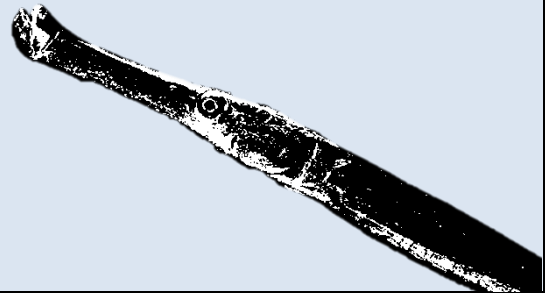


BIPARENTAL IMMUNE PRIMING IN THE BROAD-NOSED PIPEFISH (SYNGNATHUS TYPHLE)

Vorgelegt von:

Anne Beemelmans

Kiel, 2016



Dissertation

zur Erlangung des Doktorgrades

- Dr. rer. nat. -

der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

Erste Gutachterin: Dr. Olivia Roth

Zweiter Gutachter: Prof. Dr. Hinrich Schulenburg

Tag der mündlichen Prüfung: 08.04.2016

Zum Druck genehmigt: 08.04.2016

gez. Prof. Dr. Wolfgang J. Duschl, Dekan

Für meine Familie

Table of Content

Summary	11
Zusammenfassung	13
Introduction.....	15
1. Parasite mediated selection on immunity	16
1.1. Immune system of vertebrates.....	16
1.2. Evolution of the immune system	19
1.3. Immune system of fish (Teleostei)	20
2. Parental effects.....	25
2.1. Trans-generational immune priming	27
2.2. Biparental immune priming	28
2.3. Costs of immune priming	30
2.4. Epigenetic inheritance	31
2.4.1. DNA methylation.....	32
2.4.2. Histone modifications.....	34
3. The broad-nosed pipefish (<i>Syngnathus typhle</i>) as model organism	36
Thesis outline	40
Chapter I.....	43
Chapter II.....	69
Chapter III.....	99
Chapter IV	123
Synthesis.....	141
1. Biparental immune priming over generations	143
2. Paternal versus maternal immune priming effects	146
3. How is immune priming mediated over generations?	148
Future perspectives	155
1. Future project I: Paternal transmission of antimicrobial peptides	156
2. Future project II: Paternal transmission of specific microbiota	157
Author contributions.....	159
Eidesstattliche Erklärung	161
List of publications	163
Danksagung	164
Bibliography.....	166
Appendices.....	185
Appendix Chapter I	185
Appendix Chapter II	195
Appendix Chapter III	207
Appendix Chapter IV	216
Curriculum vitae.....	221

Summary

All living organisms are permanently exposed to harmful parasites and pathogens. The immune system and with it sophisticated strategies arose to face challenging pathogens such as bacteria and viruses. One outstanding strategy is the transfer of immunity from mothers to offspring known as “trans-generational immune priming” (TGIP). Through the transfer of acquired immunological protection mothers can boost the immune defense of their offspring and facilitate resistance against bacteria present in the maternal environment. Commonly, TGIP is limited to females and was considered to be a maternal trait. Yet, in the sex-role reversed pipefish *Syngnathus typhle* fathers are closely connected to the embryos during male pregnancy over a placenta-like structure. This intimate physical connection between fathers and offspring may mechanistically facilitate additional paternal immune priming and provides an exclusive chance to study biparental investment into offspring immunity. In my thesis, I explored multi-generational biparental immune priming in the sex-role reversed pipefish *S. typhle* and discussed its evolutionary and ecological consequences as well as the mechanistic basis.

In the first two chapters, I investigated parental influences on offspring immunity upon an immune challenge with two different heat-killed bacteria types (*Vibrio* spp. and *Tenacibaculum maritimum*). Therefore, I challenged two consecutive age classes of the F1-generation (one-week-old and four-month-old juveniles) with bacteria types experienced by their parents and assessed gene expression of 29 immune genes and immune cell activity. My results indicate different magnitudes of maternal and paternal immune priming. Indeed, fathers had a major impact on offspring immunity resulting in a long-lasting paternal immune priming effect in four-month-old juveniles, which came with immunological specificity. Maternal immune priming effects were only effective in one-week-old juveniles and diminished upon maturation. Yet, no additive biparental immune priming in combination with the exposure of two different bacteria types was detectable, indicating underlying complex trade-off effects. In the second chapter, I investigated potential beneficial immune priming effects in favor for specific pathogens. Whereas in one-week-old juveniles both parental bacteria challenges induced a differently altered immune gene expression profile, in four-month-old offspring protection was only maintained against prevalent *Vibrio* bacteria. Thus optimal protection against prevailing pathogen types was accomplished by the parents.

In chapter three, I investigated multi-generational biparental immune priming by continuing the main experiment until the second generation. Therefore, I crossed untreated individuals of the F1-generation and exposed one-week-old F2-juveniles with identical heat-killed bacteria types, which already their grandparents had experienced. I found evidence that both

grandparents affect the immune system of their grand-offspring. With this astonishing result I could show that TGIP in vertebrates can be sustained over two generations. This is challenging the conventional view that TGIP is only short-term and fading after maturation.

As individual investment into immunity is costly in terms of energy resources, induced immunity can lead to resource allocation trade-offs with other life history-traits. In accordance, I recorded reduced fecundity and delayed maturation time of adult F1-offspring and reduced body size of F2-juveniles in case of parental respectively grandparental bacteria exposure. These fitness detriments are pointing to a resource allocation trade-off between immune priming and fecundity indicating that costs might constrain the overall beneficial immune priming effects.

By including the expression of genes responsible for epigenetic regulation mechanism such as DNA methylation and histone acetylation, I provide evidence that long-term protection could be based on parental epigenetic marks. A significantly altered expression pattern of 15 epigenetic genes indicate that these factors might be crucial players in the regulation of immune gene expression and its non-genetic inheritance over two generations. Further, lectins, peptidoglycan recognition proteins, immunoglobulins, and histone acetyltransferases might be tightly connected in mediating the immune priming process over the paternal line.

The result of my thesis showed that the importance of TGIP as one form of non-genetic inheritance might be more wide-ranging and long-lasting than formerly recognized and may affect models of host-parasite coevolution. The involvement of epigenetic inheritance mechanisms indicate that TGIP mediated over the paternal line is not only restricted to the pipefish system with its male pregnancy but certainly could be more widespread in the animal kingdom than previously reported.

Zusammenfassung

Alle lebenden Organismen sind permanent allgegenwärtigen infektiösen Parasiten und Pathogenen ausgesetzt. Ausgelöst durch ihre schädliche Wirkung auf die Fitness ihres Wirtes evolvierten sich mit dem Immunsystem verschiedene Abwehrmechanismen, um einen Schutz zu gewährleisten. Eine raffinierte Strategie ist die Übertragung von erworbener Immunität von Müttern auf die Nachkommen über die Plazenta oder Muttermilch, bekannt als „generationsübergreifende Immunprägung“. Mit der Weitergabe von erworbenem Immunschutz können somit Mütter die Immunabwehr ihrer Nachkommen in der frühen embryonalen Entwicklung gewährleisten. Zudem ermöglicht es den Nachkommen schneller eine Resistenz gegen die in der mütterlichen Umgebung vorhandenen Krankheitserreger aufzubauen. Daher wurde bisher davon ausgegangen, dass Immunprägung nur durch das weibliche Geschlecht verwirklicht wird. Doch in dem seltenen Fall der Grasnadel *Syngnathus typhle* ist die Rollenverteilung der Geschlechter vertauscht. Bei dieser besonderen Fischart brüten die Männchen während einer männlichen Schwangerschaft ihre Embryonen in einer Bruttasche und sind über eine plazenta-ähnliche Struktur so eng mit ihnen verbunden, dass mechanistisch eine väterliche Immunprägung möglich wäre. In meiner Doktorarbeit habe ich die Aspekte der biparentalen Immunprägung über zwei Generationen in der Grasnadel *S. typhle* erforscht und ihre evolutionären und ökologischen Folgen, sowie mechanistische Grundlage näher untersucht.

In den ersten beiden Kapiteln untersuchte ich die elterlichen Einflüsse auf die Immunität ihrer direkten Nachkommen hinsichtlich einer Impfung mit zwei verschiedenen hitze-getöteten Bakterien (*Vibrio* spp. und *Tenacibaculum maritimum*). Dazu behandelte ich die erste Filialgeneration (Jungtiere im Alter von eine Woche und vier Monaten) mit denselben Bakterien, denen auch ihre Eltern ausgesetzt wurden und evaluierte die Genexpression von 29 Immungenen und Immunzellaktivitäten. Meine Ergebnisse des ersten Kapitels zeigen ein unterschiedliches Ausmaß an mütterlicher und väterlicher Immunprägung in den Jungtieren. Hierbei hatten Väter einen sehr ausgeprägten und langanhaltenden Einfluss auf die Immunität ihrer Nachkommen. Väterliche Immunprägungs-effekte konnten auch noch in vier-Monate alten Jungtieren festgestellt werden und diese wiesen sogar immunologische Spezifität gegen *Vibrio* Bakterien auf. Mütterliche Immunprägungseffekte waren nur in sehr jungen Tieren (eine Woche alt) nachweisbar, die sich im Laufe der Entwicklung verringerten. Dennoch konnte ich keine additiven biparentalen Immunprägungseffekte feststellen, was auf zugrundeliegende komplexe „trade-off“ Effekte zurückzuführen sein könnte. Im zweiten Kapitel analysierte ich detailliert parentale Immunprägungseffekte zu Gunsten von spezifischen Krankheitserregern. Hier zeigte sich, dass spezifisch gegen lokal vorkommende

Vibrio Bakterien ein langanhaltender Immunschutz von den Eltern übertragen wurde und somit ein optimaler Schutz gegen prävalente Bakterienarten gewährleistet wurde.

In meinem dritten Kapitel untersuchte ich die Möglichkeit von biparentaler Immunprägung über zwei Generationen. Dazu kreuzte ich unbehandelte F1-Nachkommen miteinander und injizierte F2-Jungtiere mit den identischen hitzegetötete Bakterien, die bereits ihre Großeltern erfahren hatten. Hier fand ich Hinweise darauf, dass beide Großeltern das Immunsystem ihrer Enkel beeinflussen. Die Erhaltung von elterlicher Immunprägung über zwei Generationen in Wirbeltieren könnte die herkömmliche Ansicht, dass dieses Phänomen nur von kurzer Dauer ist, und nach der Reife des Immunsystems verschwindet, in Frage stellen.

