA-48	Koce	Kher	FLO	GUA	0.955064055	0.385643254	23.12120594
IA-17	IA-49	Koce	Kher	FLO	FUN	0.985512326	0.385247637	23.05829097
IA-17	IA-50	Koce	Kher	FLO	FUN	0.91325001	0.340373538	23.18463872
IA-17	IA-51	Koce	Kher	FLO	FUN	0.950780733	0.307435081	23.12120594

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-17	IA-52	Koce	Kher	FLO	FUN	0.938169217	0.343073634	23.64378505
IA-18	IA-19	Koce	Koce	FLO	FLO	0.920869193	0.264707735	30.1739952
IA-18	IA-23	Koce	Koce	FLO	FLO	0.891428794	0.230154811	31.12335615
IA-18	IA-24	Koce	Koce	FLO	SFR	0.979270574	0.261209929	31.87146029
IA-18	IA-25	Koce	Koce	FLO	SFR	0.968986207	0.309167221	32.28625672
IA-18	IA-26	Koce	Koce	FLO	SFR	0.961424332	0.268713816	33.94935
IA-18	IA-32	Koce	Koce	FLO	GUA	0.995244777	0.534014566	39.12549979
IA-18	IA-33	Koce	Koce	FLO	GUA	0.991304688	0.575123678	41.00234681
IA-18	IA-34	Koce	Koce	FLO	GUA	0.984085501	0.417585423	44.76323255
IA-18	IA-35	Koce	Koce	FLO	GUA	0.98284199	0.313764469	47.09673689
IA-18	IA-39	Koce	Kher	FLO	GUA	0.963009209	0.264993533	18.82701574
IA-18	IA-40	Koce	Kher	FLO	GUA	0.967499318	0.27700321	17.18663535
IA-18	IA-41	Koce	Kher	FLO	GUA	0.974829534	0.516187354	17.57290011
IA-18	IA-42	Koce	Kher	FLO	GUA	0.969303884	0.311587075	17.09398435
IA-18	IA-43	Koce	Kher	FLO	GUA	0.976389917	0.567969792	17.09398435
IA-18	IA-44	Koce	Kher	FLO	GUA	0.988702766	0.291771444	20.17172268
IA-18	IA-45	Koce	Kher	FLO	GUA	0.965081839	0.348247389	18.6753863
IA-18	IA-46	Koce	Kher	FLO	GUA	0.955441302	0.261691298	15.76252337
IA-18	IA-47	Koce	Kher	FLO	GUA	0.992420987	0.576943043	18.72552166
IA-18	IA-48	Koce	Kher	FLO	GUA	0.976400031	0.355836704	15.33059122
IA-18	IA-49	Koce	Kher	FLO	FUN	0.985510633	0.342147202	15.28932441
IA-18	IA-50	Koce	Kher	FLO	FUN	0.966744548	0.30090076	15.28932441
IA-18	IA-51	Koce	Kher	FLO	FUN	0.949762443	0.266442209	15.33059122
IA-18	IA-52	Koce	Kher	FLO	FUN	0.992056075	0.307946367	15.67321657
IA-19	IA-23	Koce	Koce	FLO	FLO	0.916763996	0.232525773	27.87093223
IA-19	IA-24	Koce	Koce	FLO	SFR	0.99345565	0.269637098	31.48869779

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-19	IA-25	Koce	Koce	FLO	SFR	0.980212675	0.313292205	32.66797141
IA-19	IA-26	Koce	Koce	FLO	SFR	0.982512979	0.279675937	32.31022276
IA-19	IA-32	Koce	Koce	FLO	GUA	0.97451584	0.50236732	38.90641957
IA-19	IA-33	Koce	Koce	FLO	GUA	0.974312789	0.544063337	39.52411528
IA-19	IA-34	Koce	Koce	FLO	GUA	0.972547185	0.383210155	44.49705355
IA-19	IA-35	Koce	Koce	FLO	GUA	0.970995283	0.286749727	46.71117967
IA-19	IA-39	Koce	Kher	FLO	GUA	0.985566608	0.301218928	13.68991574
IA-19	IA-40	Koce	Kher	FLO	GUA	0.979897148	0.27427212	11.1777694
IA-19	IA-41	Koce	Kher	FLO	GUA	0.963384232	0.491055649	11.44394405
IA-19	IA-42	Koce	Kher	FLO	GUA	0.984967677	0.276832356	11.11407789
IA-19	IA-43	Koce	Kher	FLO	GUA	0.993612215	0.550070557	11.14578716
IA-19	IA-44	Koce	Kher	FLO	GUA	0.990575223	0.269426492	15.71767968
IA-19	IA-45	Koce	Kher	FLO	GUA	0.976078233	0.319267181	13.61190989
IA-19	IA-46	Koce	Kher	FLO	GUA	0.985592461	0.245953974	11.47857028
IA-19	IA-47	Koce	Kher	FLO	GUA	0.973910327	0.575899973	13.61190989
IA-19	IA-48	Koce	Kher	FLO	GUA	0.955460385	0.32754904	7.881261683
IA-19	IA-49	Koce	Kher	FLO	FUN	0.967991901	0.369451457	7.85883984
IA-19	IA-50	Koce	Kher	FLO	FUN	0.950325067	0.279907968	7.903876541
IA-19	IA-51	Koce	Kher	FLO	FUN	0.959665174	0.269895133	7.881261683
IA-19	IA-52	Koce	Kher	FLO	FUN	0.945497723	0.300606227	8.01994555
IA-23	IA-24	Koce	Koce	FLO	SFR	0.971796969	0.236310207	31.77974366
IA-23	IA-25	Koce	Koce	FLO	SFR	0.974136252	0.305185554	31.04924805
IA-23	IA-26	Koce	Koce	FLO	SFR	0.963933018	0.260582334	34.64353236
IA-23	IA-32	Koce	Koce	FLO	GUA	0.995012274	0.539692385	37.48403499
IA-23	IA-33	Koce	Koce	FLO	GUA	0.997337301	0.583773471	40.1412213
IA-23	IA-34	Koce	Koce	FLO	GUA	0.985103728	0.417642523	44.35033688

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-23	IA-35	Koce	Koce	FLO	GUA	0.989474095	0.322826901	46.79259642
IA-23	IA-39	Koce	Kher	FLO	GUA	0.97425395	0.300288137	17.2808094
IA-23	IA-40	Koce	Kher	FLO	GUA	0.981533427	0.291895151	15.49906431
IA-23	IA-41	Koce	Kher	FLO	GUA	0.976472421	0.522257846	15.80775308
IA-23	IA-42	Koce	Kher	FLO	GUA	0.987070644	0.31646544	15.41413729
IA-23	IA-43	Koce	Kher	FLO	GUA	0.990262522	0.580872688	15.41413729
IA-23	IA-44	Koce	Kher	FLO	GUA	0.99571601	0.319655973	18.82701574
IA-23	IA-45	Koce	Kher	FLO	GUA	0.968208205	0.353010158	17.18663535
IA-23	IA-46	Koce	Kher	FLO	GUA	0.969549472	0.259636994	15.899393
IA-23	IA-47	Koce	Kher	FLO	GUA	0.997890955	0.562795495	17.18663535
IA-23	IA-48	Koce	Kher	FLO	GUA	0.973136072	0.366877464	13.38565741
IA-23	IA-49	Koce	Kher	FLO	FUN	0.992367898	0.374565657	13.3127105
IA-23	IA-50	Koce	Kher	FLO	FUN	0.944407677	0.302531916	13.42258343
IA-23	IA-51	Koce	Kher	FLO	FUN	0.978976056	0.274430142	13.34903447
IA-23	IA-52	Koce	Kher	FLO	FUN	0.955541714	0.327557238	13.65074566
IA-24	IA-25	Koce	Koce	SFR	SFR	0.96157144	0.26346421	32.3309287
IA-24	IA-26	Koce	Koce	SFR	SFR	0.953522887	0.221946786	32.48746792
IA-24	IA-32	Koce	Koce	SFR	GUA	0.997738615	0.532612084	39.52217549
IA-24	IA-33	Koce	Koce	SFR	GUA	0.993652784	0.5751983	38.82317018
IA-24	IA-34	Koce	Koce	SFR	GUA	0.994069218	0.4079081	44.80145381
IA-24	IA-35	Koce	Koce	SFR	GUA	0.997425495	0.32285091	47.31492768
IA-24	IA-39	Koce	Kher	SFR	GUA	0.983294301	0.321431404	22.69129209
IA-24	IA-40	Koce	Kher	SFR	GUA	0.980197248	0.311164526	22.69129209
IA-24	IA-41	Koce	Kher	SFR	GUA	0.992438711	0.521380069	23.24859646
IA-24	IA-42	Koce	Kher	SFR	GUA	0.990336282	0.321574756	22.63181285
IA-24	IA-43	Koce	Kher	SFR	GUA	0.99664835	0.580045511	22.5727989

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-24	IA-44	Koce	Kher	SFR	GUA	0.993297483	0.326991436	25.93012021
IA-24	IA-45	Koce	Kher	SFR	GUA	0.979962576	0.360965147	23.73209715
IA-24	IA-46	Koce	Kher	SFR	GUA	0.991973818	0.257678811	23.24859646
IA-24	IA-47	Koce	Kher	SFR	GUA	0.9922623	0.559243467	23.79381923
IA-24	IA-48	Koce	Kher	SFR	GUA	0.989481885	0.372905321	21.33747778
IA-24	IA-49	Koce	Kher	SFR	FUN	0.992675134	0.389112156	21.28183889
IA-24	IA-50	Koce	Kher	SFR	FUN	0.989872234	0.32069231	21.39355535
IA-24	IA-51	Koce	Kher	SFR	FUN	0.986910791	0.296798612	21.33747778
IA-24	IA-52	Koce	Kher	SFR	FUN	0.967980679	0.353141916	21.79888201
IA-25	IA-26	Koce	Koce	SFR	SFR	0.879247051	0.214331259	33.81015351
IA-25	IA-32	Koce	Koce	SFR	GUA	0.893879142	0.48780308	38.77421066
IA-25	IA-33	Koce	Koce	SFR	GUA	0.857629775	0.476154244	37.81421344
IA-25	IA-34	Koce	Koce	SFR	GUA	0.852834458	0.328354536	43.35050368
IA-25	IA-35	Koce	Koce	SFR	GUA	0.877057493	0.266966646	46.10712698
IA-25	IA-39	Koce	Kher	SFR	GUA	0.964483647	0.380740284	18.98239952
IA-25	IA-40	Koce	Kher	SFR	GUA	0.965152992	0.356736449	18.98239952
IA-25	IA-41	Koce	Kher	SFR	GUA	0.847149148	0.419016814	19.47270003
IA-25	IA-42	Koce	Kher	SFR	GUA	0.857393337	0.25775559	18.87838559
IA-25	IA-43	Koce	Kher	SFR	GUA	0.853630022	0.478125013	18.87838559
IA-25	IA-44	Koce	Kher	SFR	GUA	0.948410224	0.349417158	21.68073002
IA-25	IA-45	Koce	Kher	SFR	GUA	0.839869026	0.272790088	20.2804659
IA-25	IA-46	Koce	Kher	SFR	GUA	0.968287362	0.309924577	19.47270003
IA-25	IA-47	Koce	Kher	SFR	GUA	0.8579188	0.479630088	20.39098697
IA-25	IA-48	Koce	Kher	SFR	GUA	0.84060455	0.292124668	17.2808094
IA-25	IA-49	Koce	Kher	SFR	FUN	0.994545738	0.446398781	17.2335294
IA-25	IA-50	Koce	Kher	SFR	FUN	0.983718937	0.396096328	17.32848069

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-25	IA-51	Koce	Kher	SFR	FUN	0.989716423	0.367338481	17.2808094
IA-25	IA-52	Koce	Kher	SFR	FUN	0.988470827	0.415406394	17.67360523
IA-26	IA-32	Koce	Koce	SFR	GUA	0.936786998	0.50406905	39.61560866
IA-26	IA-33	Koce	Koce	SFR	GUA	0.934121157	0.538173273	38.84131417
IA-26	IA-34	Koce	Koce	SFR	GUA	0.93361223	0.388644641	44.90927937
IA-26	IA-35	Koce	Koce	SFR	GUA	0.92909631	0.305440047	47.04310674
IA-26	IA-44	Koce	Kher	SFR	GUA	0.948145256	0.308903384	10.899441
IA-26	IA-45	Koce	Kher	SFR	GUA	0.916796875	0.333480736	7.707068645
IA-26	IA-47	Koce	Kher	SFR	GUA	0.932333477	0.535908387	7.707068645
IA-32	IA-33	Koce	Koce	GUA	GUA	0.43499902	0.315456015	40.83643708
IA-32	IA-34	Koce	Koce	GUA	GUA	0.704070879	0.435165453	44.20983618
IA-32	IA-35	Koce	Koce	GUA	GUA	0.761354768	0.441686859	42.7678213
IA-32	IA-39	Koce	Kher	GUA	GUA	0.943073559	0.508198255	41.17427718
IA-32	IA-40	Koce	Kher	GUA	GUA	0.94361474	0.449367036	41.13427162
IA-32	IA-41	Koce	Kher	GUA	GUA	0.798510663	0.464547617	42.45973703
IA-32	IA-42	Koce	Kher	GUA	GUA	0.859208731	0.438447851	40.94160419
IA-32	IA-43	Koce	Kher	GUA	GUA	0.859576625	0.560138431	40.94160419
IA-32	IA-44	Koce	Kher	GUA	GUA	0.922429251	0.500360406	40.94160419
IA-32	IA-45	Koce	Kher	GUA	GUA	0.847917642	0.483996281	41.26903973
IA-32	IA-46	Koce	Kher	GUA	GUA	0.954711981	0.502678639	42.56367779
IA-32	IA-47	Koce	Kher	GUA	GUA	0.890975686	0.642616933	40.31650482
IA-32	IA-48	Koce	Kher	GUA	GUA	0.873587406	0.394229762	40.50623059
IA-32	IA-49	Koce	Kher	GUA	FUN	0.901083483	0.468894654	40.31650482
IA-32	IA-50	Koce	Kher	GUA	FUN	0.919264339	0.467856665	40.60210346
IA-32	IA-51	Koce	Kher	GUA	FUN	0.974125166	0.489722525	40.22263623
IA-32	IA-52	Koce	Kher	GUA	FUN	0.951137781	0.453124779	41.19199543

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-33	IA-34	Koce	Koce	GUA	GUA	0.554361468	0.309110742	42.06018624
IA-33	IA-35	Koce	Koce	GUA	GUA	0.742540093	0.449914798	43.16744805
IA-33	IA-39	Koce	Kher	GUA	GUA	0.946796406	0.546536271	35.41512063
IA-33	IA-40	Koce	Kher	GUA	GUA	0.947021944	0.492772743	35.67331448
IA-33	IA-41	Koce	Kher	GUA	GUA	0.491694092	0.180570157	37.04692629
IA-33	IA-42	Koce	Kher	GUA	GUA	0.843948296	0.451515285	35.41512063
IA-33	IA-43	Koce	Kher	GUA	GUA	0.561701794	0.274963716	35.50056133
IA-33	IA-44	Koce	Kher	GUA	GUA	0.922362803	0.539164123	34.23820822
IA-33	IA-45	Koce	Kher	GUA	GUA	0.717308672	0.374789211	35.24607374
IA-33	IA-46	Koce	Kher	GUA	GUA	0.954255277	0.546256088	37.140835
IA-33	IA-47	Koce	Kher	GUA	GUA	0.586749677	0.36001581	35.50056133
IA-33	IA-48	Koce	Kher	GUA	GUA	0.848846771	0.40512875	34.92221321
IA-33	IA-49	Koce	Kher	GUA	FUN	0.905377355	0.518571815	34.75309738
IA-33	IA-50	Koce	Kher	GUA	FUN	0.922393203	0.51665265	34.32243518
IA-33	IA-51	Koce	Kher	GUA	FUN	0.975643184	0.534352587	34.75309738
IA-33	IA-52	Koce	Kher	GUA	FUN	0.958573163	0.49660037	35.53416339
IA-34	IA-35	Koce	Koce	GUA	GUA	0.690733726	0.232827519	44.6921557
IA-34	IA-39	Koce	Kher	GUA	GUA	0.865353808	0.351821728	36.58783884
IA-34	IA-40	Koce	Kher	GUA	GUA	0.880980254	0.337508507	35.84861438
IA-34	IA-41	Koce	Kher	GUA	GUA	0.595552087	0.265685992	36.0851135
IA-34	IA-42	Koce	Kher	GUA	GUA	0.773209549	0.268941582	35.93723902
IA-34	IA-43	Koce	Kher	GUA	GUA	0.663233458	0.358440845	35.84861438
IA-34	IA-44	Koce	Kher	GUA	GUA	0.861127365	0.359853659	35.93723902
IA-34	IA-45	Koce	Kher	GUA	GUA	0.653385366	0.1903419	35.00770223
IA-34	IA-46	Koce	Kher	GUA	GUA	0.912711104	0.375261053	36.95759562
IA-34	IA-47	Koce	Kher	GUA	GUA	0.733983233	0.425659993	35.67331448