Da eine erhöhte Immunantwort für einen Organismus energetisch kostspielig ist, habe ich energetische Kosten bezüglich anderer Fitness relevanter Parameter analysiert. Entsprechend stellte ich eine verzögerte Reifezeit und Reproduktion von erwachsenen F1-Nachkommen fest und konnte zudem eine reduzierte Körpergröße der F2-Jungtiere im Falle einer elterlichen bzw. großelterlichen Bakterienbehandlung verzeichnen. Diese Fitnessnachteile, die auf eine Energieverteilungsbalance zwischen Immunprägung und Reproduktion zurückzuführen sind, zeigten, dass Immunprägung eine kostspielige Strategie darstellt. In meiner Arbeit erörtere ich mögliche Kosten und Nutzen.

Durch die Einbeziehung des Expressionsmusters von Genen, die für epigenetische Regulationsmechanismen verantwortlich sind, wie z.B. DNA-Methylierung und Histon-Acetylierung fand ich Hinweise darauf, dass die Übertragung von langfristigem Schutz über mehrere Generationen auf epigenetische Mechanismen zurückzuführen sein könnte. Weiterhin ergaben meine Analysen, dass Lektine, Peptidoglycan-Erkennungsproteine, Immunglobuline und Histonacetyltransferasen bei der Vermittlung des väterlichen Immunprägungsprozesses in *S. typhle* wichtig sein könnten.

Die Resultate dieser Arbeit zeigen, dass die Bedeutung von generationsübergreifender Immunprägung, als eine Form von nicht-genetischer Vererbung, weitreichender sind und lang anhaltendere Folgen haben können, als ursprünglich angenommen wurde. Dies könnte sogar Modelle von Wirt-Parasit-Koevolution beeinflussen. Zudem legt die Involvierung von epigenetischen Effekten nahe, dass Immunprägung vermittelt über die väterliche Linie nicht nur auf die männliche Schwangerschaft der Seenadel beruhen muss, sondern durchaus weiter im Tierreich verbreitet sein könnte als bislang bekannt war.

Introduction

The concepts of heredity and evolutionary change are presently intensively discussed. It is under debate that additional acquired information apart from genes also accounts for heredity. Non-DNA based changes have been shown not only to be transmitted between daughter cells but also between filial generations, while passing on information about actual environmental conditions. This non-genetic inheritance as one form of trans-generational plasticity may be more widespread than previously acknowledged. This is leading to claims for a more inclusive view of inheritance and adaptation into the neo-Darwinian synthesis that only allows for DNA-based changes. In my thesis I use parental effects as response to infection as one outstanding example of trans-generational phenotypic plasticity reflecting the ecological importance of pathogens, one of the strongest selection pressures in nature.

1. Parasite mediated selection on immunity

Parasites are ubiquitous (Windsor, 1998) and impose strong selection on their hosts' fitness (Hamilton *et al.*, 2008) by exploiting their resources. Parasites are classified into macroparasites, which are visible multicellular organisms, such as parasitic worms (helminths), and smaller microparasites with shorter generation time like bacteria, viruses, protozoa and fungi (Wilson & Cotter, 2013). To acquire protection against harmful infections, hosts are in constant need to adapt and improve their immune system (Van Valen, 1974; Hamilton *et al.*, 2008). Pathogens, in turn, need to overcome the host's defense and to evolve effective infection mechanisms (Boots & Bowers, 2004; Schmid-Hempel, 2011). Thus, hosts and parasites are engaged in an antagonistic arms race of constant adaptation to each other for their mutual survival (Hamilton *et al.*, 2008). Due to their shorter generation time and larger population size, parasites are predicted to adapt faster to their hosts than the hosts to their parasites (Dybdahl & Lively, 1998). This leads to constantly fluctuating interactions and complex dynamics of allele frequency changes of genes coding for parasite infectivity and host resistance (Dybdahl & Lively, 1998; Kaltz & Shykoff, 1998; Little, 2002; Hamilton *et al.*, 2008). The oscillation of host and parasite genotypes are referred to as 'Red Queen dynamics' (Van Valen, 1974) and characterized by time-lagged negative-frequency-dependent selection, whereby rare allele that result in higher fitness, have an advantage and are spread through the population by positive selection (Dybdahl & Lively, 1998; Lively & Dybdahl, 2000; Koskella & Lively, 2009). The evolution of host resistance is sharpened by parasite-mediated selection, leading to the evolution of a huge variety of defense mechanism to minimize fitness losses (Schmid-Hempel & Ebert, 2003).

1.1. Immune system of vertebrates

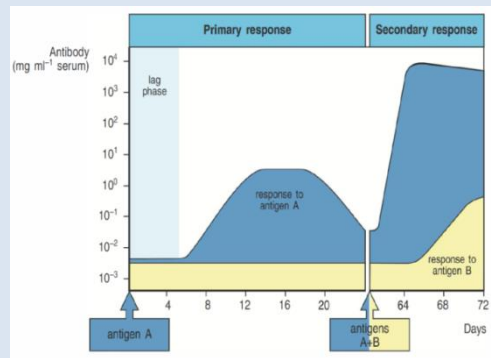
The vertebrate immune system consists of the immediately available but non-specific innate line of defense and the specific long-lasting adaptive immune protection (Wilson & Cotter, 2013). Both immune responses involve humoral and cell-mediated immunity and may be viewed as two independent systems; however, they are interconnected in many ways (Flajnik & Du Pasquier, 2004).

The innate immune defense involves mechanical barriers, specific cells and proteins that are constantly present and immediately ready to fight all invading microorganisms (Flajnik & Du Pasquier, 2004). The main components include all surface and physical epithelial barriers and immediate inflammatory responses mediated by the recruitment of immune cells through a diverse set of biochemical signaling cascades (Murphy, 2011). The most important innate

Introduction

immune cells are granulocytes, phagocytic macrophages, dendritic cells and natural killer cells (NK) cells (see Table 1 for detailed functions) (Murphy, 2011). Pattern recognition receptors (PRR) of the immune cells recognize conserved pathogen-associated molecular patterns (PAMPs) like mannose, glucan, or lipopolysaccharides that are shared by macro and microparasites (Wilson & Cotter, 2013; Leavy, 2015). Stimulation of PRR-receptors on a macrophage triggers not only phagocytosis and destruction of the pathogen, but also a simultaneous secretion of signaling molecules such as cytokines and chemokines. These signaling molecules in turn recruit and direct other immune cells to the site of infection which induces an inflammatory response (Leavy, 2015). In addition, dendritic cells transport and present pathogenic fragments to T-lymphocytes that activate the adaptive antibody mediated immune response (Murphy, 2011). On top of that, the complement system consisting of roughly 30 plasma proteins performs a vital inflammation response. Specific complement components can opsonize ("coat") the surface of pathogens and induce their direct destruction. This is either mediated by the recruitment of macrophages, by direct lysis via a membrane attack complex (MAC), or an antibody mediated adaptive response (Table 1) (Mathern & Heeger, 2015).

Adaptive immune responses are initiated by antigen presenting cells that transport antigens to the lymphoid organs and thus trigger lymphocytes to proliferate and differentiate in antigen specific effector cells (B-cells and T-cells) (Murphy, 2011). Stimulated B-cells differentiate either into antibody-producing plasma cells or migrate into the lymphoid tissue, where they undergo somatic hypermutation and affinity maturation (Murphy, 2011; Schmid-Hempel, 2011). During those processes, the specificity of binding between antigen and antibody is refined to produce clonal antibodies identical to particular targeted antigens; a process known as 'clonal selection of antigen production' (Murphy, 2011; Schmid-Hempel, 2011). T-cells develop in the thymus and are composed of two main classes depending on their surface proteins (CD8+ or CD4+). CD8+T-cells become cytotoxic T-cells, which bind to antigen-major-histocompatibility complex class I (MHC I) complex located on cell surfaces and induce the killing of infected cells (Murphy, 2011). CD4+T-cells differentiate into T-helper cells that recognize the antigen-MHC II complex of macrophages as well as B-cells and thereby activate phagocytosis that finally leads to the production of specific antibodies. A significant number of activated antigen-specific B-cells and T-cells persist as long lived memory cells for several years and are known to store an immunological memory (Kurtz, 2004). These memory cells can be activated quickly, ensure a rapid and effective response upon a second exposure, and provide long-lasting protection (see Box 1). This ability was first described by Edward Jenner (1796) who discovered the vaccination against smallpox (Murphy, 2011).

Box 1: Immune memory of mammals

The first encounter with an antigen initiates a primary response with a specific antibody A (plateau curve) after a lag phase of 4-5 days. A second exposure to a combination of two different pathogens A and B causes a rapid and intense response to antigen A. This illustrates immunological memory. It is the ability to create a more pronounced and more efficient response upon secondary contact with the same antigen.

Specificity: describes the degree to which the immune system differentiates between different antigens or pathogen types (*i.e.* recognize and mount a more effective immune response against antigen A than to antigen B after a second

exposure). **Cross-reactivity:** describes the extent to which different antigens cannot be differentiated by the immune system. Cross-reactivity of the adaptive immune system is also known from non-specific effects of vaccines. **Immunological memory:** the ability of the immune system to store and recall immunological information on previously encountered pathogen to respond quicker and more vigorously upon a secondary contact. **Specific immune memory:** immune memory that comes with specificity.

Referred from (Kurtz, 2004; 2005; Murphy, 2011; Schmid-Hempel, 2011; Benn *et al.*, 2013)

Nevertheless, specific responses and immune memory has also been found to exist in invertebrate species that only possess innate immunity (Little *et al.*, 2003; Kurtz *et al.*, 2004; Kurtz, 2005; Sadd *et al.*, 2005; Kurtz & Armitage, 2006; Roth *et al.*, 2009; Sadd & Schmid-Hempel, 2009; Wang *et al.*, 2009; Roth *et al.*, 2010; Bolte *et al.*, 2013; Musthaq & Kwang, 2014; Wu *et al.*, 2015). Indeed, Toll-like receptors (TLRs), natural killer cells (NKs), complement components and phagocytosis represent alternative mechanisms to provide specificity and memory within the innate immune system (Pham *et al.*, 2007; Wang *et al.*, 2009; Sun *et al.*, 2014; Wu *et al.*, 2015). For instance, Toll-like receptors distinguish among classes of pathogens and play an important role in orchestrating the adaptive immune responses (Palti, 2011). The huge diversity of TLRs in aquatic species and in teleosts points to their potential connection to specific innate immune reactions and additional role in adaptive immunity (Palti, 2011; Rauta *et al.*, 2014). Recently, Haase *et al.* (2014) found parasite-strain specific innate immune gene expression in the three-spined stickleback, indicating that specific responses to parasite genotypes are mediated by innate immunity. Although, so far little is known about the degree of discrimination accomplished by the innate immune system, the common division between an unspecific innate and specific adaptive immune response should not be seen as black and white, but rather as complex cross-talk between both systems (Tort *et al.*, 2003; Flajnik & Du Pasquier, 2004; Sun *et al.*, 2014).

1.2. Evolution of the immune system

The negative impact imposed by parasites has initiated the selection for genetic immune mechanisms (Boots and Bower, 2004). The immune system accomplishes a diversity of tasks, but its main function is to distinguish self from non-self, leading to the protection against diseases (Murphy, 2011). A fundamental process is allorecognition and the rejection of foreign grafts. This ability already exists in early metazoa such as sponges and cnidarian, which possess an ancient form of a histocompatibility system (“tissue compatibility”) to recognize and destruct foreign tissues. This was thought to be the very original function of preventing parasites to enter the host (Du Pasquier, 2006).

However, the strong selection imposed by parasites which constantly evolve new strategies to overcome host defenses, induced the generation of a unique diversity of immune repertoire with increasing specificity to recognize and fight back parasites (Flajnik & Kasahara, 2010). A hallmark emerged approximately 500 million years ago, with the appearance of the first adaptive immune system in jawed vertebrates (*Gnathostomes*) and was considered as ‘immunological big-bang’ (Schluter *et al.*, 1998; Flajnik & Kasahara, 2010). Individuals with adaptive immunity were able to create a specific, incredibly diverse immune response with an ‘immune memory’ that enabled a rapid response to previously encountered pathogens (Murphy, 2011). These features arose with recombination-activating genes (RAGs), somatic hypermutation and variable–diversity–joining rearrangement (VDJ rearrangement) of immunoglobulin (Ig), T-cell (TCR) and B-cell receptor (BCR) genes leading to the production of an immense collection of different antigen receptors (Kurtz, 2004; Cooper & Alder, 2006; Flajnik & Du Pasquier, 2008; Flajnik & Kasahara, 2010).

Adaptive immunity based on lymphocyte bearing antigen-specific receptors occurs across the vertebrate phylogeny, and has its evolutionarily origin in cartilaginous fish (*i.e.* sharks and rays) (Flajnik & Kasahara, 2010; Rauta *et al.*, 2012). They are the first class of jawed vertebrates that own both innate and adaptive immune response (Flajnik & Kasahara, 2010; Rauta *et al.*, 2012). The presence of recombination associated genes (RAG) enables jawed vertebrates to create a vast amount of different combinations of immunoglobulin, TCRs and BCRs by joining V, D and J gene segments through RAG1 and RAG2 enzymes (Gellert, 2002). Lampreys and hagfish (*Agnathans*, jawless vertebrates) belong to the basal group of cartilaginous fish and they assemble variable lymphocyte receptors (VLRs) by modular leucine rich repeat (LRR) modules (Alder *et al.*, 2005). They also lack, apart from other relevant immune components, major histocompatibility complex (MHC) genes (Cooper & Alder, 2006). Interestingly, in cod-like fishes and in *Syngnathidae* (pipefish), two different families of jawed vertebrates, an independent secondary loss of MHC class II and

corresponding genes was discovered which implements an absence of MHC II-mediated adaptive immune response (Star *et al.*, 2011; Haase *et al.*, 2013). These findings suggest that alternative adaptive immune strategies with convergent functions have evolved dynamically (Buonocore & Gerdol, 2015). However, to date it is not known, which alternative strategy has emerged in the *Syngnathidae* family to compensate this fundamental loss of MHC II.

1.3. Immune system of fish (Teleostei)

Fish represent a crucial phylogenetic transition point in the evolution of immune defense as they possess a rapid and efficient innate immune system and display characteristics of an adaptive antigen-specific immunity resembling that of the mammalian system (Foey & Picchietti, 2014). However, the adaptive immune system of fish is comparatively slower and simpler due to the fact that they are poikilothermic free-living organisms depending on the temperature of their environment and reveal certain physiological differences (Press & Evensen, 1999; Workenhe *et al.*, 2010; Foey & Picchietti, 2014). One of the key difference is the absence of red bone marrow and lymph nodes, the main tissue in which central immune cells, *i.e.* granulocytes, macrophages, and lymphocytes, are generated (Sunyer, 2013; Foey & Picchietti, 2014). Instead, fish possess the thymus, spleen, head kidney and mucosa-associated lymphatic tissue as major immune organs in which immune cells are generated and differentiate (Press & Evensen, 1999; Zapata *et al.*, 2006).

Teleosts have a well-developed mucosal/barrier defense system at their surfaces as they are constantly under attack in a pathogen-rich aquatic environment (Foey & Picchietti, 2014). Skin and mucus membranes of the gills and the intestinal tract have epithelial cells as first physical barrier and mucosa-associated lymphatic tissue, which are hosting a collection of non-specific immune factors to form a protective barrier against invading pathogens (Foey & Picchietti, 2014). Gut associated lymphatic tissue (GALT) for instance harbours of lymphocytes, macrophages and granulocytes in the epithelium (Salinas, 2015).

The head kidney is the major immune organ and it is morphologically similar to the red bone marrow. It also reveals hemopoietic functions and is responsible for phagocytosis, antigen processing, and production of antibodies (Press & Evensen, 1999; Workenhe *et al.*, 2010; Foey & Picchietti, 2014). Furthermore, the head kidney produces granulocytes, macrophages, dendritic cells, lymphocytes, natural killer cells and is the primary source of T- and B-cells (Whyte, 2007; Workenhe *et al.*, 2010).

Introduction

The spleen as second important immune organ is responsible for antigen presentation and the initiation of the adaptive immune response (Whyte, 2007; Workenhe *et al.*, 2010). Surprisingly, *Syngnathidae* (pipefish and seahorses) are lacking a spleen and GALT (Matsunaga & Rahman, 1998), which might be connected to their unusual adaptive immune system *i.e.* loss of MHC II (Haase *et al.*, 2013).

The thymus of fish is an aggregation of macrophages that promotes the generation of T-lymphocytes, which are involved in the stimulation of phagocytosis, allograft rejection and antibody production (Rauta *et al.*, 2012).

As fish are poikilothermic and certain factors of the adaptive immune response are negatively affected by the external temperature (Whyte, 2007; Uribe *et al.*, 2011). This results in slower proliferation and maturation time of their lymphocytes (Whyte, 2007; Uribe *et al.*, 2011). Therefore, they fundamentally rely on innate immunity for survival (Magnadóttir *et al.*, 2005; Magnadóttir, 2006), which mediates a plethora of secreted antimicrobial immune compounds and immune cells such as macrophages, granulocytes, and natural killer cells (see Table 1 for further information about innate immunity in fish).

In general, teleost are able to create an antigen-specific adaptive immune response, yet, it is rather simple and undifferentiated (Whyte, 2007; Foey & Picchiatti, 2014). They possess three major types of immunoglobulin (Ig) whereby the isotype IgM is the most ancient class and functions as soluble antibody. Although lymphocyte proliferation takes longer and with it the establishment of an adequate antibody titer, fish can develop a memory response upon a second exposure (vaccination) to the same antigen providing protection against infections (Uribe *et al.*, 2011; Foey & Picchiatti, 2014). Similar to mammals, their adaptive immune responses is characterized by immunoglobulin, T-cell receptors, cytokines, and major histocompatibility complex molecules (see Table 1 for further information about adaptive immunity in fish and Box 2 of Chapter IV for further information about Immunoglobulin of fish).

Introduction

Table 1: Innate and adaptive immune factors of the teleost's immune system. Elements of immune responses in teleost and genes / immune cells that were investigated in my thesis are listed with their specific functions as well as information about maternal / paternal transmission or presence in the brood pouch of *Syngnatidea*.

Elements of immune response in teleost	Genes of interest	Function of specific immune molecules	maternally or paternally transmitted
INNATE IMMUNE COMPONENTS			
First line of defense-inflammation response			
Vitellogenin: Egg yolk precursor (nutrition) protein but also immune relevant as it binds gram+/gram- bacteria via pathogen-associated molecular patterns such as lipopolysaccharides, and peptidoglycan. Transports bacteria cell walls into eggs (carrier of immune-priming signals).	-	-	maternal
Antimicrobial peptides: Low molecular amphipathic peptides that insert into biological membranes and lyse via pore formation a broad spectrum of pathogens such as bacteria, viruses, fungi and protozoa. They are released into the mucus of various organs (gills, liver, and intestine) but are also present in fish eggs.	-	-	maternal and present in brood pouch of <i>Syngnatidea</i>
Lysozymes: Enzymes that lyze the peptidoglycan layer of bacteria cell walls and hydrolase gram+/gram- bacteria; expressed in mucus, lymphoid tissue, and plasma.	-	-	maternal
Toll like receptors: receptors of macrophages, and dendritic cells. Recognize pathogens and detect differences between gram+ / gram- bacteria and viruses. Initiate innate immune response and alert adaptive immunity.	-	-	unknown
Pattern-recognition receptors: Bind to conserved pathogen-associated molecular patterns (PAMPs) that are shared by broad classes of microorganism.	<i>Peptidoglycan recognition protein II</i>	Recognition of peptidoglycan on bacteria cell wall (gram+ bacteria)	present in brood pouch of <i>Syngnatidea</i>
Lectins: Proteins that recognize and bind carbohydrates in a calcium-dependent manner of the pathogen cell-surfaces, which is leading to opsonization ("tagging") for phagocytosis or activation of complement component system.	<i>Lectin type I</i>	C-type lectin (CTL) with carbohydrate-recognition domain (CRD); calcium-dependent carbohydrate binding modules for pathogen recognition	maternal and present in brood pouch of <i>Syngnatidea</i>
	<i>Lectin type II</i>	Type II C-type lectin-like receptors can function as an adhesion receptor and as a phagocytic pathogen-recognition receptor	maternal and present in brood pouch of <i>Syngnatidea</i>
Cytokines: are produced by almost all cells of the immune system with the function to regulate the immune response <i>i.e.</i> signaling.	<i>Ik cytokine (RED-protein)</i>	Factor that inhibits interferon gamma mediated downregulation of MHCII (innate & adaptive immune response)	unknown
Interleukins: Cytokines made by lymphocytes that are involved in the pro-inflammatory response and have regulatory functions.	<i>Interleukin 8</i>	Cytokine for early attraction of neutrophil natural killer cells (phagocytosis, inflammatory activity)	unknown
	<i>Interleukin 10</i>	Anti-inflammatory cytokine with multiple effects in immunoregulation and inflammation. It stimulates macrophages and enhances B-cell proliferation and antibody production (innate & adaptive immune response)	unknown
Interferon: Cytokines that are induced upon infection. IFN- α , β have a non-specific antiviral function that is based on the inhibition ("interference") of viral replication in virus infected cells.	<i>Interferon induced transmembrane protein 3</i>	IFN-induced antiviral protein that inhibits the entry of viruses into the cytoplasm	unknown