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-34	IA-48	Koce	Kher	GUA	GUA	0.796591662	0.267383341	35.93723902
IA-34	IA-49	Koce	Kher	GUA	FUN	0.885408947	0.427932441	35.84861438
IA-34	IA-50	Koce	Kher	GUA	FUN	0.894638331	0.396651232	36.11647823
IA-34	IA-51	Koce	Kher	GUA	FUN	0.937448816	0.387267557	35.93723902
IA-34	IA-52	Koce	Kher	GUA	FUN	0.929031	0.389685936	36.57657931
IA-35	IA-39	Koce	Kher	GUA	GUA	0.86890625	0.260678746	31.83994949
IA-35	IA-40	Koce	Kher	GUA	GUA	0.887722857	0.288515557	32.00621667
IA-35	IA-41	Koce	Kher	GUA	GUA	0.755197566	0.400975234	33.41224893
IA-35	IA-42	Koce	Kher	GUA	GUA	0.770619662	0.193445926	31.83994949
IA-35	IA-43	Koce	Kher	GUA	GUA	0.815983463	0.4631361	31.0233447
IA-35	IA-44	Koce	Kher	GUA	GUA	0.874112784	0.244297976	31.10445163
IA-35	IA-45	Koce	Kher	GUA	GUA	0.768804619	0.231676841	32.37862929
IA-35	IA-46	Koce	Kher	GUA	GUA	0.908289792	0.285625064	33.50292005
IA-35	IA-47	Koce	Kher	GUA	GUA	0.870341494	0.512408603	30.86301627
IA-35	IA-48	Koce	Kher	GUA	GUA	0.815906433	0.239740281	31.92275834
IA-35	IA-49	Koce	Kher	GUA	FUN	0.944474772	0.410696268	31.75778174
IA-35	IA-50	Koce	Kher	GUA	FUN	0.933182598	0.332006243	31.92275834
IA-35	IA-51	Koce	Kher	GUA	FUN	0.964210526	0.324755937	31.83994949
IA-35	IA-52	Koce	Kher	GUA	FUN	0.945579292	0.345676099	32.52109502
IA-39	IA-40	Kher	Kher	GUA	GUA	0.664675801	0.211345042	12.49114519
IA-39	IA-41	Kher	Kher	GUA	GUA	0.78978182	0.479495078	11.35472064
IA-39	IA-42	Kher	Kher	GUA	GUA	0.754867913	0.272984353	12.24377128
IA-39	IA-43	Kher	Kher	GUA	GUA	0.801740624	0.53499221	11.61045674
IA-39	IA-44	Kher	Kher	GUA	GUA	0.837040506	0.259747602	13.01762716
IA-39	IA-45	Kher	Kher	GUA	GUA	0.825499688	0.337467881	20.51179368
IA-39	IA-46	Kher	Kher	GUA	GUA	0.822005462	0.265150847	11.31988231

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-39	IA-47	Kher	Kher	GUA	GUA	0.943204868	0.602329978	11.61373052
IA-39	IA-48	Kher	Kher	GUA	GUA	0.848170399	0.317917915	12.51582779
IA-39	IA-49	Kher	Kher	GUA	FUN	0.922590714	0.350612014	12.62901815
IA-39	IA-50	Kher	Kher	GUA	FUN	0.932750819	0.296209146	12.41182049
IA-39	IA-51	Kher	Kher	GUA	FUN	0.912694078	0.279860423	12.13185668
IA-39	IA-52	Kher	Kher	GUA	FUN	0.948041816	0.31416025	12.18178577
IA-40	IA-41	Kher	Kher	GUA	GUA	0.778981835	0.427522658	6.058242918
IA-40	IA-42	Kher	Kher	GUA	GUA	0.711613406	0.23601017	4.277810573
IA-40	IA-43	Kher	Kher	GUA	GUA	0.781337699	0.521824005	5.24069722
IA-40	IA-44	Kher	Kher	GUA	GUA	0.855177521	0.268398711	7.433409549
IA-40	IA-45	Kher	Kher	GUA	GUA	0.856056766	0.340909151	21.80829228
IA-40	IA-46	Kher	Kher	GUA	GUA	0.841094182	0.246864043	5.579273541
IA-40	IA-47	Kher	Kher	GUA	GUA	0.959484203	0.597714654	8.198287431
IA-40	IA-48	Kher	Kher	GUA	GUA	0.825378891	0.273021797	4.28382465
IA-40	IA-49	Kher	Kher	GUA	FUN	0.938900362	0.32249333	4.282017764
IA-40	IA-50	Kher	Kher	GUA	FUN	0.941485839	0.282510307	3.503648175
IA-40	IA-51	Kher	Kher	GUA	FUN	0.889274165	0.259086036	5.528838395
IA-40	IA-52	Kher	Kher	GUA	FUN	0.939148389	0.267183168	3.509598332
IA-41	IA-42	Kher	Kher	GUA	GUA	0.713762469	0.386226205	5.2458543
IA-41	IA-43	Kher	Kher	GUA	GUA	0.374279034	0.169656838	5.53350868
IA-41	IA-44	Kher	Kher	GUA	GUA	0.857849823	0.486864924	8.596725116
IA-41	IA-45	Kher	Kher	GUA	GUA	0.650489222	0.320549637	21.55357842
IA-41	IA-46	Kher	Kher	GUA	GUA	0.869014688	0.481209875	6.370478654
IA-41	IA-47	Kher	Kher	GUA	GUA	0.51731391	0.259289883	8.394346355
IA-41	IA-48	Kher	Kher	GUA	GUA	0.761520782	0.355784019	5.541318858
IA-41	IA-49	Kher	Kher	GUA	FUN	0.900962325	0.493494625	5.536628786

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-41	IA-50	Kher	Kher	GUA	FUN	0.901608694	0.476847272	5.819186227
IA-41	IA-51	Kher	Kher	GUA	FUN	0.933465779	0.481253163	6.551951442
IA-41	IA-52	Kher	Kher	GUA	FUN	0.943130916	0.47222611	5.272619037
IA-42	IA-43	Kher	Kher	GUA	GUA	0.698823346	0.436132691	4.623158607
IA-42	IA-44	Kher	Kher	GUA	GUA	0.789473684	0.23510431	7.010265754
IA-42	IA-45	Kher	Kher	GUA	GUA	0.721657118	0.220734621	21.66746032
IA-42	IA-46	Kher	Kher	GUA	GUA	0.810929771	0.225933101	5.852429493
IA-42	IA-47	Kher	Kher	GUA	GUA	0.842283578	0.501890096	7.416672326
IA-42	IA-48	Kher	Kher	GUA	GUA	0.71414106	0.193778811	3.499698176
IA-42	IA-49	Kher	Kher	GUA	FUN	0.93915787	0.360915504	3.496744428
IA-42	IA-50	Kher	Kher	GUA	FUN	0.922271116	0.300626118	3.918304141
IA-42	IA-51	Kher	Kher	GUA	FUN	0.904116525	0.29138311	4.945838865
IA-42	IA-52	Kher	Kher	GUA	FUN	0.939483625	0.321046886	3.039831914
IA-43	IA-44	Kher	Kher	GUA	GUA	0.843210598	0.535330113	7.839930992
IA-43	IA-45	Kher	Kher	GUA	GUA	0.645839018	0.369226254	21.46205431
IA-43	IA-46	Kher	Kher	GUA	GUA	0.862947524	0.530549331	5.854106647
IA-43	IA-47	Kher	Kher	GUA	GUA	0.361012658	0.144107325	7.624194531
IA-43	IA-48	Kher	Kher	GUA	GUA	0.738012698	0.437912248	4.630970201
IA-43	IA-49	Kher	Kher	GUA	FUN	0.940161284	0.565056551	4.628361939
IA-43	IA-50	Kher	Kher	GUA	FUN	0.94617332	0.532274205	4.294107053
IA-43	IA-51	Kher	Kher	GUA	FUN	0.930043236	0.535050648	5.248808047
IA-43	IA-52	Kher	Kher	GUA	FUN	0.960350536	0.552043045	4.646712922
IA-44	IA-45	Kher	Kher	GUA	GUA	0.817600748	0.284939999	22.08612137
IA-44	IA-46	Kher	Kher	GUA	GUA	0.861483328	0.240045041	7.713727021
IA-44	IA-47	Kher	Kher	GUA	GUA	0.938546616	0.582579809	9.113042203
IA-44	IA-48	Kher	Kher	GUA	GUA	0.810951862	0.289827223	7.652244584

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-44	IA-49	Kher	Kher	GUA	FUN	0.91243378	0.353373216	7.645744021
IA-44	IA-50	Kher	Kher	GUA	FUN	0.9373783	0.310890391	7.030158716
IA-44	IA-51	Kher	Kher	GUA	FUN	0.958560523	0.291747945	8.229572085
IA-44	IA-52	Kher	Kher	GUA	FUN	0.982397383	0.313040549	6.819530023
IA-45	IA-46	Kher	Kher	GUA	GUA	0.828150573	0.291326926	21.2192267
IA-45	IA-47	Kher	Kher	GUA	GUA	0.737805828	0.397371165	21.68932929
IA-45	IA-48	Kher	Kher	GUA	GUA	0.737629549	0.260789187	21.78036715
IA-45	IA-49	Kher	Kher	GUA	FUN	0.944587328	0.415602751	21.76150696
IA-45	IA-50	Kher	Kher	GUA	FUN	0.932834658	0.36266668	21.58476579
IA-45	IA-51	Kher	Kher	GUA	FUN	0.940076366	0.350431655	21.83974807
IA-45	IA-52	Kher	Kher	GUA	FUN	0.965014804	0.384117115	21.40521291
IA-46	IA-47	Kher	Kher	GUA	GUA	0.961140098	0.56111519	8.469880809
IA-46	IA-48	Kher	Kher	GUA	GUA	0.847196262	0.297380303	6.374140901
IA-46	IA-49	Kher	Kher	GUA	FUN	0.962533105	0.332881717	6.368649896
IA-46	IA-50	Kher	Kher	GUA	FUN	0.938088934	0.282855793	5.308951036
IA-46	IA-51	Kher	Kher	GUA	FUN	0.950311526	0.277323961	6.842991778
IA-46	IA-52	Kher	Kher	GUA	FUN	0.976014329	0.323685696	5.606634919
IA-47	IA-48	Kher	Kher	GUA	GUA	0.838744842	0.510918999	8.399082235
IA-47	IA-49	Kher	Kher	GUA	FUN	0.992134569	0.627051432	8.01994555
IA-47	IA-50	Kher	Kher	GUA	FUN	0.93234195	0.561105164	8.227242411
IA-47	IA-51	Kher	Kher	GUA	FUN	0.927041968	0.565229182	8.750477989
IA-47	IA-52	Kher	Kher	GUA	FUN	0.98699782	0.595153501	8.051770856
IA-48	IA-49	Kher	Kher	GUA	FUN	0.917679128	0.343391947	2.469795086
IA-48	IA-50	Kher	Kher	GUA	FUN	0.912637234	0.324313737	3.499205365
IA-48	IA-51	Kher	Kher	GUA	FUN	0.913409126	0.320032674	4.277810573
IA-48	IA-52	Kher	Kher	GUA	FUN	0.965350775	0.320435247	2.479203863

Table S4.4. Continued

ID1	ID2	Species1	Species2	SamplingSite1	SamplingSite2	Bray_Microbiome	Unifrac_Microbiome	Euclidean-Genetic
IA-49	IA-50	Kher	Kher	FUN	FUN	0.857799237	0.289972484	3.4972362
IA-49	IA-51	Kher	Kher	FUN	FUN	0.903426791	0.265835122	3.490370213
IA-49	IA-52	Kher	Kher	FUN	FUN	0.891441477	0.273146108	2.477453384
IA-50	IA-51	Kher	Kher	FUN	FUN	0.844584599	0.214542078	4.947230687
IA-50	IA-52	Kher	Kher	FUN	FUN	0.857598879	0.210590619	2.482715983
IA-51	IA-52	Kher	Kher	FUN	FUN	0.850424356	0.228206168	4.291681004

Table S4.5. Individual, sampling location and pairwise distances Bray-Curtis for DNA methylation and microbiome, Euclidean for genetic distance based on SNPs for 18 fish (14 *Kryotolebias hermaphroditus* and 4 *K. ocelatus*) living in sympatry.

ID1	ID2	SamplingSite1	SamplingSite2	Species1	Species2	Bray-Methylation	Bray-Microbiome	Euclidean-Genetic
IA_43	IA_47	GUA	GUA	Kher	Kher	0.035769717	0.361012658	7.624194531
IA_41	IA_43	GUA	GUA	Kher	Kher	0.033843627	0.374279034	5.53350868
IA_32	IA_33	GUA	GUA	Koce	Koce	0.039833898	0.43499902	40.83643708
IA_33	IA_41	GUA	GUA	Kher	Koce	0.046918458	0.491694092	37.04692629
IA_41	IA_47	GUA	GUA	Kher	Kher	0.03155525	0.51731391	8.394346355
IA_33	IA_34	GUA	GUA	Koce	Koce	0.033785039	0.554361468	42.06018624
IA_33	IA_43	GUA	GUA	Kher	Koce	0.051325468	0.561701794	35.50056133
IA_33	IA_47	GUA	GUA	Kher	Koce	0.054949938	0.586749677	35.50056133
IA_34	IA_41	GUA	GUA	Kher	Koce	0.051785465	0.595552087	36.0851135
IA_43	IA_45	GUA	GUA	Kher	Kher	0.035328461	0.645839018	21.46205431
IA_41	IA_45	GUA	GUA	Kher	Kher	0.035274796	0.650489222	21.55357842
IA_34	IA_45	GUA	GUA	Kher	Koce	0.05332876	0.653385366	35.00770223
IA_34	IA_43	GUA	GUA	Kher	Koce	0.052939766	0.663233458	35.84861438
IA_39	IA_40	GUA	GUA	Kher	Kher	0.039681711	0.664675801	12.49114519
IA_34	IA_35	GUA	GUA	Koce	Koce	0.035629022	0.690733726	44.6921557
IA_42	IA_43	GUA	GUA	Kher	Kher	0.036294137	0.698823346	4.623158607
IA_32	IA_34	GUA	GUA	Koce	Koce	0.033259851	0.704070879	44.20983618

Table S4.5. Continued

ID1	ID2	SamplingSite1	SamplingSite2	Species1	Species2	Bray-Methylation	Bray-Microbiome	Euclidean-Genetic
IA_40	IA_42	GUA	GUA	Kher	Kher	0.035461828	0.711613406	4.277810573
IA_41	IA_42	GUA	GUA	Kher	Kher	0.041147149	0.713762469	5.2458543
IA_42	IA_48	GUA	FUN	Kher	Kher	0.040077852	0.71414106	3.499698176
IA_33	IA_45	GUA	GUA	Kher	Koce	0.052827408	0.717308672	35.24607374
IA_42	IA_45	GUA	GUA	Kher	Kher	0.03730509	0.721657118	21.66746032
IA_34	IA_47	GUA	GUA	Kher	Koce	0.053148961	0.733983233	35.67331448
IA_45	IA_48	GUA	FUN	Kher	Kher	0.036660518	0.737629549	21.78036715
IA_45	IA_47	GUA	GUA	Kher	Kher	0.033492849	0.737805828	21.68932929
IA_43	IA_48	GUA	FUN	Kher	Kher	0.035254013	0.738012698	4.630970201
IA_33	IA_35	GUA	GUA	Koce	Koce	0.034836751	0.742540093	43.16744805
IA_39	IA_42	GUA	GUA	Kher	Kher	0.036957482	0.754867913	12.24377128
IA_35	IA_41	GUA	GUA	Kher	Koce	0.045964848	0.755197566	33.41224893
IA_32	IA_35	GUA	GUA	Koce	Koce	0.042343349	0.761354768	42.7678213
IA_41	IA_48	GUA	FUN	Kher	Kher	0.038470925	0.761520782	5.541318858
IA_35	IA_45	GUA	GUA	Kher	Koce	0.05359191	0.768804619	32.37862929
IA_35	IA_42	GUA	GUA	Kher	Koce	0.052961983	0.770619662	31.83994949
IA_34	IA_42	GUA	GUA	Kher	Koce	0.05584007	0.773209549	35.93723902
IA_40	IA_41	GUA	GUA	Kher	Kher	0.036874557	0.778981835	6.058242918

Table S4.5. Continued

ID1	ID2	SamplingSite1	SamplingSite2	Species1	Species2	Bray-Methylation	Bray-Microbiome	Euclidean-Genetic
IA_40	IA_43	GUA	GUA	Kher	Kher	0.030785407	0.781337699	5.24069722
IA_42	IA_44	GUA	GUA	Kher	Kher	0.047288125	0.789473684	7.010265754
IA_39	IA_41	GUA	GUA	Kher	Kher	0.035354412	0.78978182	11.35472064
IA_34	IA_48	FUN	GUA	Kher	Koce	0.056243549	0.796591662	35.93723902
IA_32	IA_41	GUA	GUA	Kher	Koce	0.059527032	0.798510663	42.45973703
IA_39	IA_43	GUA	GUA	Kher	Kher	0.041845922	0.801740624	11.61045674
IA_42	IA_46	GUA	GUA	Kher	Kher	0.051734849	0.810929771	5.852429493
IA_44	IA_48	GUA	FUN	Kher	Kher	0.04777325	0.810951862	7.652244584
IA_35	IA_48	FUN	GUA	Kher	Koce	0.053629509	0.815906433	31.92275834
IA_35	IA_43	GUA	GUA	Kher	Koce	0.053753588	0.815983463	31.0233447
IA_44	IA_45	GUA	GUA	Kher	Kher	0.043446724	0.817600748	22.08612137
IA_39	IA_46	GUA	GUA	Kher	Kher	0.054321604	0.822005462	11.31988231
IA_40	IA_48	GUA	FUN	Kher	Kher	0.035091966	0.825378891	4.28382465
IA_39	IA_45	GUA	GUA	Kher	Kher	0.037585918	0.825499688	20.51179368
IA_45	IA_46	GUA	GUA	Kher	Kher	0.043053374	0.828150573	21.2192267
IA_39	IA_44	GUA	GUA	Kher	Kher	0.03833418	0.837040506	13.01762716
IA_47	IA_48	GUA	FUN	Kher	Kher	0.032809831	0.838744842	8.399082235
IA_40	IA_46	GUA	GUA	Kher	Kher	0.043210437	0.841094182	5.579273541

Table S4.5. Continued

ID1	ID2	SamplingSite1	SamplingSite2	Species1	Species2	Bray-Methylation	Bray-Microbiome	Euclidean-Genetic
IA_42	IA_47	GUA	GUA	Kher	Kher	0.035903021	0.842283578	7.416672326
IA_43	IA_44	GUA	GUA	Kher	Kher	0.05615761	0.843210598	7.839930992
IA_33	IA_42	GUA	GUA	Kher	Koce	0.058147553	0.843948296	35.41512063
IA_50	IA_51	FUN	FUN	Kher	Kher	0.045403751	0.844584599	4.947230687
IA_46	IA_48	GUA	FUN	Kher	Kher	0.047198763	0.847196262	6.374140901
IA_32	IA_45	GUA	GUA	Kher	Koce	0.055091999	0.847917642	41.26903973
IA_39	IA_48	GUA	FUN	Kher	Kher	0.039806721	0.848170399	12.51582779
IA_33	IA_48	FUN	GUA	Kher	Koce	0.060042049	0.848846771	34.92221321
IA_51	IA_52	FUN	FUN	Kher	Kher	0.047025965	0.850424356	4.291681004
IA_40	IA_44	GUA	GUA	Kher	Kher	0.047271921	0.855177521	7.433409549
IA_40	IA_45	GUA	GUA	Kher	Kher	0.033360221	0.856056766	21.80829228
IA_50	IA_52	FUN	FUN	Kher	Kher	0.033873563	0.857598879	2.482715983
IA_49	IA_50	FUN	FUN	Kher	Kher	0.039124119	0.857799237	3.4972362
IA_41	IA_44	GUA	GUA	Kher	Kher	0.054223002	0.857849823	8.596725116
IA_32	IA_42	GUA	GUA	Kher	Koce	0.058445172	0.859208731	40.94160419
IA_32	IA_43	GUA	GUA	Kher	Koce	0.057040765	0.859576625	40.94160419
IA_34	IA_44	GUA	GUA	Kher	Koce	0.066465138	0.861127365	35.93723902
IA_44	IA_46	GUA	GUA	Kher	Kher	0.05874384	0.861483328	7.713727021

Table S4.5. Continued

ID1	ID2	SamplingSite1	SamplingSite2	Species1	Species2	Bray-Methylation	Bray-Microbiome	Euclidean-Genetic
IA_43	IA_46	GUA	GUA	Kher	Kher	0.040770323	0.862947524	5.854106647
IA_34	IA_39	GUA	GUA	Kher	Koce	0.053917639	0.865353808	36.58783884
IA_35	IA_39	GUA	GUA	Kher	Koce	0.048150688	0.86890625	31.83994949
IA_41	IA_46	GUA	GUA	Kher	Kher	0.049664777	0.869014688	6.370478654
IA_35	IA_47	GUA	GUA	Kher	Koce	0.047463136	0.870341494	30.86301627
IA_32	IA_48	FUN	GUA	Kher	Koce	0.057138229	0.873587406	40.50623059
IA_35	IA_44	GUA	GUA	Kher	Koce	0.062272854	0.874112784	31.10445163
IA_34	IA_40	GUA	GUA	Kher	Koce	0.05298501	0.880980254	35.84861438
IA_34	IA_49	FUN	GUA	Kher	Koce	0.055392564	0.885408947	35.84861438
IA_35	IA_40	GUA	GUA	Kher	Koce	0.054543368	0.887722857	32.00621667
IA_40	IA_51	GUA	FUN	Kher	Kher	0.037786735	0.889274165	5.528838395
IA_32	IA_47	GUA	GUA	Kher	Koce	0.05694865	0.890975686	40.31650482
IA_49	IA_52	FUN	FUN	Kher	Kher	0.038274317	0.891441477	2.477453384
IA_34	IA_50	FUN	GUA	Kher	Koce	0.06635413	0.894638331	36.11647823
IA_41	IA_49	GUA	FUN	Kher	Kher	0.031294817	0.900962325	5.536628786
IA_32	IA_49	FUN	GUA	Kher	Koce	0.05984973	0.901083483	40.31650482
IA_41	IA_50	GUA	FUN	Kher	Kher	0.052162082	0.901608694	5.819186227
IA_49	IA_51	FUN	FUN	Kher	Kher	0.026998553	0.903426791	3.490370213

Table S4.5. Continued

ID1	ID2	SamplingSite1	SamplingSite2	Species1	Species2	Bray-Methylation	Bray-Microbiome	Euclidean-Genetic
IA_42	IA_51	GUA	FUN	Kher	Kher	0.034279115	0.904116525	4.945838865
IA_33	IA_49	FUN	GUA	Kher	Koce	0.054926982	0.905377355	34.75309738
IA_35	IA_46	GUA	GUA	Kher	Koce	0.069016683	0.908289792	33.50292005
IA_44	IA_49	GUA	FUN	Kher	Kher	0.056435425	0.91243378	7.645744021
IA_48	IA_50	FUN	FUN	Kher	Kher	0.041834479	0.912637234	3.499205365
IA_39	IA_51	GUA	FUN	Kher	Kher	0.030301472	0.912694078	12.13185668
IA_34	IA_46	GUA	GUA	Kher	Koce	0.06643636	0.912711104	36.95759562
IA_48	IA_51	FUN	FUN	Kher	Kher	0.031575802	0.913409126	4.277810573
IA_48	IA_49	FUN	FUN	Kher	Kher	0.030175465	0.917679128	2.469795086
IA_32	IA_50	FUN	GUA	Kher	Koce	0.06694322	0.919264339	40.60210346
IA_42	IA_50	GUA	FUN	Kher	Kher	0.048927821	0.922271116	3.918304141
IA_33	IA_44	GUA	GUA	Kher	Koce	0.070886886	0.922362803	34.23820822
IA_33	IA_50	FUN	GUA	Kher	Koce	0.0673381	0.922393203	34.32243518
IA_32	IA_44	GUA	GUA	Kher	Koce	0.065918883	0.922429251	40.94160419
IA_39	IA_49	GUA	FUN	Kher	Kher	0.041619196	0.922590714	12.62901815
IA_47	IA_51	GUA	FUN	Kher	Kher	0.026886424	0.927041968	8.750477989
IA_34	IA_52	FUN	GUA	Kher	Koce	0.064057356	0.929031	36.57657931
IA_43	IA_51	GUA	FUN	Kher	Kher	0.032759965	0.930043236	5.248808047

Table S4.5. Continued

ID1	ID2	SamplingSite1	SamplingSite2	Species1	Species2	Bray-Methylation	Bray-Microbiome	Euclidean-Genetic
IA_47	IA_50	GUA	FUN	Kher	Kher	0.051992163	0.93234195	8.227242411
IA_39	IA_50	GUA	FUN	Kher	Kher	0.060289463	0.932750819	12.41182049
IA_45	IA_50	GUA	FUN	Kher	Kher	0.047368524	0.932834658	21.58476579
IA_35	IA_50	FUN	GUA	Kher	Koce	0.067319688	0.933182598	31.92275834
IA_41	IA_51	GUA	FUN	Kher	Kher	0.032193812	0.933465779	6.551951442
IA_44	IA_50	GUA	FUN	Kher	Kher	0.064272277	0.9373783	7.030158716
IA_34	IA_51	FUN	GUA	Kher	Koce	0.054311386	0.937448816	35.93723902
IA_46	IA_50	GUA	FUN	Kher	Kher	0.05115673	0.938088934	5.308951036
IA_44	IA_47	GUA	GUA	Kher	Kher	0.03938621	0.938546616	9.113042203
IA_40	IA_49	GUA	FUN	Kher	Kher	0.036676304	0.938900362	4.282017764
IA_40	IA_52	GUA	FUN	Kher	Kher	0.042192115	0.939148389	3.509598332
IA_42	IA_49	GUA	FUN	Kher	Kher	0.039755925	0.93915787	3.496744428
IA_42	IA_52	GUA	FUN	Kher	Kher	0.04860397	0.939483625	3.039831914
IA_45	IA_51	GUA	FUN	Kher	Kher	0.038700822	0.940076366	21.83974807
IA_43	IA_49	GUA	FUN	Kher	Kher	0.03008983	0.940161284	4.628361939
IA_40	IA_50	GUA	FUN	Kher	Kher	0.044325624	0.941485839	3.503648175
IA_32	IA_39	GUA	GUA	Kher	Koce	0.058843152	0.943073559	41.17427718
IA_41	IA_52	GUA	FUN	Kher	Kher	0.048262735	0.943130916	5.272619037

Table S4.5. Continued

ID1	ID2	SamplingSite1	SamplingSite2	Species1	Species2	Bray-Methylation	Bray-Microbiome	Euclidean-Genetic
IA_39	IA_47	GUA	GUA	Kher	Kher	0.024750091	0.943204868	11.61373052
IA_32	IA_40	GUA	GUA	Kher	Koce	0.05503473	0.94361474	41.13427162
IA_35	IA_49	FUN	GUA	Kher	Koce	0.051936634	0.944474772	31.75778174
IA_45	IA_49	GUA	FUN	Kher	Kher	0.038443057	0.944587328	21.76150696
IA_35	IA_52	FUN	GUA	Kher	Koce	0.06601349	0.945579292	32.52109502
IA_43	IA_50	GUA	FUN	Kher	Kher	0.042398316	0.94617332	4.294107053
IA_33	IA_39	GUA	GUA	Kher	Koce	0.056156624	0.946796406	35.41512063
IA_33	IA_40	GUA	GUA	Kher	Koce	0.054659184	0.947021944	35.67331448
IA_39	IA_52	GUA	FUN	Kher	Kher	0.059061033	0.948041816	12.18178577
IA_46	IA_51	GUA	FUN	Kher	Kher	0.051207076	0.950311526	6.842991778
IA_32	IA_52	FUN	GUA	Kher	Koce	0.063937664	0.951137781	41.19199543
IA_33	IA_46	GUA	GUA	Kher	Koce	0.065162351	0.954255277	37.140835
IA_32	IA_46	GUA	GUA	Kher	Koce	0.069704873	0.954711981	42.56367779
IA_44	IA_51	GUA	FUN	Kher	Kher	0.047352373	0.958560523	8.229572085
IA_33	IA_52	FUN	GUA	Kher	Koce	0.062963361	0.958573163	35.53416339
IA_40	IA_47	GUA	GUA	Kher	Kher	0.034698648	0.959484203	8.198287431
IA_43	IA_52	GUA	FUN	Kher	Kher	0.039069309	0.960350536	4.646712922
IA_46	IA_47	GUA	GUA	Kher	Kher	0.048890508	0.961140098	8.469880809

Table S4.5. Continued

ID1	ID2	SamplingSite1	SamplingSite2	Species1	Species2	Bray-Methylation	Bray-Microbiome	Euclidean-Genetic
IA_46	IA_49	GUA	FUN	Kher	Kher	0.048971843	0.962533105	6.368649896
IA_35	IA_51	FUN	GUA	Kher	Koce	0.047458627	0.964210526	31.83994949
IA_45	IA_52	GUA	FUN	Kher	Kher	0.044008512	0.965014804	21.40521291
IA_48	IA_52	FUN	FUN	Kher	Kher	0.041799779	0.965350775	2.479203863
IA_32	IA_51	FUN	GUA	Kher	Koce	0.05808675	0.974125166	40.22263623
IA_33	IA_51	FUN	GUA	Kher	Koce	0.05467108	0.975643184	34.75309738
IA_46	IA_52	GUA	FUN	Kher	Kher	0.046765871	0.976014329	5.606634919
IA_44	IA_52	GUA	FUN	Kher	Kher	0.06275269	0.982397383	6.819530023
IA_47	IA_52	GUA	FUN	Kher	Kher	0.050660662	0.98699782	8.051770856
IA_47	IA_49	GUA	FUN	Kher	Kher	0.031560562	0.992134569	8.01994555

Table S4.6. Results from non-parametric regression analyses of microbiome alpha diversity analyses. A) Species and location as predictors (N=42), B) Species, location and individual heterozygosity as predictors, species removed due to collinearity (N=28, see Table S4.1.)