Introduction

Tumor necrosis factors (TNF): Cytokine receptors involved in apoptosis but also in inflammation response and lymphoid organ development.	<i>LPS-induced TNF-α factor (LITAF)</i>	Expression in LPS-induced response while mediating cytokine TNF- α , an important activator of macrophages leading to increased phagocytosis	unknown
Chemokine: Cytokines that direct chemotaxis; are also pro-inflammatory cytokines and recruit T-cells of immune system to the site of infection.	<i>Chemokine 7</i>	Chemotaxis for leukocytes, monocytes, neutrophils, blood cells	Present in brood pouch of Syngnatidae
Transferrin: Glycoprotein with iron chelator activity. It causes withholding of nutrients (iron) to prevent bacterial outgrowth in a process termed nutritional immunity.	<i>Transferrin</i>	Iron withholding a process that prevents bacterial outgrowth	Present in brood pouch of Syngnatidae
Chaperone: assist in folding or unfolding of proteins and the assembly or disassembly of other macromolecular structures.	<i>Heat shock protein 60</i>	Chaperone, help folding of proteins, part of general stress response	unknown
	<i>Calreticulin</i>	Chaperone, promote phagocytosis and clearance of apoptotic cells	Present in brood pouch of Syngnatidae
Blood clotting: it inhibits blood loss and reduces flow draining from the site of infection to prevent the distribution of pathogens.	<i>Coagulation factor II</i>	It is mediating the interplay between coagulation (blood clotting) and inflammation	unknown
Cellular innate defense			
Natural killer cells: large granular cells that kill virus infected and tumor cells			
Granulocytes: migrate toward the sites of infection to perform phagocytosis and destroy invaded microorganisms.			
Monocytes/Macrophages: cells that perform phagocytosis. They remove pathogens with the production of reactive oxygen species with respiratory burst and release of hydrolytic lysozymes. Macrophages derive from monocytes.	<i>Natural resistance-associated macrophage protein</i>	Activation of macrophages	unknown
	<i>Allograft inflammation factor</i>	Activation of macrophages	unknown
	<i>Translocator protein</i>	Immunomodulation such as oxidative bursts by neutrophils and macrophages	unknown
	<i>Chemokine 7</i>	Chemotaxis (see above)	(see above)
	<i>Interleukin 10</i>	Regulation of macrophage activity, enhances B-cell proliferation	unknown
	<i>Monocytes</i>	(Measurements of monocyte cells with flow cytometer)	
Complement System			
<i>Complements the adaptive pathway and acts via classical, alternative and lectin pathway</i>			
Complement system: Composed of >30 plasma and cell surface proteins that act together to attack pathogens. It causes opsonisation of pathogens, chemotaxis and activation of leukocytes, as well as direct killing of pathogens via lysis. It is engaged both by innate immunity and as one of the main effector mechanisms of antibody-mediated immunity. There are three types of complement pathways: <i>i.</i>) classical pathway (activated by antibody-antigen complex – C1q), <i>ii.</i>) lectin pathway (activated by lectins), <i>iii.</i>) alternative pathway (opsonization with C3, forming of membrane attack complex MAC)	<i>C3</i>	Key molecule for regulation of effectors; opsonization of bacteria and activator of complement system that is followed by the lytic pathway during which the membrane-attack complex (MAC) is formed	maternal
	<i>C9</i>	Last component of the membrane attack complex (MAC) and induces pores in the membrane of pathogens; part of the lytic pathway	unknown
	<i>C1q</i>	Recognition sub-component (C1q) of antigen-antibody complex; directly binds to the antigen and helps in forming the antibody complex; induces the classical pathway and enhances phagocytosis	unknown

Introduction

ADAPTIVE IMMUNE SYSTEM			
<i>Acquired immune defense over antigen recognition</i>			
<p>MHC: highly polymorphic genes that can bind to peptide fragments derived from pathogens and display them on the cell surface (antigen presentation).</p> <p>MHC class I: are found on all cells and display epitopes (peptides) on the cell surface to cytotoxic CD8+ T-cells which trigger the infected cell to undergo cell death (apoptosis).</p> <p>MHC class II: molecules are restricted only to antigen presenting cells which activates DC4+ T-helper cells via presentation of extracellular pathogens (see "T-cells").</p>	<p><i>Tap-binding protein (Tapsin)</i></p>	<p>Transmembrane glycoprotein which mediates peptide loading on MHC I and transport of antigenic peptides across the endoplasmic reticulum membrane</p>	<p>unknown</p>
	<p><i>Human immunodeficiency virus type 1 enhancer-binding protein 2,3</i></p>	<p>Zink finger transcription factor with function in a variety of biological and developmental processes, such as HIV-1 gene expression, cell fate specification during embryogenesis, V(D)J recombination of immunoglobulins and in MHC enhancer binding</p>	<p>unknown</p>
<p>Immunoglobulins (Ig): B-cell membrane bound antigen receptors or soluble antibody molecules released by B-cells. Antibodies are large Y-shape proteins whose arms form two identical antigen binding sites which are highly variable, while providing huge diversity for specific antigen recognition. Igs can participate in three pathways: (i.) direct neutralization, (ii.) opsonization ("coating") to tag pathogen for phagocytosis via magrophages, or (iii.) complement activation leading to lysis and degradation.</p>	<p><i>Immuno-globulin light chain</i></p>	<p>Recognition of antigen (pathogen). Light chain is composed of constant part and variable part which is creating a variable region that contains the antigen binding site (See Box 2 Chapter IV)</p>	<p>maternal</p>
<p>Integrin: membrane receptors involved in cell adhesion, important in adhesive interactions between lymphocytes and antigen-presenting cells, immune patrolling, and cell migration.</p>	<p><i>Integrin-beta 1</i></p>	<p>Fibronectin receptor mediates cell signaling; it can bind to immunoglobulin and helps to form the clot matrix to stop blood loss</p>	<p>Present in brood pouch of Syngnataidae</p>
Cellular adaptive defense			
<p>Dendritic cells: Antigen uptake, migrate centrally and perform antigen-presentation in the context of MHC to T lymphocytes (transition between innate and adaptive immune response).</p>	-	-	-
<p>Lymphocytes: a class of white blood cells that bear variable cell-surface receptors for antigens and are responsible for all adaptive immune responses. Upon antigen recognition they differentiate into antigen-specific effector cells.</p> <p>The two major forms are B - and T -cells, which mediate humoral and cell-mediated immunity:</p>	<p><i>Chemokine 7</i></p>	<p>Chemotaxis (see above)</p>	
	<p>B-lymphocytes (B-cells): produce specific antibodies upon antigen detection via BCR receptor. Memory B-cells provide immune memory.</p>	<p><i>Lymphocyte antigen 75</i></p>	<p>Acts as an endocytic receptor to direct captured antigens from the extracellular space to a specialized antigen-processing compartment.</p>
<p>T-lymphocytes (T-cell): mature in thymus and own a T-cell receptor which recognizes antigen-MHC complex on cell surfaces. T-helper cells activate macrophages and help antibody production in B-cells. Cytotoxic T-cells kill infected cells. Memory T-cells provide immune memory.</p>	<p><i>Lymphocyte cytosolic protein 2</i></p>	<p>Positive role in promoting T-cell development and activation</p>	<p>unknown</p>
	<p><i>CD45 (leukocyte common antigen)</i></p>	<p>Transmembrane tyrosine phosphatase found on all leukocytes (white blood cells) and plays a crucial role in the function of these cells. It positively regulates T-cell and B-cell antigen receptor signaling.</p>	<p>unknown</p>
	<p><i>Interleukin 10</i></p>	<p>Cytokine produced by regulatory T-cells and tends to supress lymphocyte response (controls over-reaction of immune response)</p>	<p>unknown</p>
	<p><i>Tyroprotein kinase</i></p>	<p>Enzyme that specifically phosphorylates tyrosine residues in proteins. They are critical in the cytokine receptor signaling pathways that lead to T- and B-cell activation</p>	<p>unknown</p>
	<p><i>Lymphocytes</i></p>	<p>(Measurements of lymphocyte cells with flow cytometer)</p>	
<p>Referred from (Press & Evensen, 1999; Tort <i>et al.</i>, 2003; Magnadóttir <i>et al.</i>, 2005; Magnadóttir, 2006; Zapata <i>et al.</i>, 2006; Swain & Nayak, 2009; Workenhe <i>et al.</i>, 2010; Murphy, 2011; Uribe <i>et al.</i>, 2011; Rauta <i>et al.</i>, 2012; Small <i>et al.</i>, 2013; Sunyer, 2013; Foey & Picchietti, 2014; Whittington <i>et al.</i>, 2015) (Uniprot, Wikigenes)</p>			

2. Parental effects

While the genotype of an offspring is determined by the maternal and paternal DNA sequence that merge together in the zygote, the offspring's phenotype is influenced by a plethora of environmental factors and has evolved with much greater plasticity (Bonduriansky & Day, 2009; Bonduriansky, 2012; Scheiners, 2014). Responsible for this developmental plasticity is the non-Mendelian parental contribution initiated by environmental variation in the parental generation (Bonduriansky & Day, 2009; Bonduriansky, 2012; Burgess & Marshall, 2014). Parental effects are, thus, defined as inherited environmental effects known to arise from the mother (maternal effect) and the father (paternal effect) apart from the nuclear genes, shaping the phenotype, life-history and fitness of their progeny (Kirkpatrick & Lande, 1989; Rossiter, 1996; Mousseau & Fox, 1998a). As in most species mothers and offspring share the same environment, the maternal phenotype is considered to be a central environmental condition, experienced during early development of an organism (Wade, 1998). The phenotype of the mother does not only define the time and place of offspring production, but also offspring density, size, condition, behaviour, and vulnerability to diseases (Wade, 1998). Since the paternal investment or father-offspring influence is low in the majority of species (Kokko & Jennions, 2008), paternal effects were usually assumed to be predominantly defined through Mendelian transmission of genes, or were regarded as rare phenomenon of species with strong paternal care (Crean & Bonduriansky, 2014). However, this view is challenged, as nowadays a rapidly growing number of examples indicate that paternal effects are occurring in a variety of organisms (Heijmans *et al.*, 2008; Carone *et al.*, 2010; Roth *et al.*, 2010; Curley *et al.*, 2011; Crean *et al.*, 2012; Rando, 2012; Jiang *et al.*, 2013; Crean & Bonduriansky, 2014; Eggert *et al.*, 2014; Kaufmann *et al.*, 2014; Stein & Bell, 2014; Gutierrez-Galve *et al.*, 2015). Hence, fathers are found to shape the offspring's phenotype to a higher extent than initially expected (Kaufmann *et al.*, 2014; Curley *et al.*, 2011; Crean *et al.*, 2012). Non-genetic paternal effects might be facilitated by the transmission of epigenetic, somatic, morphological and behavioural cues (see §2.4 and Crean & Bonduriansky, 2014). The mechanisms of paternal transmission are still not well understood. But sperm and seminal-fluid (*i.e.* spermatozoa and associated proteins) can convey more non-genetic (epigenetic, sncRNA, proteins, lipids) factors, that might facilitate paternal effects than originally believed (Chow *et al.*, 2003; Crean *et al.*, 2012; Jiang *et al.*, 2013; Kumar *et al.*, 2013; Eggert *et al.*, 2014).