Chao1- lm(formula = chao1 ~ Species + Location + SL, data = Alpha_diversity_noNA)

term	mean_boot_p	med_boot_p	p.value
(Intercept)	0.339	0.258	0.306
LocationFLO	0.412	0.379	0.502
LocationFUN	0.598	0.638	0.982
LocationGUA	0.366	0.296	0.362
LocationPIC	0.578	0.622	0.882
LocationSFR	0.433	0.41	0.847
SL	0.402	0.367	0.49
SpeciesKoce	0.0765	0.0073	0.0136

Chao1- lm(formula = chao1 ~ Location + H.indiv, data = Alpha_div_He)

term	mean_boot_p	med_boot_p	p.value
(Intercept)	0.472	0.457	0.812
H.indiv	0.0886	0.011	0.0205
LocationFUN	0.604	0.631	0.941
LocationGUA	0.121	0.0392	0.0637
LocationSFR	0.217	0.0333	0.0325

Table S4.6. Continued

Faith_pd- lm(faith_pd ~ Species + Location + SL,

data = Alpha_diversity_noNA)

term	mean_boot_p	med_boot_p	p.value
(Intercept)	0.378	0.324	0.379
LocationFLO	0.479	0.472	0.865
LocationFUN	0.544	0.564	0.854
LocationGUA	0.212	0.0966	0.128
LocationPIC	0.55	0.588	0.937
LocationSFR	0.411	0.385	0.618
SL	0.467	0.454	0.882
SpeciesKoce	0.0502	0.0034	0.0057

Faith_pd- lm(faith_pd ~ Location + H.indiv,

data = Alpha_div_He)

term	mean_boot_p	med_boot_p	p.value
(Intercept)	0.412	0.36	0.418
H.indiv	0.0556	0.0048	0.0083
LocationFUN	0.464	0.442	0.493
LocationGUA	0.127	0.0496	0.0663
LocationSFR	0.163	0.0294	0.0327

Table S4.6. Continued

Shannon- lm(formula = shannon ~ Species + Location + SL,

data = Alpha_diversity_noN

A)

term	mean_boot_p	med_boot_p	p.value
(Intercept)	0.0037	0	0
LocationFLO	0.503	0.523	0.854
LocationFUN	0.469	0.457	0.67
LocationGUA	0.351	0.274	0.302
LocationPIC	0.394	0.326	0.391
LocationSFR	0.329	0.238	0.343
SL	0.0915	0.0147	0.0211
SpeciesKoce	0.38	0.323	0.512

Shannon- lm(shannon ~ Location + SL, data = Alpha_div_He)

term	mean_boot_p	med_boot_p	p.value
(Intercept)	0	0	0
LocationFUN	0.251	0.138	0.175
LocationGUA	0.317	0.24	0.236
LocationSFR	0.0275	0.0028	0.0038
SL	0.0279	0.001	0.0018

Table S4.6. Continued

Simpson- lm(formula = simpson_e ~ Species + Location + SL,

data = Alpha_diversity_n

oNA

term	mean_boot_p	med_boot_p	p.value
(Intercept)	0.0094	0.0006	0.0009
LocationFLO	0.567	0.584	0.648
LocationFUN	0.31	0.237	0.23
LocationGUA	0.0056	0.0004	0.0006
LocationPIC	0.0977	0.0459	0.0526
LocationSFR	0.349	0.286	0.277
SL	0.203	0.11	0.124
SpeciesKoce	0.473	0.463	0.587

Simpson- lm(simpson_e ~ Location + H.indiv + SL, data = Alpha_div_He)

term	mean_boot_p	med_boot_p	p.value
(Intercept)	0.0065	0.0002	0.0003
H.indiv	0.222	0.179	0.182
LocationFUN	0.325	0.227	0.251
LocationGUA	0.0161	0.0006	0.001
LocationSFR	0.561	0.577	0.976
SL	0.238	0.151	0.151

Table S4.7. Results of two-way ANOVA analysis of fluctuating asymmetry including side (left or right) and individual as factors, and F-ratio tests of significance, following Palmer (1994) and Tocts et al. (2016).

Ocellus

Source	Df	SS	MS
Side (DA)	1	0.029	0.0286
Individual (among individuals)	20	20.96	1.0483
Side:Individual (FA)	20	0.536	0.0268
Error	41	0.398	0.0097

Significance test	F ratio	F ratio	df num	df denom	P
FA relative to masurement error	2.76	0.0268/0.0097	20	41	0.003
DA	1.07	0.0286/0.0268	1	20	0.313
Among individuals	39.12	1.0483/0.0268	20	20	<0.0001

Pupil

Source	Df	SS	MS
Side (DA)	1	0.031	0.030
Individual (among individuals)	20	1.756	0.088
Side:Individual (FA)	20	0.561	0.028
Error	41	0.489	0.012

Significance test	F ratio	F ratio	df num	df denom	P
FA relative to masurement error	2.35	0.028/0.012	20	41	0.01
DA	1.09	0.030/0.028	1	20	0.308
Among individuals	3.13	0.088/0.028	20	20	0.007

Table S4.8. Repeat measurements, fluctuating asymmetry, and corrected values (following Torcs et al 2016) for three morphometric traits (ocellus area, distance from eye to snout and eye diameter) of *Kryptolebias hermaphroditus* and *K. ocellatus* in sympatric locations (FUN and GUA).

			1st measurement						2nd measurement						FA1			FA2			FA1-FA2			FA1 corrected			FA2 corrected		
Fish ID	**Species**	**Location**	**Left pupil diameter (mm)**	**Right pupil diameter (mm)**	**Distance from left eye to snout (mm)**	**Distance from right eye to snout (mm)**	**Area of left ocellus (mm^2)**	**Area of right ocellus (mm^2)**	**Left pupil diameter (mm)**	**Right pupil diameter (mm)**	**Distance from left eye to snout (mm)**	**Distance from right eye to snout (mm)**	**Area of left ocellus (mm^2)**	**Area of right ocellus (mm^2)**	**Signed FA Pupil**	**Signed FA snout**	**Signed FA ocellus**	**Signed FA Pupil**	**Signed FA snout**	**Signed FA ocellus**	**Signed FA Pupil**	**Signed FA snout**	**Signed FA ocellus**	FAcorr Pupil	FAcorr Snout	FAcorr Ocellus	FAcorr Pupil	FAcorr Snout	FAcorr Ocellus
IA-11	Koce	FLO	0.725	0.800	1.350	1.350	0.912	0.799	0.775	0.800	1.000	1.250	0.813	0.760	-0.075	0.000	0.113	-0.025	-0.250	0.053	-0.050	0.250	0.060	0.000	0.166	0.126	0.000	0.121	0.114
IA-12	Koce	FLO	0.550	0.700	0.850	1.100	0.473	0.478	0.550	0.675	0.750	0.875	0.412	0.645	-0.150	-0.250	-0.005	-0.125	-0.125	-0.233	-0.025	-0.125	0.228	0.072	0.000	0.008	0.105	0.000	0.172
IA-13	Koce	FLO	0.725	0.650	0.700	0.700	0.176	0.433	0.500	0.750	0.700	0.900	0.304	0.537	0.075	0.000	-0.257	-0.250	-0.200	-0.233	0.325	0.200	-0.024	0.133	0.166	0.244	0.235	0.013	0.172
IA-18	Koce	FLO	0.300	0.550	1.500	0.600	0.221	0.222	0.450	0.575	0.950	0.425	0.227	0.232	-0.250	0.900	-0.001	-0.125	0.525	-0.005	-0.125	0.375	0.004	0.179	1.097	0.012	0.105	0.594	0.056
IA-19	Koce	FLO	0.750	0.625	1.200	0.600	0.483	0.591	0.650	0.750	1.300	0.550	0.506	0.518	0.125	0.600	-0.108	-0.100	0.750	-0.012	0.225	-0.150	-0.096	0.185	0.795	0.095	0.077	0.823	0.049
IA-23	Koce	FLO	0.750	0.525	1.700	0.700	0.681	0.570	0.775	0.600	1.300	0.475	0.528	0.531	0.225	1.000	0.111	0.175	0.825	-0.003	0.050	0.175	0.114	0.287	1.198	0.124	0.180	0.898	0.058
IA-25	Koce	SFR	0.900	1.100	0.700	1.550	1.671	2.114	1.000	1.200	0.600	1.100	1.293	1.644	-0.200	-0.850	-0.443	-0.200	-0.500	-0.351	0.000	-0.350	-0.092	0.127	0.635	0.430	0.184	0.402	0.290
IA-26	Koce	SFR	0.550	1.200	0.550	1.150	1.059	1.224	0.700	0.900	1.425	0.675	0.865	1.082	-0.650	-0.600	-0.165	-0.200	0.750	-0.217	-0.450	-1.350	0.052	0.583	0.378	0.152	0.184	0.823	0.156
IA-32	Koce	GUA	0.950	1.075	1.150	2.800	1.593	1.868	1.075	1.000	1.350	2.700	1.554	1.854	-0.125	-1.650	-0.275	0.075	-1.350	-0.300	-0.200	-0.300	0.025	0.039	1.442	0.262	0.073	1.263	0.239
IA-33	Koce	GUA	0.875	0.800	1.500	1.700	2.091	1.942	1.100	0.650	1.600	1.625	1.810	1.762	0.075	-0.200	0.149	0.450	-0.025	0.048	-0.375	-0.175	0.101	0.133	0.000	0.162	0.459	0.000	0.109
IA-34	Koce	GUA	0.700	0.750	1.025	1.500	2.122	1.725	0.550	0.675	0.800	1.425	1.890	1.648	-0.050	-0.475	0.397	-0.125	-0.625	0.242	0.075	0.150	0.155	0.000	0.244	0.410	0.105	0.531	0.303
IA-40	Kher	GUA	0.650	0.700	0.450	1.250	0.367	0.528	0.800	0.500	0.600	1.000	0.383	0.457	-0.050	-0.800	-0.161	0.300	-0.400	-0.074	-0.350	-0.400	-0.087	0.000	0.585	0.148	0.307	0.296	0.013
IA-44	Kher	GUA	0.550	0.650	0.800	1.350	0.574	0.523	0.625	0.750	0.600	0.750	0.435	0.499	-0.100	-0.550	0.051	-0.125	-0.150	-0.064	0.025	-0.400	0.115	0.000	0.326	0.064	0.105	0.000	0.003
IA-45	Kher	GUA	0.700	0.850	0.825	1.675	0.808	0.772	0.875	0.600	0.650	0.950	0.667	0.757	-0.150	-0.850	0.036	0.275	-0.300	-0.090	-0.425	-0.550	0.126	0.072	0.635	0.049	0.282	0.184	0.029
IA-46	Kher	GUA	0.675	0.650	0.925	1.000	0.362	0.403	0.650	0.675	0.900	0.925	0.378	0.364	0.025	-0.075	-0.041	-0.025	-0.025	0.014	0.050	-0.050	-0.055	0.079	0.050	0.028	0.000	0.000	0.075
IA-47	Kher	GUA	0.875	0.550	1.675	0.650	0.500	0.512	0.600	0.700	1.325	0.650	0.466	0.456	0.325	1.025	-0.012	-0.100	0.675	0.010	0.425	0.350	-0.022	0.388	1.223	0.001	0.077	0.747	0.071
IA-48	Kher	GUA	0.650	1.025	1.100	2.025	1.000	0.921	0.750	1.075	0.925	1.625	1.013	1.215	-0.375	-0.925	0.079	-0.325	-0.700	-0.202	-0.050	-0.225	0.281	0.306	0.712	0.092	0.311	0.608	0.141
IA-49	Kher	FUN	0.800	0.700	1.100	1.500	0.714	0.751	0.800	0.700	0.900	1.150	0.748	0.833	0.100	-0.400	-0.037	0.100	-0.250	-0.085	0.000	-0.150	0.048	0.159	0.157	0.024	0.101	0.121	0.024
IA-50	Kher	FUN	0.950	0.850	1.350	1.300	0.949	0.684	0.900	0.850	1.300	1.125	1.065	0.819	0.100	0.050	0.265	0.050	0.175	0.246	0.050	-0.125	0.019	0.159	0.224	0.278	0.041	0.227	0.307
IA-51	Kher	FUN	1.150	1.250	1.600	1.150	0.932	0.932	0.850	0.775	1.325	0.975	0.882	0.910	-0.100	0.450	0.000	0.075	0.350	-0.028	-0.175	0.100	0.028	0.000	0.643	0.013	0.073	0.414	0.033
IA-52	Kher	FUN	0.750	0.900	0.900	1.575	0.692	0.663	0.800	0.800	0.775	1.625	0.712	0.702	-0.150	-0.675	0.029	0.000	-0.850	0.010	-0.150	0.175	0.019	0.072	0.456	0.042	0.000	0.760	0.071

Figure S4.9. Predicted community metagenomic profiling using PICRUSt2 v2.5.2 displaying the 20 most abundant MetaCyc pathways represented across all communities.

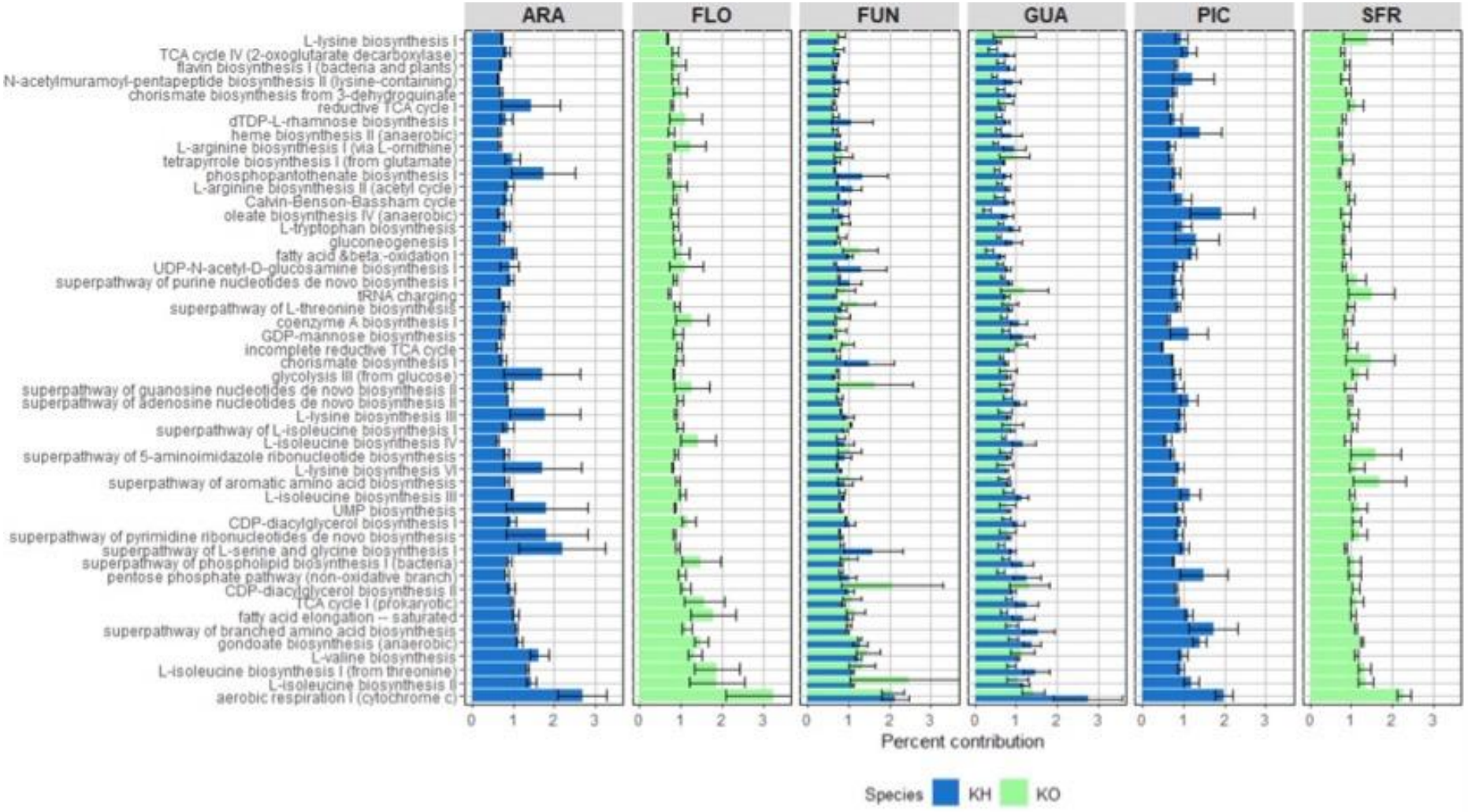


Figure S4.10. Comparison of raw data and predicted CIs for non-parametric bootstrapped and linear models of microbiome alpha diversity.

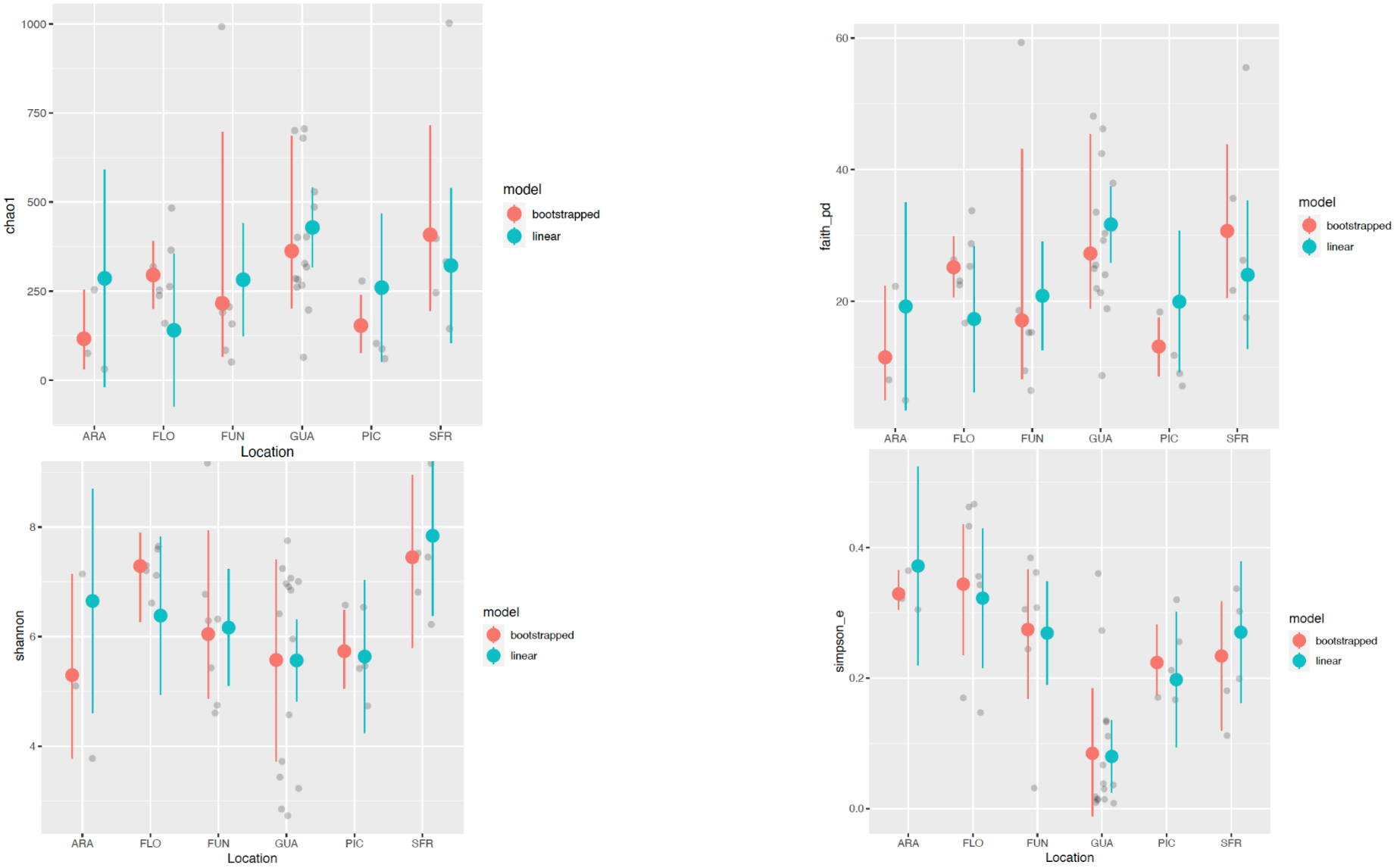


Figure S4.11. Microbiome alpha diversity estimates for *Kryptolebias hermaphroditus* and *K. ocellatus* in sympatry or allopatry.

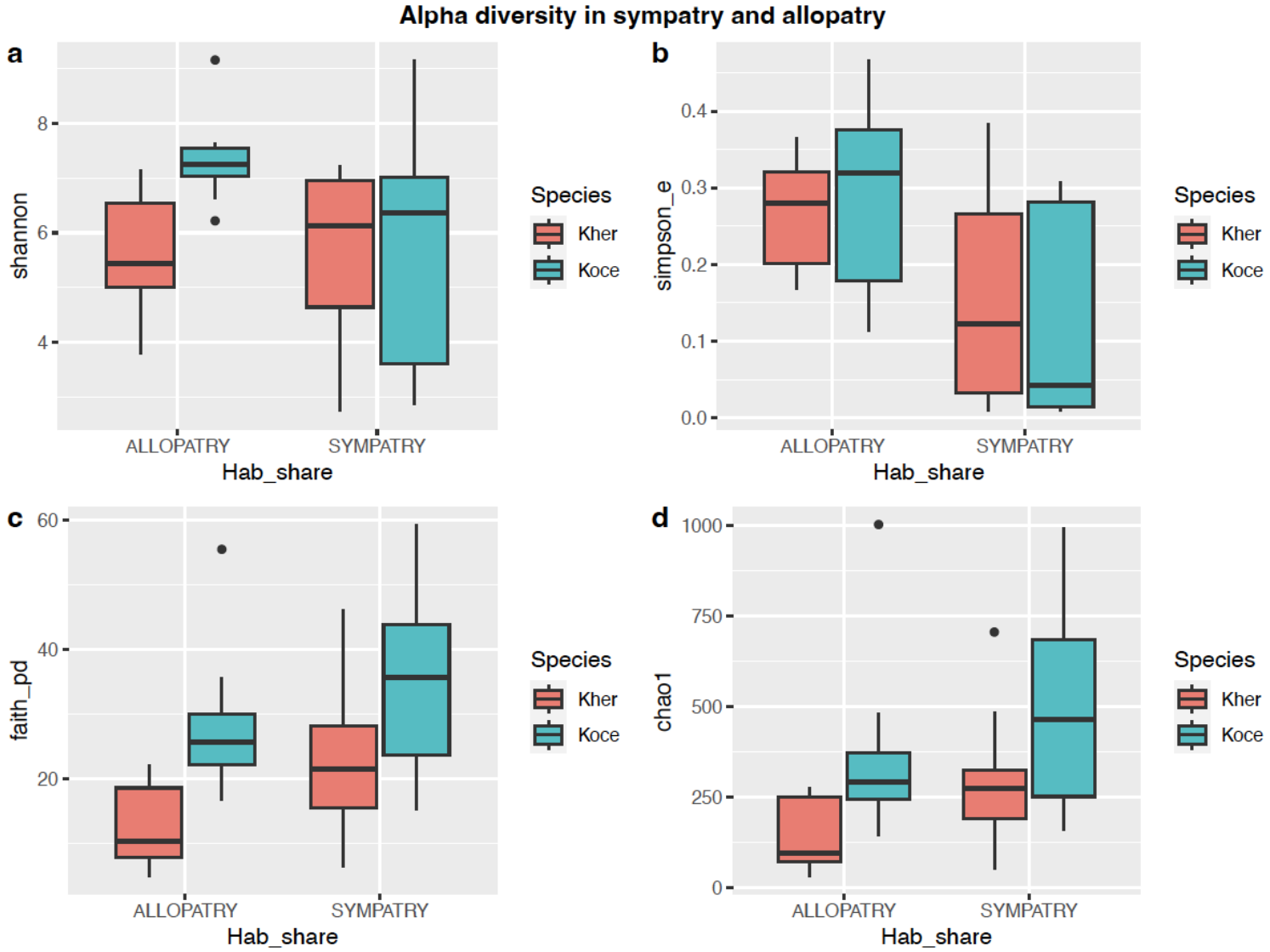


Figure S4.12. Heterozygosity estimates for *Kryptolebias hermaphroditus* and *K. ocellatus* in sympatry and allopatry.

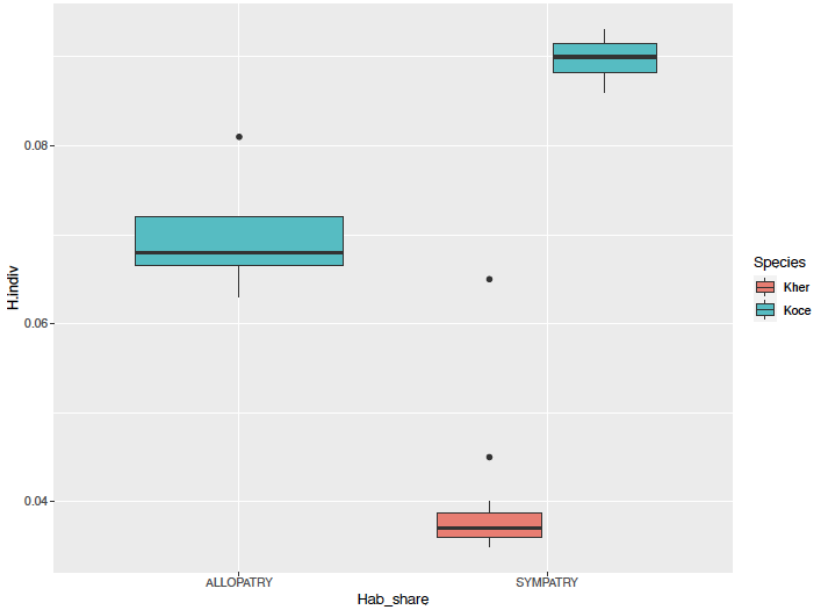


Figure S4.13. Relationship between genetic (Euclidean distance) and microbiome dissimilarity (Bray-Curtis and weighted Unifract distance).

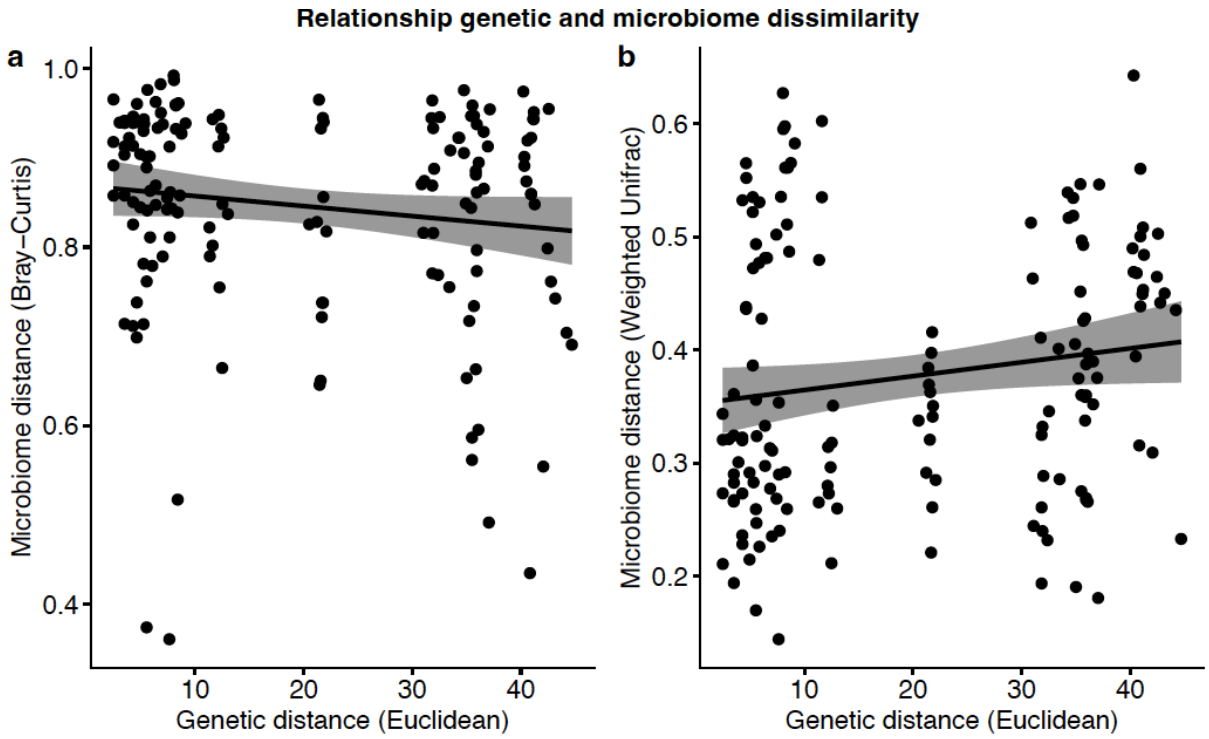
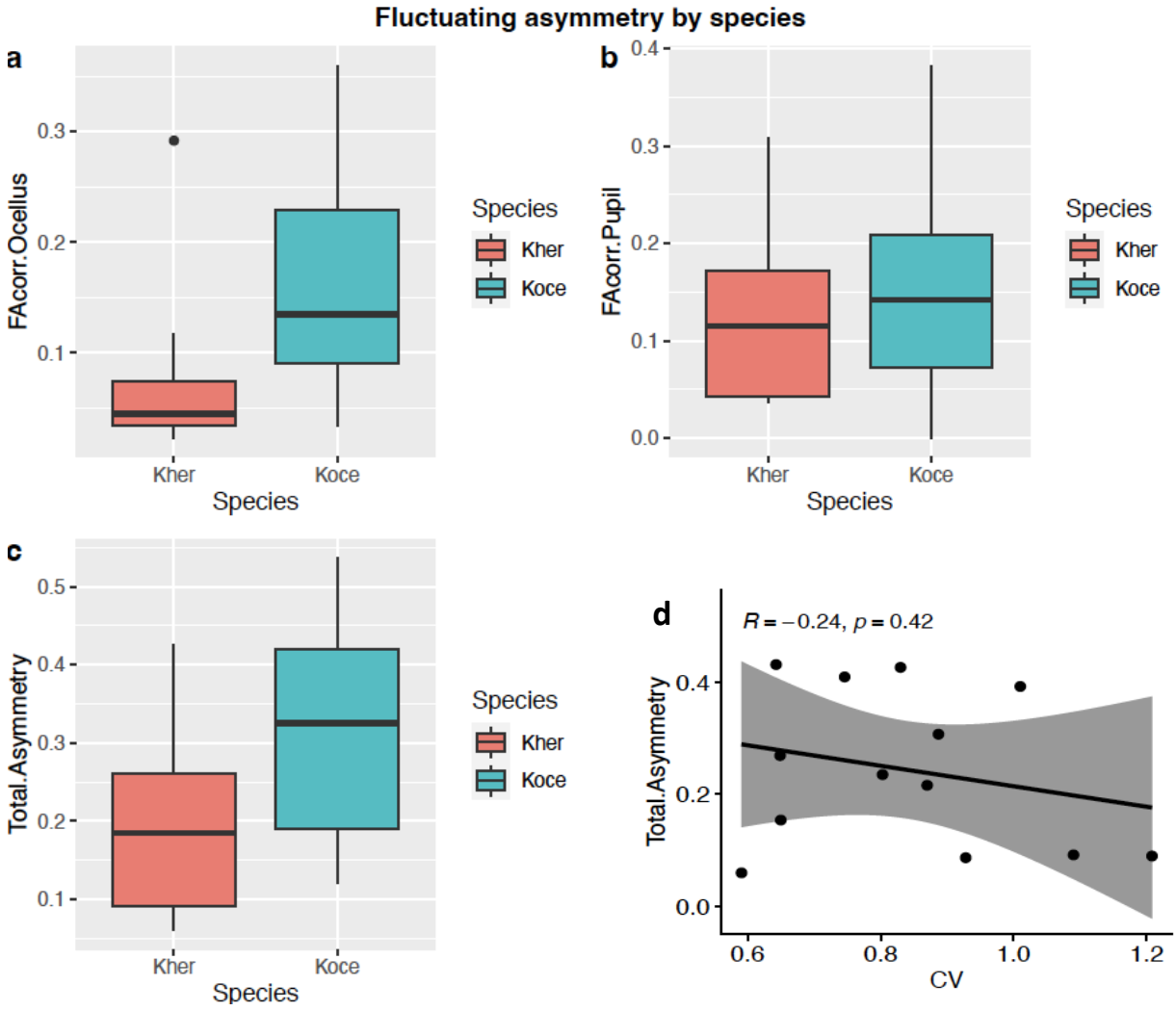


Figure S4.14. Fluctuating asymmetry in ocellus area, eye diameter and both combined (total asymmetry) in *Kryptolebias hermaphroditus* and *K. ocellatus* (a-c) and relationship between total asymmetry and epigenetic diversity (methylation coefficient of variation, CV) (d).



X. APPENDIX II: PUBLISHED MANUSCRIPT

Published manuscript

Anka, I. Z., Uren Webster, T. M., Berbel-Filho, W. M., Hitchings, M., Overland, B., Weller, S., de Leaniz, C. G. & Consuegra, S. (2024). Microbiome and epigenetic variation in wild fish with low genetic diversity. *Nature Communications*, 15(1), 4725.