Such parental effects are a key source of trans-generational phenotypic plasticity (TGP) that allow for immediate responses to present or future environmental disturbances (Uller, 2008; Bonduriansky & Day, 2009; Burgess & Marshall, 2014) and is an important mechanism to

Introduction

buffer populations against environmental stressors (Sunday *et al.*, 2012; Reusch, 2014). For instance, parents in stressful environments can prime their offspring for experienced stressor leading to offspring with a higher phenotypic plasticity that perform better in response to particular stressor (Herman *et al.*, 2012). As such, parental effects might become adaptive when genetic effects (mutations) do not respond fast enough to changing ecological selection pressures (*i.e.* parasites) (Marshall & Uller, 2007).

Yet, little is known about time frames of non-genetic parental effects and if they are removed or accumulate over generations (Herman *et al.*, 2012). So far, it has been reported that environmentally (abiotic stressors) induced parental effects persisting beyond the border of one generation exists in vertebrates (Pembrey *et al.*, 2005; Youngson & Whitelaw, 2008; Pembrey, 2010; Donelson *et al.*, 2012; Shama & Wegner, 2014), invertebrates (Magiafoglou & Hoffmann, 2003; Hafer *et al.*, 2011; Lock, 2012; Bitume *et al.*, 2014), and plant species (Molinier *et al.*, 2006; Herman *et al.*, 2012). However, controlled experiments and empirical data that investigate parental and filial phenotypes under biotic stress (pathogen exposure) over time are still rare. To address the evolution and impact of multi-generational parental effects on infectious disease resistance and host-parasite coevolution, we need to experimentally test how non-genetic effects over more than one generation interact, *i.e.* if both parents and all four grandparents contribute to the display of the offspring phenotype, as these effects may be of synergistic impact (see §2.1 and §2.2).

The vast diversity of parental effects is of crucial importance for a successful upbringing of the progeny and their reproductive future. Parental effects are responsible for effective predator defense mechanisms (Agrawal *et al.*, 1999), acclimation to abiotic environmental changes (Sunday *et al.*, 2012; Munday *et al.*, 2013; Shama & Wegner, 2014), and induced disease resistance in offspring (Mitchell & Read, 2005; Goellner & Conrath, 2008). The magnitude of disease resistance is thereby indirectly influenced by the nutritional status, behavioural cues, as well as immune experience of their parents (Rosengaus *et al.*, 1998; Mitchell & Read, 2005; Goellner & Conrath, 2008; Frost *et al.*, 2010). The investigation of parental impact on disease resistance continuously kept scientists busy for decades (Ehrlich, 1892; Brambell, 1969; Brambell, 1970a; Kowalczyk *et al.*, 1985; Schmid-Hempel, 2005a; Roth *et al.*, 2009; Roth *et al.*, 2010) leaving a diversity of open questions and phenomena to be discovered.

2.1. Trans-generational immune priming

Parents (mothers and/or fathers) and offspring share the same or similar environments and most likely are exposed to similar parasitic and pathogenic environments. Immune priming over generations can, thus, prepare offspring for the parasite environment experienced by their parents and can be beneficial when parasite presence is predictable (Agrawal *et al.*, 1999). The transmission of parental parasite experience that subsequently leads to an enhanced offspring immune defense is known as trans-generational immune priming (TGIP) (Grindstaff *et al.*, 2003; Moret, 2006; Hasselquist & Nilsson, 2009; Moreau *et al.*, 2012; Roth *et al.*, 2012). TGIP is the most important non-genetic effector mechanism to cope with the current parasite fauna and allows a much faster response than genetic effectors, indicating its adaptive characteristics (Agrawal *et al.*, 1999).

The well-known concept of passive transfer of maternal immunity (passive immunity) is more than 120 years old and was investigated by Paul Ehrlich for the first time in 1892. He studied the immunity transfer (antibodies) from mothers to their offspring over the placenta, milk or eggs (Brambell, 1958; Brambell, 1969; Brambell, 1970a). He was the first, to find that maternal immune priming is enabling the offspring to handle pathogens of the maternal environment, an important trade during early life stages when the adaptive immune system of newborn is naïve and most susceptible (Grindstaff *et al.*, 2006; Boulinier & Staszewski, 2008; Hasselquist & Nilsson, 2009). As this phenotypic plastic effect is enhancing the immune status of the offspring and is also affecting their growth, fecundity and survival, TGIP can ultimately even result in the facilitation of genetic fixation and become adaptive (Marshall & Uller, 2007; Uller, 2008; Burgess & Marshall, 2014). It was later discovered that TGIP evolved independently in mammals, birds, and fish (Patterson *et al.*, 1962; Hasselquist & Nilsson, 2009; Swain & Nayak, 2009; Jiménez de Oya *et al.*, 2011). Bird and fish females provide maternal antibodies over the egg yolk, where they enhance early offspring immune response. It was shown that the amount of antibodies transferred to chicks corresponds to the antibody concentration in the blood during the pre-laying period (Gasparini *et al.*, 2002). Surprisingly, also invertebrates, accomplish TGIP, yet, by other mechanisms than antibody-mediated immunity (Little *et al.*, 2003; Sadd *et al.*, 2005; Watson *et al.*, 2005; Freitak *et al.*, 2009; Roth *et al.*, 2010; Tidbury *et al.*, 2011; Freitak *et al.*, 2014). Recently, it was discovered in honey bees that the nutrition protein vitellogenin acts as a carrier of immune priming signals by transporting bacteria fragments into the eggs (Salmela *et al.*, 2015). As in conventional sex roles, the physiological connection between father and offspring is rather restricted, and sperm cells were considered to be too small to carry globular immune proteins (Wassarman *et al.*, 2001), TGIP was determined as a pure maternal trait (Boulinier &

Staszewski, 2008; Hasselquist & Nilsson, 2009). Astonishingly, despite the physical barrier and the general opinion that offspring parasite environment will be more similar to the mothers, immune priming effects have also been discovered to originate from fathers (Roth *et al.*, 2010; Zanchi *et al.*, 2011). This is making the study of paternal immune priming and its underlying mechanisms a completely new area of investigation (see § 2.2 - 2.4).

Since TGIP facilitates passive protection for the period when the immune system is developing, selection for adaptive TGIP is supposed to be strongest during early life stages (Hasselquist & Nilsson, 2009). So far it was considered to last until the maturation of the acquired immune system of new-borns is achieved (Rossiter, 1996). When parasite dispersal is limited due to environmental barriers and assemblages of parasite genotypes are stable over generations, it merges in a high probability to encounter the same parasite genotypes. If these conditions are met, long-lasting TGIP is highly beneficial as it allows a faster reaction towards the current parasite assemblage in the environment based on the immunological experience transferred by the parents (Lui, 2000). Hence, ecological conditions such as stable virulence and pathogenic environments may select for persistent or even multigenerational TGIP. To date, only rare evidence supports the existence of TGIP effects in vertebrates beyond the establishment of adaptive immunity during maturation (Lemke *et al.*, 1994; Reid *et al.*, 2006), while in invertebrates and plants immune priming effects are multigenerational maintained (Luna *et al.*, 2012; Norouzitallab *et al.*, 2014). Long-lasting TGIP in vertebrates may challenge the current view about host-parasite coevolution dynamics (Mostowy *et al.*, 2012; Tidbury *et al.*, 2012), reproductive processes like mate choice and sexual selection, reproductive strategy, and investment into immune defense.

2.2. Biparental immune priming

Fish eggs and juveniles strongly depend on maternally derived nutrition and immune factors as they are released in the aquatic environment and develop externally, exposed to an environment rich of microbial pathogens during a period when their adaptive immune system is not established (Magnadóttir *et al.*, 2005; Swain & Nayak, 2009; Foey & Picchiatti, 2014). As the formation of lymphoid tissue, B-cells and T-cells takes time to mature, the ability to synthesize adaptive immunity via antibodies is delayed (Magnadóttir *et al.*, 2005; Swain & Nayak, 2009; Foey & Picchiatti, 2014). Depending on the fish species and environmental conditions, it can take several weeks post hatch (2-4 weeks) to reach full immune competence (Swain *et al.*, 2002; Swain *et al.*, 2006; Zapata *et al.*, 2006). To overcome this sensitive time period of high vulnerability, females provide their eggs with immune

Introduction

components of innate and adaptive immune system such as IgM (antibodies), complement factors, macroglobulin, lectins and antimicrobial peptides (Magnadóttir *et al.*, 2005; Magnadóttir, 2006; Swain *et al.*, 2006; Swain & Nayak, 2009; Zhang *et al.*, 2013) (Table 1). However, as free living larvae are extremely susceptible to a range of biotic and abiotic factors during critical post larval stages, which are connected with high mortality (May, 1974), several fish species have evolved parental care strategies (nesting behavior, mouth breeding) (Clutton-Brock, 1991) and viviparity (live birth) in various forms (Guillette Jr, 1987; Wourms & Lombardi, 1992; Blackburn, 2014). For instance, mothers of mouth brooding *Chichlids* can transfer antibodies to their fry through mucus from their mouth cavity (Mor & Avtalion, 1990; Sin *et al.*, 1994). Likewise, in fish species with paternal care or male sexually dimorphic traits (anal glands), also fathers provide their eggs or fry with immune components (antibodies/antimicrobial peptides in mucus) facilitating biparental protection of their offspring (Giacomello *et al.*, 2006; Buckley *et al.*, 2010; Pizzolon *et al.*, 2010).