XI. APPENDIX III: PUBLISHED BOOK CHAPTER

11

Microbiome, Epigenetics and Fish Health Interactions in Aquaculture

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11.1 Introduction

Increasing understanding of the critical role of the microbiome (the complex community of endogenous microorganisms present in the gut and other mucosal surfaces, and their genes; Box 11.1) for human health has changed our perspective of health, disease, well-being, nutrition, and even behavior [1]. The Human Microbiome Project, launched in 2007 [2], helped uncover the bidirectional interaction between host and microbiome, and is opening the door to personalized medicine and nutrition [3]. More recently, the capacity of the microbiome to epigenetically regulate host gene expression, and the influence of environmental factors (like diet) on microbiome composition and function has further contributed to the development of precision medicine [4]. Following this trend, research on the microbiome in aquaculture has also increased exponentially for the last 10 years, with a large focus on disease, nutrition, and probiotics (Box 11.1), reflecting the growing importance of the fish-farming industry and the need for alternatives to both antimicrobials and fishmeal [5] to make its intensification sustainable. Epigenetics research in aquaculture has also increased in importance over the same period, although the integration of both epigenetics and microbiome is severely lacking compared to research in mammals and terrestrial model organisms (Figure 11.1), in part due to the much larger diversity of fish species, habitats, and diets. Recent reviews have comprehensively summarized the current knowledge about the fish microbiome and its importance for aquaculture [6–10]. This chapter, therefore, highlights the potential of combining research on microbiome and epigenetics for aquaculture in terms of sustainability and production.

11.2 The Fish Microbiome in Aquaculture

Fish represent 20% of the daily animal protein intake for a growing human population [11] and are key for the food security of millions of people. Thus, the importance of the aquaculture sector has grown continuously for the last 40 years [12]. However, aquaculture-intensive rearing conditions often result in high levels of stress in fish due to confinement, crowding, handling, and environmental mismatch, which can impair immunocompetence and increase disease susceptibility [13]. Increasing awareness of the consequences of antimicrobial resistance, as well as concerns regarding fish welfare, has prompted a search for alternative disease management approaches that allow aquaculture sustainable growth with a lower ecological footprint. As a result, there has been an exponentially increasing interest in the role of the fish microbiome on the health and performance of cultured fish over the last 10 years, particularly in the use of alternatives to fishmeal and fish oil, dietary additives, and pre/probiotics aiming to enhance growth, performance, and disease resistance [8, 14].

Epigenetics in Aquaculture, First Edition. Edited by Francesc Piferrer and Han-Ping Wang.

Box 11.1 Definitions Used in this Chapter

Alpha and beta microbiome diversity: Two measures used to describe organisms' microbiome diversity individually (alpha) or as community heterogeneity between two samples (beta). Alpha diversity measures include richness (number of taxa) or distribution (evenness) while beta diversity quantifies the differences in the overall taxonomic composition between samples. Phylogenetic information can be included in measures of both alpha and beta diversity.
Core microbiome: A group of microbial taxa commonly associated with a host taxon, usually present in a defined proportion of individuals and/or above a given abundance.
DNA methylation: The addition of methyl groups to cytosine bases in DNA, most often at CpG dinucleotides. DNA methylation in regulatory and coding regions alters associated gene transcription. Bacterial metabolites are known to alter the activity and availability of enzymes and cofactors essential for the regulation of host DNA methylation.
Dysbiosis: Alteration of the microbiome homeostasis with modifications in composition, diversity, or function (e.g., a reduction in diversity or an imbalance in the community composition with dominance of particular taxa). Dysbiosis modifies microbiome metabolic activity and results in changes in host disease susceptibility and/or maladaptation.
Fish microbiome: The fish microbiota and their genomes found in a particular environment.
Fish microbiota: The community of microorganisms (bacteria, viruses, eukaryotes, and protists) associated with the gastrointestinal tract, skin, gills, and olfactory mucosal tissues of fish. They can be resident (permanently associated with the organism) or transient (temporarily associated).
Gnotobiotic fish: Fish that are germ-free (axenic) or derived from germ-free eggs and colonized by well-characterized microbial lineages used as models for microbiome experiments. Comparisons between gnotobiotic fish and those conventionally raised (exposed to normal microbiota) are used to understand the mechanisms of host-microbiome interactions.
Histone modification: Posttranslational modifications to histone proteins that collectively alter chromatin state and the accessibility of genes for transcription. Metabolites of microbial origin (such as SCFAs) induce modifications in the chromatin structure of the host animal.
Microbiome engineering: Selection of the microbiome to modify its function and improve host performance. This can be done by indirect selection or by artificial selection applied to the host [91]. It is a very promising, but still underused, avenue to increase resilience to stress and disease in aquaculture.
Microbiome-epigenome crosstalk: A complex series of interactions mediated by microbial and host gene expression and metabolite production. Together, metabolites of both microbial and host origin regulate aspects of the host epigenome and transcriptome and the microbiome.
Noncoding RNA regulation: Collectively various ncRNAs (including micro RNAs and long noncoding RNAs) that regulate gene expression at transcriptional and posttranscriptional levels. Host ncRNAs are differentially expressed in the presence of microbiota and their metabolites.
Prebiotic: Nondigestible food ingredient (dietary fiber) that favors the growth of particular groups of commensal microorganisms that exert beneficial effects on the host's health or well-being.
Probiotic: Feed supplements consisting of live microorganisms that confer nutritional and immunological benefits to the host by manipulating their microbial community. The use of probiotics has increased in aquaculture as substitutes for antimicrobials.
SFCAs: Short-chain (less than six carbon atoms) fatty acids produced by commensal bacteria in the intestine by fermenting carbohydrates and fiber that play a role in the host neuroendocrine and immune regulation, potentially through epigenetic mechanisms.

11.2.1 The Fish Microbiome Diversity and Composition

Animals are associated with a diverse community of microorganisms that colonize all body surfaces exposed to environmental factors, including skin, respiratory, and gastrointestinal systems, influencing their immunity, extending their metabolic repertoire, and affecting their nutritional status, behavior [15], and ultimately fitness [16]. In vertebrates, the microbiome influences both host physiological activity and health through the release of metabolites [17], including neurotransmitters [18], hormones [19], vitamins [20], and short-chain fatty acids (SFCAs). As for other vertebrates, the fish microbiota (Box 11.1) consists of a community of bacteria, viruses, eukaryotes, and protists associated with mucosal tissues in the gastrointestinal tract [21], skin [22], and gills [23]. The diversity and composition of the gut microbiome, and

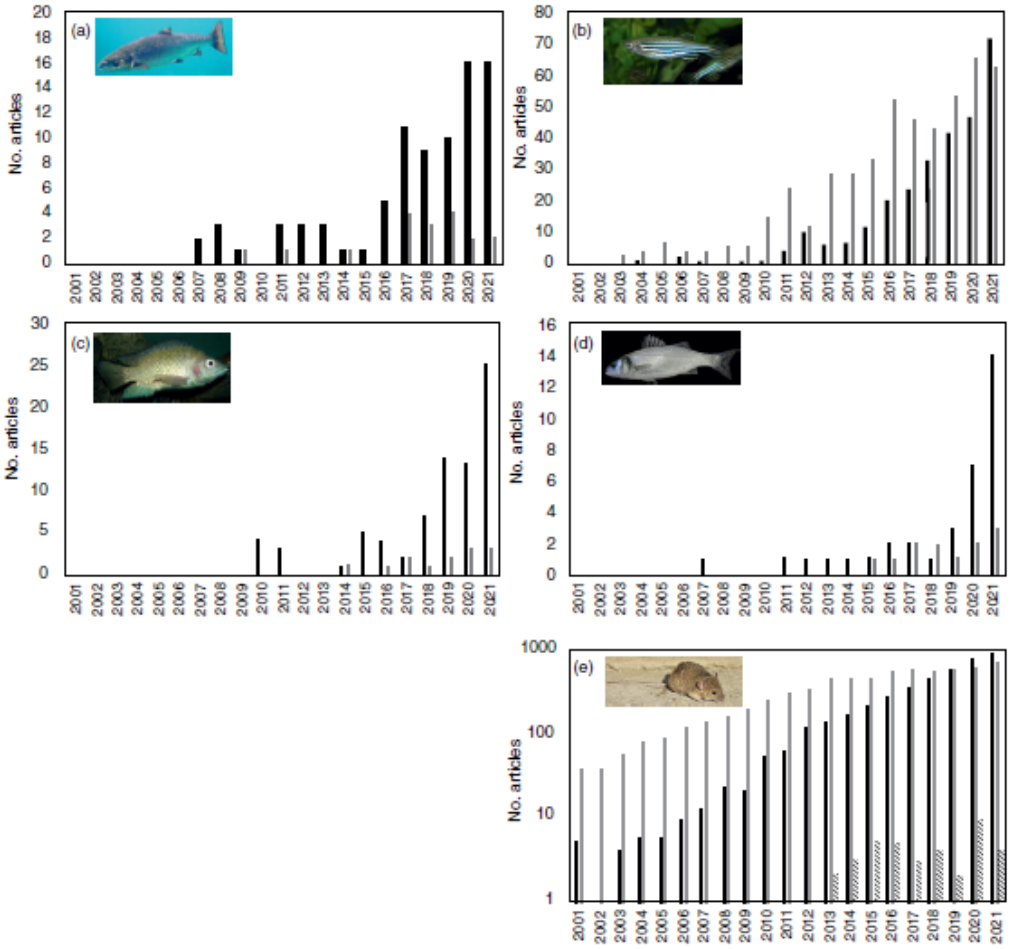

Figure 11.1 Number of articles published on the microbiome or epigenome for select species up until the end of 2021. (a) Atlantic salmon *Source:* Hans-Petter Fjeld/Wikimedia Commons/CC BY SA 2.5, (b) zebrafish *Source:* Azul/Wikimedia Commons/public domain, (c) Nile tilapia *Source:* Bjørn Christian Tørrissen/Wikimedia Commons/CC BY SA 3.0, (d) European sea bass *Source:* Hans Hillewaert/Wikimedia Commons/CC BY SA 4.0, and (e) mouse *Source:* Amirekul/Wikimedia Commons/CC BY SA 4.0. Searches were conducted in PubMed (3 November 2021) for terms in the Title or Abstract as follows: (((Common name) OR (Species name)) AND ((Microbiome) OR (Microbiota))); (((Common name) OR (Species name)) AND ((Epigenome) OR (Epigenetic))); and (((Common name) OR (Species name)) AND ((Microbiome) OR (Microbiota)) AND ((Epigenome) OR (Epigenetic))). Review articles were excluded. Black: microbiome, Gray: epigenome; Hatched: microbiome and epigenome.

increasingly that of the skin and gills, has been described in many fish species. An influence on immunity and nutrient absorption is widely assumed, although the mechanisms underneath this bidirectional relationship are largely still to be determined [21].

Compared to terrestrial animals, the outer mucosal surfaces (gills and skin) of fish and their food items are constantly in contact with water, which provides increased opportunities for microbial colonization [21]. Fish are also much more diverse than any other vertebrate group, in a number of species, habitat conditions, and diet adaptations, potentially contributing to microbiome diversity as well. Most of the available information refers to a reduced number of model species with genome

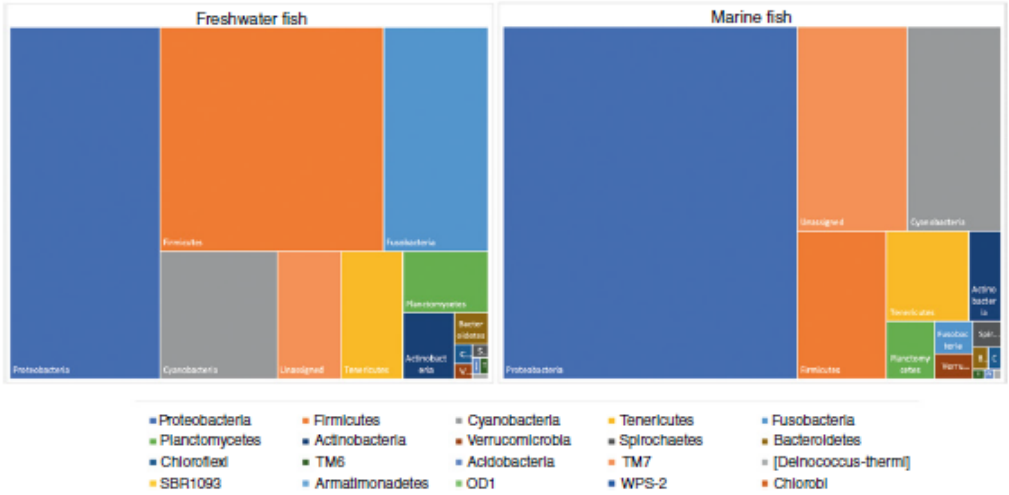

Figure 11.2 Relative abundance of different bacterial taxa in the gut microbiota of fish from freshwater and marine habitats based on data from 14 orders, 42 families, 79 genera, and 85 species. *Source:* Adapted from data in Kim et al. [35].

availability, e.g., zebrafish (*Danio rerio*), three spine stickleback (*Gasterosteus aculeatus*) [24], or species of economic value (mainly salmonids and some cyprinids like carps [25], although the number of studies exploring wild fish populations (e.g., [26–28]) and additional aquaculture species (e.g., [29, 30]) is increasing. Fish microbiome research has been largely focused on marine fish [31], predominantly on their symbiotic bacteria [32]. Current studies on freshwater fish literature refer to less than 1% of freshwater fish species, predominantly on Cyprinidae, followed by Salmonidae and Cichlidae, reflecting the most common and economically important aquaculture species including common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and Nile tilapia (*Oreochromis niloticus*) [25]. Different fish organs (gut, skin, gills) have specialized microbiome compositions, with the skin being more influenced by water bacterioplankton and abiotic factors than the gut, which tends to be mainly determined by diet and life stage.

Despite the diversity of species and environmental conditions, 16S rRNA sequencing has identified that Proteobacteria, Firmicutes, and *Bacteroidetes* represent up to 90% of the gut microbiota in most species [33]. This is also supported by a comparison of the gut microbiome of 12 wild fish and three shark species that identified the dominant phyla including Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Planctomycetes, Proteobacteria, and Tenericutes [34], and by a broad species comparison of freshwater and marine fish [35] (Figure 11.2). In contrast with the mammalian gut, which is dominated by Firmicutes and Bacteriodetes, Proteobacteria predominate in the fish gut, with Firmicutes and Bacteriodetes in much lower abundance [34]. *Terenicutes*, typically *Mycoplasma* sp., have commonly been found to dominate the gut microbiome of adult farmed salmonids, although their role in the microbial community is largely unknown [36]. Also, in contrast to mammals and birds, the dominant indigenous lactic acid-producing bacteria in fish include *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, *Carnobacterium*, *Weissella*, and *Pediococcus* [37], but not *Bifidobacterium*. Some of these, such as bacteria from the genus *Carnobacterium* [38], are common in healthy fish and widely used as probiotics because of their beneficial effects on gut health and ability to inhibit the growth of fish pathogens [39, 40], but pathogenic species from the genera *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Carnobacterium*, and *Lactococcus* have been identified to cause mortalities in aquaculture settings and need to be controlled [37].

11.2.2 Extrinsic and Intrinsic Factors that Affect Fish Microbiome Composition

The abundance and composition of the fish microbiome are affected by environmental factors including temperature, season, and diet, as well as by the host genetics [23], although the species-specific core gut microbial communities seem largely resilient to the rearing environment [41]. Here, we summarize some of the main extrinsic and intrinsic factors influencing the composition of the fish microbiome, with particular emphasis on aquaculture conditions.