The internal reproduction and provision of developing embryos via different forms of placentation in a sterile environment, as it is the case for several viviparous fish species (23 species), even increases the protection and survival of the fry (Guillette Jr, 1987; Blackburn, 2014). *Syngnathid* fishes (seahorses, pipefishes) are the only known examples of sex-role reversed fish species with male pregnancy (Stölting & Wilson, 2007). In the family of *Syngnatidea* (pipefish) females equip their eggs with nutrition and immunity before they transfer them into the paternal brood-pouch in which they are nursed with additional nutrients until they are released as independent free swimming juveniles (Berglund *et al.*, 1986; Carcupino *et al.*, 1997; 2002; Ripley & Foran, 2006; 2009; Kvarnemo *et al.*, 2011).

In the sex-role reversed pipefish selection for biparental immune priming is very likely due to the intimate paternal connection and the fact that the offspring are born and exposed to paternal parasite pressure. The pipefish *Syngnathus typhle* serves as a unique model system, because it shares an intense paternal physical connection to its progeny (see §3). Hence, it is possible to test whether and to which degree fathers are able to additionally transfer immunity to their offspring during male pregnancy. A biparental transfer of immune protection could synergistically enhance the immune competence of the offspring strengthening their abilities to face harmful pathogens.

2.3. Costs of immune priming

Mounting an immune response upon infection carries energetic costs and is demanding an organism to allocate energy resources between development, maturation, reproduction, and growth (Sheldon & Verhulst, 1996; Lochmiller & Deerenberg, 2000; Schmid-Hempel, 2005b; Ardia *et al.*, 2011; Ardia *et al.*, 2012; Contreras-Garduño *et al.*, 2014). Immune responses thus, come at a cost for other life-history traits as its activation might raise the metabolic rate by up to 32% of resting metabolic rate (Martin *et al.*, 2008). This indicates that a steady state of induced immunity through TGIP must be a costly strategy, as the parentally transferred immune products stimulate the juveniles to mount a costly immune response (Lochmiller & Deerenberg, 2000). Parents in turn are facing a resource allocation trade-off, especially under extreme environmental conditions (*e.g.* extreme temperature, starvation, high parasite load) by either investing into their own survival, or in a costly transfer of substances into the eggs (Grindstaff *et al.*, 2003).

In addition, when parasite genotypes are not stable and fluctuate frequently over generations, trans-generational immune priming comes at an additional cost (von Schantz *et al.*, 1999), as the investment into current parasite genotypes might be unused. For instance, bumblebees exposed to a mismatching pathogen environment, primed offspring were more susceptible to different parasites than the control (Sadd & Schmid-Hempel, 2009). Hence, costs of TGIP need to be taken into account as they potentially shape the net benefit of immune priming effects within and across generations (Roth *et al.*, 2010; Zanchi *et al.*, 2011; Contreras-Garduño *et al.*, 2014). Theoretical studies predicted that, trans-generational immune priming, especially when it harbors costs in terms of reduced reproduction, it might increase parasite prevalence and destabilize population dynamics on the long-term (Tidbury *et al.*, 2012). Thus, experimental and theoretical work is required to understand the complex interplay between costs and benefits of TGIP.

2.4. Epigenetic inheritance

Emerging studies revealed evidence that extreme environmental conditions such as nutrition, temperature, chemicals or radiation but also behavior (parental care, aggressions) can induce 'epigenetic' changes in an organism leading to an altered phenotype that might be maintained during following generations (Clutton-Brock, 1991; Campos *et al.*, 2014; Jablonka & Lamb, 2014; 2015; Ragunathan *et al.*, 2015; Rassoulzadegan & Cuzin, 2015; Szyf, 2015). The term 'epigenetic' ('over'-genetics) was firstly used by Waddington (1953) to explain how genotypes produce phenotypes by interacting with the environment. To date 'epigenetics' refer to all non-genetic heritable changes apart from DNA-based changes (mutations) that may lead to altered gene expression and could create phenotypic differences between individuals (Berger *et al.*, 2009).

Two multi-generational flag studies found incredible epidemiological evidence for the transmission of epigenetic memory upon dietary stress in humans. Offspring of Dutch mothers who experienced a famine during war (1944) while being pregnant were less obese than their siblings that were not exposed to the famine (Ravelli *et al.*, 1976). Sixty years later, a comparison of blood samples from their offspring (F1-generation) showed that famine exposure was associated with hypermethylation of an insulin gene, directing to the possibility that DNA methylation might be involved (Heijmans *et al.*, 2008; Veenendaal *et al.*, 2013). Further, Pembrey *et al.* (2005, 2010) examined harvest, birth and death records of an isolated town in Northern Sweden and unexpectedly found a correlation between food supply during the early life of paternal grand-parents and mortality rate caused by diabetic disease in their grand-children (Pembrey *et al.*, 2005; Pembrey, 2010). The following emerging studies confirmed that maternal and paternal life experiences induce epigenetic changes, which could be inherited over several generations (Hunt & Simmons, 2000; Rassoulzadegan *et al.*, 2006; Heijmans *et al.*, 2008; Youngson & Whitelaw, 2008; Curley *et al.*, 2011; Rando, 2012; Jablonka & Lamb, 2014; Gutierrez-Galve *et al.*, 2015; Jablonka & Lamb, 2015; Szyf, 2015). Such non-genetic transmission of parental experience might result in stable, new traits to responses of new environmental conditions (Szyf, 2015). Non-DNA based inheritance as one form of multi-generational plasticity might be more widespread than previously thought, leading to claims for a more wide-ranging view of inheritance and adaptation apart from the neo-Darwinian synthesis that only allows DNA-based changes (Danchin *et al.*, 2011). Hence, the concepts of heredity and evolutionary change are currently under discussion (Danchin *et al.*, 2011; Jablonka & Lamb, 2014; Pigliucci & Finkelman, 2014).

To what extent these epigenetic effects cause adaptive advantages and play a role in driving phenotypic variation of traits within several generations is so far not well understood. Additionally, little knowledge exists about epigenetic mechanisms that could potentially regulate host-pathogen interactions and the development of host immune defense strategies (Gómez-Díaz *et al.*, 2012). Numerous studies indicate that epigenetic mechanisms fulfill a crucial role in the regulating the transcription of immune-related genes upon infections (Huang & Wells, 2014; Marr *et al.*, 2014; Okamoto *et al.*, 2014; Smale *et al.*, 2014). Pathogen exposure of the parental generation might mediate epigenetic marks that could be passed on as protective cues to the offspring and subsequent generations. How epigenetic effects are involved in trans-generational immune priming is still unresolved and empirical data that follow parental and filial phenotypes though time in controlled experiments are rare.

2.4.1. DNA methylation

DNA methylation is the process of adding a methyl group (CH₃) to the 5' carbon of cytosine bases (Bird, 2002). This chemical modification of the DNA occurs in general at regions where a cytosine comes together with guanine, at so called CpG sites, which are spread all over the genome (Bird, 2002; Jaenisch & Bird, 2003). Specifically in promotor regions such CpG sites are highly accumulated forming 'CpG islands' of 200–4000 bp (Craig & Bickmore, 1994; Baron, 2012). A hypermethylation of CpG islands (70-80%) located within or next to promotor regions of specific genes (*i.e.* immune gens) initiates packing of chromatin structure over heterochromatin remodeling, which is resulting in the silencing of gene activity (Grewal & Moazed, 2003). Active promoters were shown to only cover a low percentage of methylation (4-7%). This indicates that suppression of gene activity through DNA hypermethylation depends on the methylation status of CpG islands in front of the promotor region (Weber *et al.*, 2007). DNA methylation negatively regulates gene expression and is necessary for all cell differentiation processes such as stem cell differentiation during embryogenesis (Gama-Sosa *et al.*, 1983; Monk *et al.*, 1987; Razin & Shemer, 1995; Lee *et al.*, 2015). This implements that DNA methylation patterns are tissue specific and frequently rearranged during early developmental processes (Gama-Sosa *et al.*, 1983; Monk *et al.*, 1987). The chemical reaction of DNA methylation is mediated by enzymatic action of several evolutionarily-conserved DNA methyltransferases (DNMTs) (Bestor, 2000) (Table 2). Vertebrates possess a maintenance DNA methyltransferase *DNMT1*, which copies complementary marks of newly-replicated DNA by recognizing the hemimethylated sequences inherited from daughter strands (Bestor, 2000). On the contrary, *DNMT3a* and

Introduction

DNMT3b conduct *de novo* methylation, which allows the formation of new methylation marks on unmethylated DNA (Okano *et al.*, 1999; Bestor, 2000; Mitchell *et al.*, 2014). As *de novo* methylation via DNMT3a/b causes new chemical modifications of the DNA (Okano *et al.*, 1999; Mitchell *et al.*, 2014) and play an essential role in maternal and paternal imprinting (Kaneda *et al.*, 2004) they are potentially a crucial factor for epigenetic changes based on environmental stress. In contrast to mutations, DNA methylation patterns are reversible, highly dynamic and can change several times throughout the life of an organism (Monk *et al.*, 1987; Bird, 2002; Lee *et al.*, 2015).

According to exciting recent studies, such methylation marks can not only be passed on to daughter cells during DNA replication but are even heritable across generations when epigenetic changes are manifested during meiosis in germ cells (Jablonka & Lamb, 2015; Szyf, 2015). Still, primordial germ cells and shortly after fertilization DNA methylation marks are completely erased before being newly established during embryogenesis and development (Reik *et al.*, 2001). Due to that reason it was initially thought that trans-generational transmission through DNA methylation marks would be impossible (Reik *et al.*, 2001; Szyf, 2015). However, current evidence claims that certain elements escape DNA methylation erasure, while establishing persistent trans-generational inheritable DNA methylation marks (Lane *et al.*, 2003). So far, epigenetic inheritance of immune priming upon pathogen exposure (biotic stress) within and across generations involving differential DNA methylation marks was only found in plants (Dorantes-Acosta *et al.*, 2012). Interestingly, Luna *et al.* (2012) could confirm that *Arabidopsis thaliana* transmitted acquired immunity against *Pseudomonas syringae* between several plant generations through hypermethylation of defense-related genes. Whether the same mechanism could also apply for maternal and paternal trans-generational immune priming observed in vertebrates is still unexplained.