11.2.2.1 Extrinsic Factors (Habitat, Diet, and Stress)

A meta-analysis of 12 fish species representing different habitats (marine, freshwater, or estuarine) and trophic levels (herbivorous, carnivorous, and omnivorous) identified marked differences in gut microbiota composition determined both by *salinity* and type of diet, and also by the method of sampling, although not by the fish-rearing environment (laboratory, aquaculture, or natural habitat) [26]. A comprehensive study including 14 orders, 42 families, 79 genera, and 85 fish species identified fish habitat as the major determinant of the gut microbiome composition, differing significantly from that of other vertebrates [35]. Habitat-related gut microbiome differences between marine and freshwater fish can also be identified in single species, for example, in gnotobiotic (colonized only by known microorganisms or germ-free; Box 11.1) threespine stickleback (*Gasterosteus aculeatus*) [42]. In this case, while marine and freshwater sticklebacks shared the core intestinal microbiota (consisting of 12 different genera from the Proteobacteria, Firmicutes, and Actinobacteria phyla), marine fish mounted a stronger immune response to resident microbiota compared to their freshwater counterparts, potentially reflecting the adaptation to the less environmentally variable freshwater environment [42]. Temperature also has an important effect on the diversity of the fish gut microbiota in salmonids [43, 44] and seems to affect alpha diversity (individual) and, particularly, beta diversity (community differentiation) of fish skin communities, which can be also influenced by dissolved oxygen [45].

Diet strongly influences the microbiome; a varied diet is consistent with a more varied gut microbiome, with omnivorous species having the highest richness and phylogenetic diversity [34]. This is also reflected in the metabolic role of the microbiome, as the functional profile of the gut microbiome in the omnivorous freshwater pufferfish (*Tetraodon cutcutia*) was similar in protein metabolism to carnivorous salmon, but to herbivorous grass carp in carbohydrate metabolism [46]. This has important implications for aquaculture. On the one hand, wild individuals seem to have a more diverse microbiome than their captive-bred counterparts, although sharing a core microbiome, as seen both in zebrafish [41] and mummichogs (*Fundulus heteroclitus*) [34]. On the other hand, in addition to the reduction of disease susceptibility and the search for alternatives to antimicrobials, the sustainability of aquaculture also depends on finding alternatives to the use of fishmeal and fish oil in commercial feeds, particularly for carnivorous species. Plant-derived components, soybean or grains, and more recently microalgae and yeast, have been used as alternatives or supplements to cover the fish's nutritional needs [30] but some of them cause enteropathy and inflammation of the fish intestine [47], as well as potential imbalance in the microbiome [30], which can have unpredictable effects. Soybean, for example, alters the proportion of *Bacteroidetes* in the gut, which can be compensated for by using amino acid supplementation with alanine-glutamine that reduces the relative abundance of *Bacteroides* spp., and the inflammation effects. While Ala-Glu supplementation can alleviate the inflammatory effects of soybean, decrease the expression of the proinflammatory cytokine (TNF-α), and enhance the digestive and absorptive ability, it also reduces the microbiome diversity, which could result in the loss of the barrier effect provided by the microbiome [48]. The diet can also have a synergic effect with other aquaculture conditions, as shown in the yellowtail kingfish (*Seriola lalandi*), where the substitution of fish meal for soya protein concentrate combined with housing at high temperature increased the abundance of *Photobacterium* sp. in the skin, affecting growth and immune defense mechanisms [49].

The *aquaculture environment* results in differences in the fish microbiome compared to the natural environment of the species, despite the maintenance of the core microbiome in many cases. In the yellowtail kingfish, for example, Proteobacteria was the most abundant phylum in the wild while Firmicutes was the most abundant phylum in the aquaculture-reared populations, with differences between both rearing environments related to amino acid and carbohydrate metabolism, strongly linked to the different diets [29]. Marked differences were also found in the skin and mostly the gut of wild and hatchery Atlantic salmon, with the hatchery fish displaying not only different composition, but also lower bacterial diversity, as could be expected from farming conditions consisting of less variable diet and environment [50]. However, a temporal comparison of the water and biofilm of semiclosed containment systems (S-CCS) in the sea and on land recirculating aquaculture systems (RAS) for Atlantic salmon identified changes in the microbiota between both types of systems, depending on salinity, and importantly found reservoirs of potential pathogens that could affect the health and performance of the fish held in those systems [51].

Fish farming intensification is often associated with increased *stress*, not only mainly due to crowding and confinement, but also handling or disruption of social behaviors. Stress impairs immunocompetence, both in wild and farmed fish [52], and can have long-lasting effects on the immune system, potentially increasing susceptibility to disease [53, 54]. Early life stages are particularly sensitive, reflecting developmental plasticity during the maturation of the vertebrate nervous and immune system, as well as periods of epigenetic reprogramming and microbiome assembly [55]. The microbiome–brain–gut axis is essential for maintaining fish homeostasis through the previously mentioned interaction between mutualistic gut microbes and the host physiological processes, modulating immunoregulation and maintaining metabolic

equilibrium [56]. The fish microbiome has been shown to be disrupted by a number of different aquaculture-related stressors. Given the importance of the microbiome to many aspects of host health, dysbiosis (i.e., disruption of the microbiome homeostasis; Box 11.1) of the host-microbiome relationship is likely to compound the adverse impacts of stress directly on the animal. An increase in cortisol has been related to changes in the diversity and structure of the fish gut microbiome [57], which may, in turn, contribute to the adverse effects of stress on animal health [56]. Stress disrupts microbial communities associated both with the gut and skin [58, 59], but with different levels of resilience. The skin microbiome seems less impacted by changes in localized cortisol concentration [57], while the gut microbiome recovers more quickly from pH stress [60]. Different types of stress, acute and chronic, also have different responses in the gut and skin microbiome. While acute stress during late embryogenesis has been shown to have a lasting effect on the skin microbiome, likely due to disruption of initial seeding communities, the effect is not so clear on the gut microbiome, while chronic stress seems to have a more pronounced effect in the gut [61]. This suggests that the indigenous microbiota can be a good indicator of physiological stress and potential immunocompromise [62]. Although it is difficult to generalize among species and conditions, changes in the abundance of particular microorganisms could also be used as indicators of stress. For example, in Atlantic salmon, an increase in the levels of fecal cortisol resulting from experimental stress was associated with significant changes in the gut microbiome (although not in the skin), in particular with the pronounced decline of the *Carnobacterium* sp. [57], while in the Eurasian perch (*Perca fluviatilis*), both predator and low food ration stress resulted in an increase in *Fusobacteria*, particularly *Cetobacterium* [59]. In salmon, some microbiome changes in response to acute and chronic stress have common signatures (e.g., increases in representatives from *Acinetobacter* and *Aeromonas*, which include several important opportunistic fish pathogens), which could be useful as general microbial biomarkers of stress [57].

However, the microbiome is also involved in the host's adaptation to stress [63]. For example, temporal changes in the microbiome of the milkfish (*Chanos chanos*) subjected to high temperatures, which correlated to changes in the hepatosomatic index (HSI, as a proxy for fish energy status), were attributed to habituation to high-temperature stress, potentially mediated by microbiome effects [64]. Osmotic stress in the Japanese rice fish (*Oryzias melastigma*) indicated that the resulting changes in the microbiota induced the production of different metabolites and the activation of different metabolic pathways that helped the resident bacteria survive the stress, and potentially maintain the biological function of the gut [65], contributing to the resilience of the host. The gut microbiome has been hypothesized to play a role in the response to crowding stress in the snout bream (*Megalobrama amblycephal*), for which high densities affected not only the growth but also the composition and abundance of the gut microbiome and the metabolites it produced [66]. Effects of the microbiome seem to be generalized even in distant taxa. Thus, the microbiome, together with epigenetic adaptation, has also been proposed to aid in the transgenerational acclimatization of coral reef organisms to environmental stress due to climate change, though changes in abundance that could be selected and vertically transmitted [67].

11.2.2.2 Intrinsic Factors (Host Genetics, and Age)

The influence of host genetics on the microbiome is variable and depends on the host species, the organ analyzed, and the environmental conditions where species live [27]. The individual genotype has been shown to influence the composition of the gut microbiome, and also to some extent the skin and gill microbiome [58, 68]. In brook trout (*Salvelinus fontinalis*), for example, three QTLs (quantitative trait loci) were found to have a major effect on the abundance of three genera in the skin, *Lysobacter*, *Rheinheimera*, and *Methylobacteriumin*, all were involved in the production of antimicrobial compounds. The QTLs explained between 17 and 41% of the variance in the abundance of the three genera, with *Methylobacteriumin* presence being negatively correlated with the pathogen *Flavobacterium psychrophilum*, indicating a role of the host genetics in controlling the microbiome composition in relation to its own fitness [58]. However, there seems to be weak evidence in fish of microbiota phylosymbiosis (higher intraspecific than interspecific similarity in the structuring of the microbial communities) [69, 70]. Thus, while in Amazonian fishes, the gut (but not the skin) microbiome is mainly modulated by species-specific factors [27], the gut microbiome composition of species from the *Gadidae* and *Sparidae* families displays a larger influence of environmental factors (such as diet or temperature) than of their phylogenetic relationship [70, 71].

The microbiome colonization of the gut is species-specific and initially originates from the egg chorion, then is influenced by the surrounding water and, strongly, by first feeding [31, 72]. Afterward, changes in the microbiome composition are observed with *age* and the development of the immune system, although some interact with physiological changes induced by migration, for example. In wild Arctic char (*Salvelinus alpinus*), the main driver of gut microbiota changes seems to be seasonal salinity changes, but an influence of age was also found in fish samples at the same time [73]. Similarly,

a comparison of chinook salmon (*Oncorhynchus tshawytscha*) farmed under freshwater and marine conditions revealed higher microbial diversity in freshwater, but also an effect of fish age on the beta diversity in both the marine and freshwater environments [74].

11.2.3 Microbiome Interaction with Fish Health and Immunity

Aquatic species are, by nature, often exposed to pathogenic and opportunistically pathogenic microorganisms in the surrounding water [75]. Interactions between the host and its microbiome play a key role in maintaining long-term fish health [76]. This is because, not only does the composition of the microbiota affect the host immune response and its susceptibility to disease, but environmental conditions, diet, health, and the genetic background of the host also influence their microbiome diversity. In mammals, the normal functioning of the innate and adaptive immune systems has been related to the maintenance of healthy intestinal microbiota, potentially through epigenomic mechanisms involving host epithelial and immune cells, and the microbiome [77]. However, the understanding of the functional interplay between host immunity and the microbiome in fish is more restricted, despite increasing information about fish microbiota composition [78]. The disruption of the host-microbiome functional relationship (dysbiosis) has been associated with human chronic diseases [79], and interactions between innate and adaptive immune responses and the gut microbiome are increasingly well-known in humans and rodents [20]. In mice, for example, certain strains of *Clostridium* that are part of the intestine indigenous microbiota control the number and function of $CD4^+$ regulatory T cells, which are known to be needed for maintaining immune homeostasis in mice and humans [80]. Similarly, in zebrafish, the disruption of the intestinal microbiome by chemical exposure to plastic-derived phthalates (DEHP) suggests a link between the microbiome and the adaptive immune mechanisms in the host intestine. Exposure to DEHP increased Bacteroidetes and Gammaproteobacteria and decreased Verrucomicrobia in both the male and female gastrointestinal system, as well as resulted in other sex-specific microbiome changes. These alterations could modify their metabolite production, potentially mediating the response of the adaptive immune system to DEHP, which includes increasing Th1/Th2 immune response [81]. The microbiome of the skin and gills can also reflect gut health. For example, the dysbiosis produced by enteritis in the yellowtail kingfish (*S. lalandi*) results in a reduction in diversity and an enrichment in Proteobacteria and Actinobacteria, mainly associated with the early stages of the disease [82]. Resident bacteria are essential for maintaining fish individual health, by creating an environment that excludes pathogens through competitive exclusion mechanisms [83, 84], such as the exclusion of *Aeromonas* by lactic acid bacteria in salmonids [85]. Resident microbiota also protect the host against the development of inflammatory diseases. Research in germ-free zebrafish indicates that colonization by commensal microorganisms in newly hatched fish induces proinflammatory and antiviral gene expression, increasing the resistance to viral infections [86]. One of the mechanisms for this interaction is through microbial-derived SCFAs that are produced by commensal bacteria in the intestine by fermenting carbohydrates and fiber, such as acetate and propionate produced by the *Bacteroidetes* phylum. These SCFAs can bind and activate the G-protein-coupled receptor GPR43, which in humans and mice is highly expressed in neutrophils and eosinophils, and whose expression is closely linked to receptors important for innate immunity [87]. This relationship between gut microbiome and immunity can be exploited to develop preventive and therapeutic measures against fish disease [84]. For example, it has been suggested that this SCFA–GPR43 pathway can be related to the effects of diet and pre- and probiotics on the immune response [87].

11.2.4 Microbiome Engineering

The extensive knowledge on the relationship between microbiome and human health has paved the way for potential applications of microbiome engineering in farmed animal systems that have the advantage of diet and genetic diversity being more standardized and susceptible to be manipulated [88]. This can be particularly important to reduce the use of antibiotics in intensive aquaculture, as many antimicrobial drugs currently used have been classified as critically important or highly important for human use by the World Health Organization and there is a strong positive correlation between human clinical antimicrobial resistance and aquaculture-derived multi-antibiotic resistance indices [89]. An increase in awareness about the risks of antimicrobial resistance has played an important role in the development of new measures of disease control and prevention, including microbiome engineering [90, 91]. While vaccination has been effective in reducing antimicrobial use for the Norwegian salmon industry [92], vaccines are not available for many diseases and are unsuitable when animals are infected by multiple opportunistic pathogens [92]. Modulation of the microbiome

(and epigenome; see later) is already being recognized as mediating lasting adverse effects of stress on immunity, offering a promising way of promoting animal resilience to stress and disease, but this has yet to be widely exploited in fish farming [93, 94]. Nutritional additives, such as pre- and probiotics, are increasingly used to enhance the immune system, improve growth, and can be used for disease prevention by enhancing animal resilience to disease and stress [95]. Probiotics (live exogenous microorganisms) that enhance the composition of resident communities can be used to maintain host health along with prebiotics (nondigestible food ingredients that favor the growth of particular groups of commensal microorganisms), although in fish, the detailed knowledge about their mechanisms of action is still limited [96]. Probiotics applied to feeds colonize the animal intestinal tract, ensuring varied benefits to the host [97]. For example, the intake of probiotics improves the digestibility of nutrients through enhanced enzymatic activity. Moreover, probiotic bacteria can compete with potentially pathogenic microorganisms for nutrients and adhesion sites within the gut of the host, being able to positively modulate the microbial community of the gut. Besides that, a diverse range of probiotic species can produce antimicrobial molecules, minimizing the growth of pathogenic bacteria, stimulating an immune response, and increasing fish resistance against diseases. Probiotics have proved to be beneficial for the fish immune response by helping modified microbiota to return to their normal status and also by activating the complement system or modulating the secretion and anti-inflammatory cytokines [84].

Probiotics are also effective at minimizing the stress effect of handling [98] and can enhance growth [99], representing a strong and bio-secure alternative to the use of antibiotics for the industry [100]. The growing interest in probiotics within the aquaculture industry has been extensively reviewed (see References [95, 101, 102] for examples). Some common probiotics like *Bacillus* and *Lactobacillus* can stimulate the expression of inflammatory cytokines in the fish gut [103] or increase phagocytic activity and resistance against particular pathogens [104]. Thus, the use of host-associated probiotics has been identified as one of the promising avenues to avoid dependence on those derived from nonaquatic sources (e.g., *Bacillus*, *Lactobacillus*, *Enterococcus*, and *Carnobacterium*) [101].

11.3 Microbiome-Epigenome Interactions

The link between environmentally induced epigenetic modifications, the microbiome composition and function, and how they can influence the host remains an open question in fish [105]. Early-life acute stress has been shown to have stimulatory effects on immunity [54], and modify the epigenome in a developmental-stage-specific manner [54, 106]. There is also growing evidence that epigenetic modifications, either through the nutrition of the parents or during early development, can affect fish growth, muscle development, and meat quality, suggesting that epigenetic programming could be applied to enhance production and disease resistance in aquaculture [107]. Moreover, it is now clear that the farming environment results in rapid epigenetic changes that can affect more than one generation [108]. Thus, the same environmental factors that affect the microbiome (temperature, diet, host genotype, and age) are influencing the epigenome, with consequences for the health and performance of the host, suggesting a common ground for incorporating microbiome and epigenetic engineering in management practices to improve animal fitness as a promising solution to achieve sustainable aquaculture intensification [109] (Figure 11.3).