2.4.2. Histone modifications

Chromatin in its condensed form consists of nucleosomes that are linked together like beads on a string, while the DNA is wrapped tightly around an octamer of core histones (two for each H2A, H2B, H3 and H4) (Berger, 2002). The accessibility of the DNA sequence for transcription processes is regulated by addition or removal of acetyl groups to histone tails, which in turn changes the positive charge of the histones and its affinity to negatively charged DNA (Wade *et al.*, 1997; Zhang & Reinberg, 2001; Berger, 2002). When histones are acetylated the chromatin structure is loose and accessible for the transcription machinery. In turn deacetylated histones bind DNA more tightly and transcription is silenced (Perry & Chalkley, 1982; Berger, 2002). Histone acetylation is catalyzed by histone acetyltransferase (HAT) and removed by histone deacetylase enzymes (HDAC) (Holbert & Marmorstein, 2005) (Table 2). The balance between HAT and HDAC activity has a significant impact on gene regulation, especially throughout development and also during diseases (Mukherjee *et al.*, 2015). Further, N-terminal tails of histones are additionally modified by methylation, phosphorylation, and ubiquitination, which are all necessary to accomplish specific functions during the gene transcription process (Zhang & Reinberg, 2001; Berger, 2002; Holbert & Marmorstein, 2005). Histone methylation is catalyzed specifically at lysine residues by histone methyltransferases (HKMT) (Peters & Schübeler, 2005), or removed over histone demethylases (Kooistra & Helin, 2012) (Table 2). For instance, trimethylation of lysine 4 on histone 3 (H3me3K4) is usually associated with promotor regions of active genes, while trimethylation in H3me2K9 and H3meK27 acts as a suppressive signal of gene expression (Gaydos *et al.*, 2014; Szyf, 2015).

Histone methylation and acetylation patterns are heritable and latest studies suggest that histones pass on epigenetic signals across generations (Campos *et al.*, 2014; Gaydos *et al.*, 2014; Jones, 2015) and that “histones act as carriers of epigenetic information” (Ragunathan *et al.*, 2015). Further, Youngblood *et al.* (2010) suggested that histone modifications may be associated with immune memory following a viral infection in CD8+ T-cells. Still, to date it remains unclear to which extent histone modifications can be maintained in the gametes of vertebrates, and which mechanisms are responsible for heritable changes mediated by histones.

Introduction

Table 2: Investigated genes responsible for epigenetic regulation mechanism are listed with their specific functions and impact on gene expression activity.

Gene	“Writer”	“Reader”	Specific Functions	Gene expression
DNA methylation C5-methylcytosine (5mC)				
DnMt1	DNA methyltransferase 1	maintenance methylation	methylation of hemimethylated DNA strands; maintenance of DNA methylation during replication; negative regulation of gene expression; establishment and regulation of tissue-specific methylation patterns	Repressing marks; Silencing of gene activity
DnMt3a	DNA methyltransferase 3a	<i>de novo</i> methylation	involved in embryo development and gamete generation; hypermethylation of CpG islands; methylation-dependent chromatin silencing; negative regulation of gene expression; regulation of genetic imprinting in germ cells and sperm	Repressing marks; Silencing of gene activity Genomic imprinting
DnMt3b	DNA methyltransferase 3b	<i>de novo</i> methylation	DNA methylation on CpG islands; transcription corepressor activity; inactivation of X chromosome by DNA methylation; methylation-dependent chromatin silencing	
DNA and protein modifications N6-methyladenine (6mA)				
N6-admet	N(6)-adenine-specific DNA methyltransferase	DNA methylation on adenine (6mA)	controls development in higher eukaryotes; regulates gene expression (mechanism in vertebrates unknown)	Unknown (Huang & Chen, 2015)
HemK2	HemK methyltransferase	DNA MTase and protein-(glutamine-N5) MTase reader domain	methylation of polypeptide chain release factors; translational termination	Unknown (Nakahigashi <i>et al.</i> , 2002)
Histone tail modifications				
Histone acetylation and deacetylation				
BROMO	Histone acetyltransferase (HAT)	acetylation (KAT2B-bromodomain)	histone acetyltransferase activity with core histones; positive regulation of transcription	Activating marks (open chromatin structure)
MYST1	Histone acetyltransferase 1	acetylation (MYST1-domain)	histone acetyltransferase activity; transcriptional activation; transcription factor binding	Gene activating
HDAC1	Histone deacetylase 1	hydrolysis of N(6)-acetyl-lysine residues	histone deacetylase activity specific for lysine residues; chromatin modification & organization; circadian regulation of gene expression; negative regulation by host of viral transcription; negative regulation of gene expression	Deactivating marks (condensed chromatin structure)
HDAC3	Histone deacetylase 3	hydrolysis of N(6)-acetyl-lysine residues		Gene silencing
HDAC6	Histone deacetylase 6	de-acetylation of core histones	histone deacetylase activity on the N-terminal part of core histones (H2A,H2B,H3,H4); negative regulation of gene expression	
Histone methylation and demethylation				
ASH2	Histone methyltransferase	methylation (SET1-ASH2 complex)	histone methyltransferase activity (H3-K4 specific); cell differentiation; embryo development; positive regulation of transcription	Gene activating
JmjC-PHD	Lysine-specific-demethylase 5B	demethylation of 'Lys-4' (H3K4me)	histone H3-K4 demethylation activity; trimethyl-H3-K4-specific; negative regulation of transcription; transcription corepressor activity	Gene silencing
NO66	Lysine-specific-histone demethylase	demethylation of 'Lys-4' (H3K4me)(H3K36me)	histone H3-K4 demethylation; histone H3-K36 demethylation; negative regulation of transcription	Gene silencing
TPR	Lysine-specific demethylase 6A	demethylation of (H3K27me3)	histone demethylase activity (H3-K27 specific); chromatin organization; embryonic development; mesodermal cell differentiation; regulation of gene expression	Gene activating
References: (Perry & Chalkley, 1982; Wade <i>et al.</i> , 1997; Okano <i>et al.</i> , 1999; Zhang & Reinberg, 2001; Berger, 2002; Bird, 2002; Kaneda <i>et al.</i> , 2004; Peters & Schübeler, 2005; Mitchell <i>et al.</i> , 2014; Huang & Chen, 2015; Szyf, 2015)				

3. The broad-nosed pipefish (*Syngnathus typhle*) as model organism

The model organism of my thesis is the broad-nosed pipefish *Syngnathus typhle* that lives in eelgrass (*Zostera marina*) meadows along the coast of the Baltic Sea (Figure 1). *S. typhle* belongs to the order of *Syngnathiformes* and family *Syngnathidae* (pipefishes and seahorses) and they are the only known animals that have evolved vivipary in males (male pregnancy) (Stölting & Wilson, 2007; Ahnesjö & Craig, 2011; Wilson & Orr, 2011).



Figure 1: Original painting of the broad-nosed pipefish *Syngnathus typhle*
Library & Archives of Royal Ontario Museum Original Image British
Zoology. Class III. Reptiles IV. Fish. London. Printed in 1726.

Pipefish females transfer their eggs into the male's brood pouch (Figure 2, 3) in which they are fertilized and nursed for approx. 45 days until they are released as free living juveniles several weeks later (Berglund *et al.*, 1986; Vincent *et al.*, 1995; Wilson *et al.*, 2003). A male can receive eggs from several females and a female may give eggs to several males (polygynandrous mating) (Berglund *et al.*, 1989). Thereby it was suggested that females have a higher potential reproductive rate as they produce more eggs than males are able to breed (Berglund *et al.*, 1989). As life-history strategies are reversed, females are under stronger sexual selection than males since they can increase their lifetime fitness over the number of mating, whereas males invest more into higher immunity to enhance longevity (Roth *et al.*, 2011). It was commonly assumed that the pipefish females deposit nutrition proteins (vitellogenin) and immune protection (antibodies) into the egg yolk and believed that the males provide only a protective environment for their offspring during their highly sensitive life stage (Ripley, 2009).

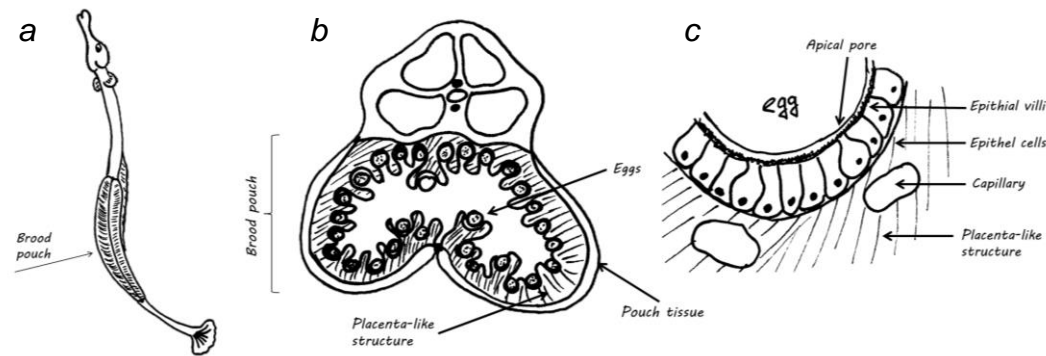


Figure 2: Brood pouch morphology of broad-nosed pipefish *Syngnathus typhle* (a) pregnant male pipefish with developed and enclosed brood pouch (b) transverse section of a pregnant male pipefish, showing the placenta-like structure with capsules for individual embryos incorporated into the epithelium (c) zoom into the placenta-like structure. The egg lies on the surface of villous epithelial cells with specialized transporter cells that have their base close to the capillary and an apical pore opening into the lumen. These pores may be used as conduit by which ions, oxygen and nutrition proteins are transported out of the placenta-like structure to the eggs/embryos to maintain their development (Figure is adapted from Kornienko (2001)).

Several studies demonstrate that the paternal brood pouch of *Syngnathids* takes over the functioning of gas exchange, osmoregulation and waste removal (Azzarello, 1991; Carcupino *et al.*, 1997; 2002; Ripley, 2009). Remarkably, fathers also supply their developing embryos with essential ions, oxygen and nutrition proteins over a placenta-like structure (Azzarello, 1991; Carcupino *et al.*, 1997; 2002; Ripley, 2009). Although the mechanism by which the nutrition transfer over a placenta-like structure occurs remains unanswered, a few studies described the brood pouch tissue of pregnant males as a fleshy, highly vascularized structure in which eggs are surrounded by an epithelium with a complex network of blood vessels (Figure 2-3) (Carcupino *et al.*, 1997; 2002). Each egg lies on the surface of villous epithelial cells with transporting cells that have their base close to the capillary and an oval-shaped apical pore opening into the lumen (Figure 2 b, c) (Kornienko, 2001). These apical pores may be used as conduit by which essential factors are transported out of the paternal blood stream into the egg lumen (Figure 2 c) (Azzarello, 1991; Ripley, 2009; Ripley & Foran, 2009; Kvarnemo *et al.*, 2011). On top of that, the brood pouch tissue of pipefish and seahorses also expresses immunological active compounds, enabling an immune protection of the developing embryos (Melamed *et al.*, 2005; Small *et al.*, 2013; Whittington *et al.*, 2015). *Syngnathids* are frequently infected with a variety of pathogens such as *Vibrio* bacteria which are causing detrimental *Vibriosis* (Alcaide *et al.*, 2001; Balcázar *et al.*, 2010). The “pseudo-placenta” like structure and close connection between embryos and paternal blood vessels most likely could mechanistically also allows a paternal transfer of immunity.