11.3.1 Mammals and Model Species

Characterizing the interactions between the microbiome and the epigenome in humans and mammalian models is an active area of current research. However, the complexity of these interactions means that much is still unknown, with new links constantly being identified. The predominant focus has been on the impacts of gut microbiota, and their metabolites, on the epigenome and the epitranscriptome of different tissues (including gut, brain, and liver) in the host animal, although epigenetic regulation of microbiome composition and function is also well-documented [110–112], and recent evidence of epigenetic changes within microbiotas themselves is increasing [113]. Together, a highly complex landscape of interactions between the microbiome and the epigenome and transcriptome is being uncovered, with metabolites of both bacterial and host origin regulating the crosstalk between host epigenome/transcriptome and the microbiome [112, 114, 115]. Diet fundamentally influences the nature of these interactions in mammals, and the disruption of this crosstalk is clearly implicated in the etiology of many diseases, particularly metabolic and inflammatory-type conditions [110, 116–118]. Recent reviews cover how the microbiome interacts with the host epigenome via chromatin remodeling, DNA methylation, and noncoding RNAs in detail, and identify key knowledge gaps [111, 119, 120].

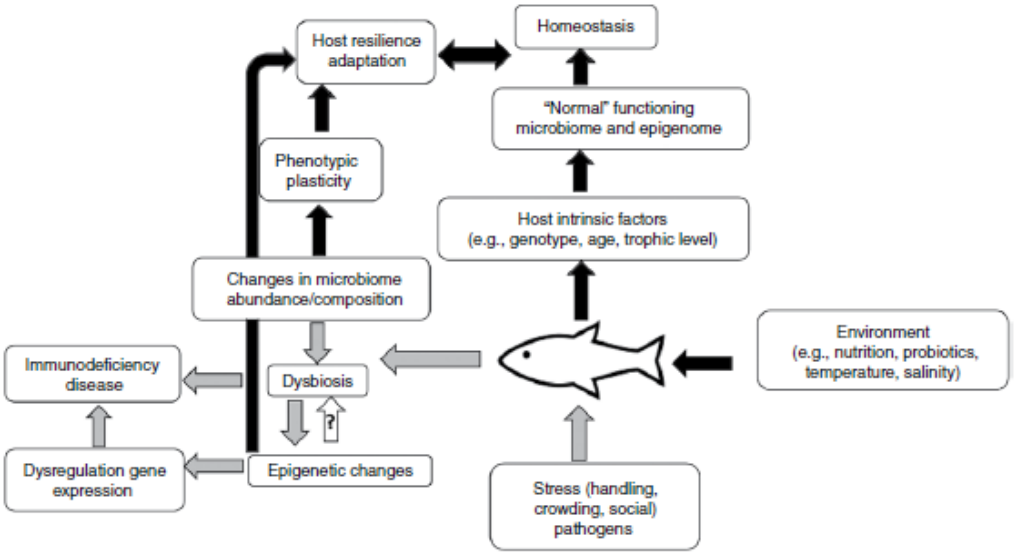

Figure 11.3 Schematic representation of the different host-related extrinsic (environmental) and intrinsic factors affecting the fish microbiome in aquaculture settings, the potential interaction with the epigenome and the health consequences for the host. Gray/black arrows indicate negative and positive impacts on the host's health respectively.

11.3.1.1 Histone Modifications

Perhaps the best understood and most widely characterized interaction is the modification of histones by microbial metabolites [121]. This reflects not only a long history of study, but also more opportunities for interaction with diverse microbial metabolites. In particular, microbial SCFA production has been well documented to alter histone acetylation via the inhibition of histone deacetylases (HDACs). SCFAs, including acetate, propionate, and butyrate, are produced through the breakdown of complex carbohydrates by gut bacteria, including *Bifidobacterium* sp., a common probiotic member of human microbiota [122]. SCFAs have diverse and important impacts on many aspects of host physiology, including energy metabolism, tissue proliferation, and inflammatory responses, with histone modifications thought to be one of the key ways that SCFAs induce these physiological impacts [121]. Using gnotobiotic models, dietary-induced microbial SCFA production has been shown to alter histone acetylation in the gut, and more recently, in the liver and adipose tissues, where it has been associated with considerable changes in the transcription of metabolic genes [123]. Manipulation of microbiota in rodent models has been used to demonstrate the impact of microbial-derived SCFAs on other histone modifications too, with histone crotonylation appearing to be a particularly important, recently discovered mechanism for microbiome-host crosstalk in the gut [119, 121]. Changes in histone methylation also demonstrate how gut microbes can induce epigenetic changes associated with the induction of inflammatory conditions in the intestine [124]. Apart from SCFAs, intestinal microbiota secrete a suite of other metabolites, some of which are also known to directly influence chromatin modification. These include folate, which is synthesized by bacteria and needed for methylation of histones and DNA [125], and some polyphenols that alter host HDAC and histone acetylase (HAT) activity whilst also regulating microbial presence [115]. HDACs also play an important role in regulating the presence of gut bacteria, with HDAC-null mice showing considerably altered microbiomes, as well as intestinal epithelial integrity [126]. The stimulation of histone acetylation of the FoxP3 locus mediated by SCFAs produced by the microbiota of the large intestine seem to be also involved in T-cells' anti-inflammatory effects [77].

11.3.1.2 DNA Methylation

DNA methylation patterns have also been strongly associated, in some cases causally, with the occurrence of various microbiota (pathogenic and commensal) within the intestinal microbiome. For example, in gnotobiotic mice, the presence of

commensal microbiota induces genome-wide changes in DNA methylation, including localized changes at TET2/3-dependent regulatory regions that are necessary for the proper expression of a suite of developmental genes crucial for the normal development and maintenance of intestinal homeostasis [127]. In humans, microbiota composition, especially ratios of Bacteroidetes to Firmicutes, has been associated with widespread differential methylation of genes involved in glucose and energy metabolism and obesity [128]. As with histone modifications, it appears that microbial regulation of DNA methylation is due to either alteration in the availability of chemical donors or modulation of enzymes responsible for methylation [111]. S-Adenosylmethionine (SAM) is the main methyl-donating substrate for methylation enzymes, DNA methyltransferases (DNMTs), and histone methyltransferases (HMTs). Its generation is dependent on microbially derived molecules including folate and other B vitamins. Modulation of diet and microbiota alters folate availability for SAM production and, hence, DNA methylation. This has been well associated with obesity and metabolic-type diseases [118, 129]. Recently, stimulation of folate production by *Bacteroides* sp., and subsequent SAM availability, has been shown to alleviate hepatic steatosis in rats [130]. Alteration of DNMT activity has also been shown to be directly associated with microbial metabolism of a number of bioactive dietary compounds, including some flavonoids and polyphenols [131].

11.3.1.3 Noncoding RNAs

Host microbiome interactions mediated by noncoding RNAs (ncRNA) have recently received considerable attention. Microbial regulation of micro-RNAs (miRNAs) and long noncoding RNAs (lncRNAs) has been the most widely documented [110, 120]. Germ-free and conventional rodent models have been used to show that both miRNAs and lncRNAs are differentially expressed in the presence of gut microbiota; however, while some studies identified specific bacteria involved, the exact mechanisms of this microbial regulation are not well known [110, 132–134]. Disruption of the microbiome, and subsequent dysregulation of miRNAs and lncRNAs, have also been implicated in several diseases including obesity, diabetes, and colon cancer [110, 134, 135]. Diet-induced changes in the microbiome are also known to modulate host miRNAs, which are differentially expressed in humans with omnivorous, vegetarian, and vegan diets [136]. The role of circular RNAs (circRNAs), which "sponge" and inhibit the activities of miRNAs, in modulating the interactions between miRNAs and microbiota adds an additional layer of complexity to this crosstalk [120]. In the brain, interactions between circRNA, miRNA, and circulating microbial metabolites have been studied to some extent and implicated in several neurological disorders, but this has been identified as a considerable knowledge gap and priority for future research [120]. In addition, the potential role of other ncRNAs in mediating host-microbiome crosstalk is yet to be widely explored. A recent study, using gnotobiotic models, was the first to demonstrate how tRNA expression profiling is remodeled in a tissue-specific manner in the presence of microbiota [137]. Few studies have examined the impacts of endogenously expressed host ncRNAs on microbiota. However, a landmark study by Liu et al. [138] showed that miRNAs secreted by gut epithelial cells enter and regulate gene expression in gut bacteria, modulating their abundance.

11.3.2 Microbiome and Epigenetic Interactions in Aquaculture

Independently, the role of the microbiome and the epigenome on fish health, nutrition, and development are active foci of research in aquaculture. It is becoming clear that both the host-associated microbiome and the epigenome are sensitive to environmental factors, including diet, stress, and other conditions experienced in aquaculture, and that both are likely to have an extensive impact on animal health and productivity. Emerging research based on mammalian models suggests that these interactions may be extensive and help to explain previously unaccountable complexity.

Studies on diet and nutritional conditioning constitute a major focus for aquaculture research. Both the microbiome and the epigenome are considered key mechanisms by which diet can be used to promote animal health, welfare, and productivity. Perhaps surprisingly, potential interactions between these mechanisms have not been directly studied, and only two recent studies on zebrafish have administered nutritional supplements, designed to alter the gut microbiome, to examine potential epigenetic effects. Cuomo et al. [139] administered a probiotic (*Lactobacillus rhamnosus*) for 28 days and characterized some small changes in promoter DNA methylation and transcription of *BDNF* and *Tph1A* in the brain and gut associated with behavioral changes. Shirani et al. [140] administered a yeast-based prebiotic for 90 days and found some evidence of differential global DNA methylation. However, neither of these studies confirmed that specific probiotic-induced changes in the microbiome were associated with epigenome modulation. While there is a lack of direct evidence for diet-microbiome-epigenome interactions, there are several examples by which similar dietary treatments have been shown to induce both microbiome and epigenetic changes, for example, the inclusion of plant-based products such as soybean in zebrafish diets [141, 142]. As for mammalian systems, there is likely to be a fundamental interaction between

diet, gut microbiome, and epigenome, with SCFAs representing a critical link. The beneficial effects of SCFAs on intestinal health, as well as wider fish health, together with the essential role of microbiota in synthesizing them, is well recognized [143, 144]. A considerable amount of recent research has focused on developing diets and nutritional supplements (i.e., prebiotics such as immunosaccharides FOS, GOS, and XOS) that promote SCFA-producing bacteria (such as *Lactobacillus* and *Bifidobactrium*) or directly introduce these probiotic bacteria with the same aim [144]. Increasing SCFA circulation has been shown to reduce intestinal inflammation, promote immune response, and improve feed utilization and growth rates [96, 143]. While the likely interactions between SCFAs and the epigenome have been recognized [143, 144], these interactions have not been investigated to date.

An improved understanding of how environmental conditions, including stressors, experienced in aquaculture affect animal health is another driver for research examining microbiome-epigenome interactions. Intensive aquaculture is often associated with stress, for example, due to crowding, handling, pollutant exposure, or lack of natural stimuli [145, 146]. As with diet, a number of aquaculture-relevant stressors have been shown to induce changes in both the microbiome and the epigenome, but the potential for interactions between them, and how this contributes to animal health effects, has not been considered. Thermal and pollutant-associated stressors have been widely studied and therefore give the clearest indication of likely interactions. For example, exposure to thermal stress has been shown to induce lasting epigenetic changes and, in some cases, were associated with various health effects including thermal tolerance, immunocompetence, and performance in a number of fish species [54, 147–150]. Thermal modulation of the microbiome has also been widely demonstrated in fish and shellfish, in some cases, with lasting disruptive effects on the host-microbiome symbiosis, even after the original stressor has subsided [61, 64, 151, 152]. Early life stages are particularly sensitive to environmental stressors and represent windows of heightened sensitivity for modulation of both the epigenome and microbiome through periods of epigenetic reprogramming and initial microbial colonization and proliferation. Exposure to stressors during early life is known to induce particularly marked and/or lasting effects on the epigenome and microbiome with associated animal health effects [153, 154].

11.4 Gaps in Knowledge and Future Research Avenues

Modulation of both the microbiome and epigenome can contribute to both positive and negative lasting effects in the host, with windows of heightened environmental sensitivity in early life. Crucially, this means that both mechanisms represent promising avenues for nutritional programming or other means of environmental conditioning, with the aim of improving health, welfare, and productivity of farmed aquatic species. But despite these commonalities, in terms of effects and goals, there has been a striking lack of research examining interactions between the microbiome and epigenome in farmed aquatic species. To our knowledge, no studies have examined direct interactions between the microbiome and epigenome in an aquatic-cultured species. For example, a better understanding of how microbially derived SCFAs can benefit fish health, welfare, and ultimately, aquaculture sustainability, via epigenetic interactions represents a unique opportunity for future research. Improved knowledge of interactions between the microbiome and epigenome will enable a better understanding of the impacts of aquaculture-related stressors on animal health, disease susceptibility, and productivity/sustainability (Figure 11.4). On the other hand, highly regulated hatchery-rearing systems may also represent a considerable opportunity for environmental conditioning in early life, the development of which will also be benefited from improved knowledge of epigenetic and microbiome mechanisms and their interactions.

11.5 Conclusions

Research on the importance of epigenetics and microbiome as separate factors in the context of aquaculture has hugely increased over the last 5–10 years. Yet there is a striking lack of knowledge regarding their interactions. In contrast to terrestrial animals, aquatic species are constantly exposed to aquatic microorganisms. As well as posing challenges, like constant exposure to potential pathogens, this also offers unique opportunities to aquaculture, such as conditioning the microbiome via the rearing water. Aquaculture conditions, including diet, water treatment, or stress due to handling and crowding influence both the microbiome and the epigenome, further highlighting the opportunities for likely interactions, conditioning, and selection. Pre- and probiotics have shown beneficial effects on host immunity and nutritional status

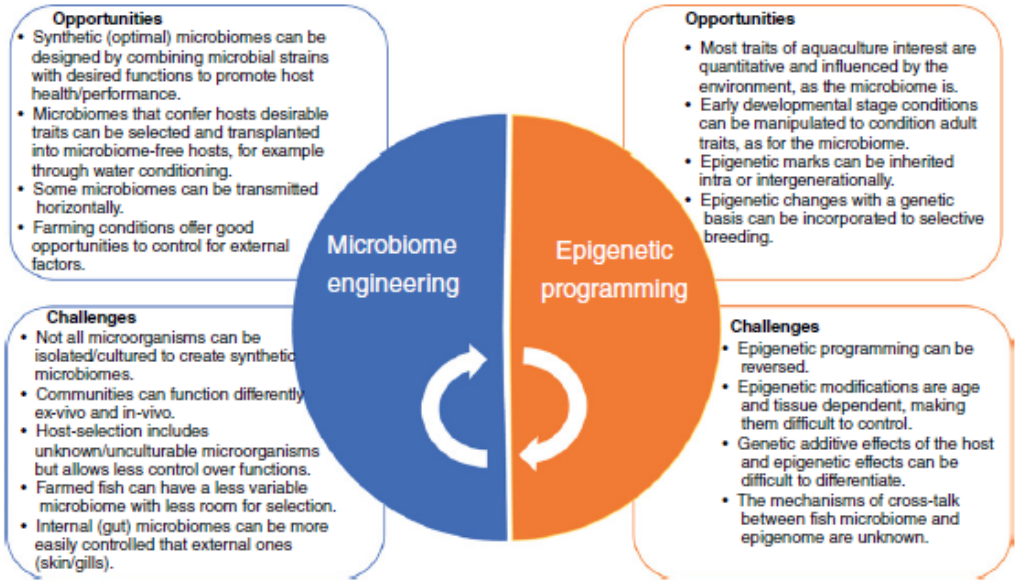

Figure 11.4 Challenges and opportunities for aquaculture of epigenetic programming and microbiome engineering; arrows indicating the epigenetics-microbiome crosstalk.

through the modification of microbiome communities and the metabolites they produce that, in turn, influence host gene expression. Research in mammals indicates that some of these metabolites (e.g., SCFAs) influence the host metabolism, energy status, and immunity through epigenetic regulatory mechanisms. Aquaculture-controlled conditions and the current, rapidly expanding knowledge about fish microbiome and epigenome suggest a great potential for the application of combined epigenetic and microbiome conditioning to improve fish health and performance under farmed conditions. Such an approach is showing promising results in medicine and agriculture. However, there are important differences between fish and mammalian microbiomes, and it is not possible to fully translate results from nonfish model species to aquaculture. Research on mechanisms of microbiome-epigenome crosstalk and their influence on host health and performance is emerging as the next challenge for research on sustainable aquaculture.

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