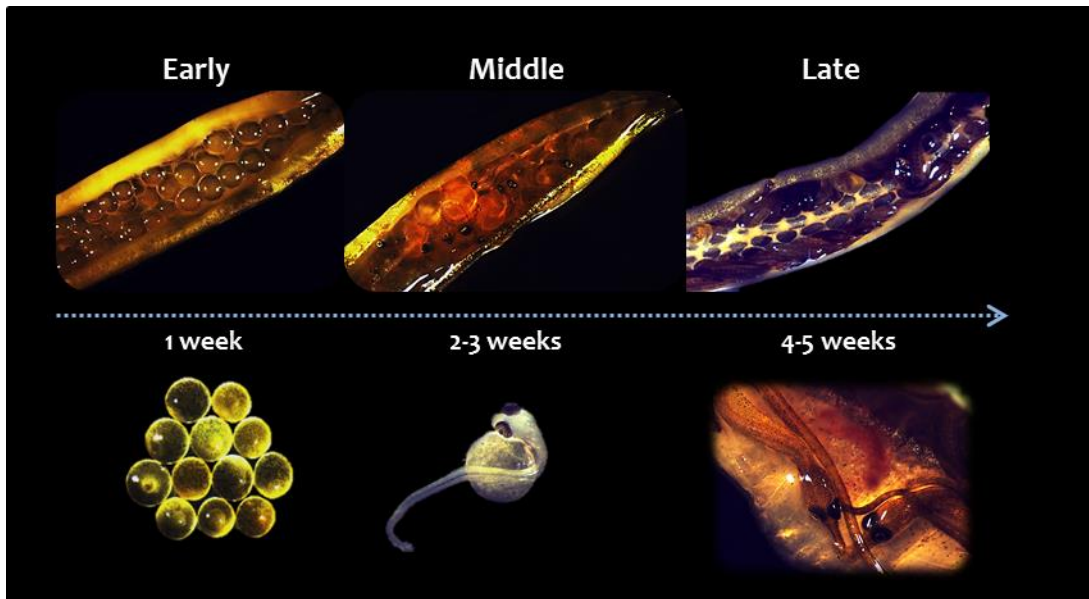


Figure 3: Brood pouch and embryo stages broad-nosed pipefish *Syngnathus typhle* with increasing male pregnancy (Early - 1 week, Middle - 2/3 weeks, and Late - 4/5 weeks).

S. typhle is established as a comparatively new model organism in the context of evolutionary ecology with emphasis on its unique sex-role reversal, evolution of male pregnancy, host-parasite interaction and its cryptic adaptive immunity (Roth *et al.*, 2011; Birrer *et al.*, 2012; Roth *et al.*, 2012a; Haase *et al.*, 2013). Recent studies have investigated adaptation and coevolution of *S. typhle* with local bacteria (*Vibrio*) phylotypes (Roth *et al.*, 2012a) and characterized its immune gene repertoire (Haase *et al.*, 2013). Interestingly, a loss of an important immune organ the spleen, MHC II and other essential adaptive immune effectors were discovered, which are leading to open questions concerning their alternative defense strategies (Haase *et al.*, 2013). Further studies showed a sexual immune dimorphism and a reversal of Batemans principle, as males instead of females possess a higher immunity (Roth *et al.*, 2011). Males with a higher immune status could use this advantage to transfer immunity to the offspring during male pregnancy. In the study of Roth *et al.* (2012) we could show that pipefish males are able to transfer immune protection to their progeny upon immune challenge with heat-killed *Vibrio* bacteria. This unique possibility of combined maternal and paternal immune priming facilitated an additive biparental immune priming effect, which would likely lead to a higher protection and survival upon pathogen infections. In this thesis I explored this fascinating strategy of biparental immune protection in *S. typhle* more in detail.

Thesis outline

The results of my thesis are structured in four chapters whereby each chapter is written in a form of a manuscript including introduction, material & methods, results and discussion. The first chapter is accepted to be published in the journal “Zoology” (in the special issue “Host-parasite coevolution”). The second chapter is in the submission process at the “Journal of Evolutionary Biology”. The third chapter is in review at “Molecular Ecology” (in the special issue “Epigenetic Inheritance”). The fourth chapter sums up the results of two conducted pilot studies and is in preparation for Short Communications at the journal “Fish and Shellfish Immunology”.

The aim of my thesis was to explore evolutionary and ecological benefits and costs as well as the underlying mechanism of multigenerational biparental immune priming in the sex-role reversed pipefish *Syngnathus typhle*. Using this unique model organism permitted me to examine maternal and paternal investment beyond the provisioning of the DNA. I could disentangle the specificity and interactive extent of biparental immunological provisioning and potential improvement of offspring adaptive plasticity to cope with the local pathogen fauna.

Chapter 1

Initially, I experimentally investigated maternal, paternal and combined biparental influences on offspring immunity and life-history traits to evaluate associated costs and benefits of TGIP in *S. typhle*. As such, I exposed the parental generation to heat-killed bacteria, which served as immune challenge, and bred individuals in a fully reciprocal mating design. Furthermore, I included two different bacteria types (*Vibrio* and *Tenacibaculum*) to unravel maternal and paternal investment into offspring immunity over maturation time. Therefore, I challenged two consecutive age classes of the F1-generation (one-week-old juveniles and four-month-old juveniles) with heterologous and homologous parental bacteria types and measured gene expression of 29 immune genes, 15 genes associated to epigenetic regulation, immune cell activity and life-history traits. With this study I intended to investigate whether both parents equally or asymmetrically affect the immune system of their offspring via sex-specific effector mechanism. I primarily focused on the question whether maternal and paternal immune priming would result in an additive biparental immune priming effect, which would lead to an induced offspring protection. As individual investment into immunity and immune response is energetically costly, resource allocation trade-offs with other life-history parameters were expectable. To include potentially energetic costs of biparental immune priming I further evaluated life-history traits such as size, mass, hepatosomatic index and sexual reproduction

throughout four consecutive life stages (one-week-old, four-month-old and six-month old F1-juveniles and one-week-old F2-juveniles). In addition, I assessed epigenetic regulation genes, which allowed me to address the involvement of epigenetic effects that could facilitate biparental immune priming.

Chapter 2

The second chapter is tightly linked to the first chapter since I performed the statistics on a subset of the data generated by the initially explained experiment but focused solely on bacteria-specific immune priming effects. By using this approach I could address the transmission of maternal and/or paternal bacteria specificity and the impact of bacteria specific parental immune priming on offspring immunity. As such, I assessed whether immune priming was favourable conducted for specific bacteria types and its lastingness throughout maturation. Likewise, I analysed life-history parameters using the focus on bacteria specific parental life-history effects and evaluated potential associated trade-offs. Here, I also investigated genes connected to epigenetic regulation mechanisms which might be crucial players in the regulation of bacteria specific immune gene expression and its non-genetic inheritance across one generation.

Chapter 3

In the third chapter I investigated multi-generational biparental immune priming in the sex-role reversed pipefish *S. typhle*. Following the first filial generation (F1-juveniles) I continued the main experiment and bred individuals until the second generation (F2-juveniles). By leaving the F1-generation untreated (naïve) and exposing the F2-juveniles with the identical heat-killed bacteria types as their grandparents, I was able to assess grandparental effects on immune gene expression and the life-history trait size of grand-offspring. At the same time, the expression of epigenetic regulation genes should give rise to the predominant question whether epigenetic modulation is involved in grandparental immune priming.

Chapter 4

The fourth chapter focuses on two pilot studies, which I conducted to assess the mechanistic aspect of paternal immune priming in *S. typhle*. In the first pilot study I analysed the expression of immune and epigenetic regulation genes in the brood pouch tissue of non-pregnant versus pregnant males to evaluate key genes mediating protection and paternal immune priming. In the second pilot study I examined the presence or absence of soluble immunoglobulin (IgM) in the pipefish plasma and its potential paternal transfer to the embryos.

Chapter I



Chapter I

Title picture of Chapter I: Close-up picture of developing embryos in the paternal brood pouch of *Syngnathus typhle*; approximately 2 weeks post hatch (Copyright: Anne Beemelmans).

Biparental immune priming in the pipefish *Syngnathus typhle*

Anne Beemelmans¹ and Olivia Roth¹

¹*Helmholtz-Centre for Ocean Research Kiel (GEOMAR), Evolutionary Ecology of Marine Fishes, Düsternbrooker Weg 20, 24105 Kiel, Germany*

Accepted for Zoology (Special Issue “Host-parasite coevolution”), January 2016

Cited in this thesis as *Beemelmans & Roth 2016a*

Abstract

The transfer of immunity from parents to offspring (trans-generational immune priming (TGIP)) boosts offspring immune defense and parasite resistance. TGIP is usually a maternal trait. However, if fathers have a physical connection to their offspring, and if offspring are born in the paternal parasitic environment, evolution of paternal TGIP can become adaptive. In *Syngnathus typhle*, a sex-role reversed pipefish with male pregnancy, both parents invest into offspring immune defense. For the connection between TGIP and parental investment, we need to know how parents share the task of TGIP, whether TGIP is asymmetrically distributed between the parents, and how the maternal and paternal effects interact in case of biparental TGIP.

We experimentally investigated the strength and differences but also the costs of maternal and paternal contribution, and their interactive biparental influence on offspring immune defense over offspring maturation. To disentangle maternal and paternal influence, two different bacteria were used in a fully reciprocal design for parental and offspring exposure. In offspring, we measured gene expression of 29 immune genes, 15 genes associated to epigenetic regulation, immune cell activity and life-history traits.

We identified asymmetric maternal and paternal immune priming with a dominating, long-lasting paternal effect. We could not detect an additive adaptive biparental TGIP impact. However, biparental TGIP harbours additive costs as shown in delayed sexual maturity. Epigenetic regulation may play a role both in maternal and paternal TGIP.

Key words: Maternal and paternal effects; Trans-generational immune priming; Epigenetic effects; Host-parasite interactions

Introduction

The transfer of non-genetic information from parents to offspring is a phenotypic plastic trait that can have fast and long-lasting effects independent of genetic inheritance (Mousseau & Fox, 1998c). Such parental effects are a key source of trans-generational phenotypic plasticity that allow for immediate responses to novel environmental challenges (Boulinier & Staszewski, 2008; Bonduriansky & Day, 2009). For instance, trans-generational immune priming (TGIP), the transfer of immunity from parents to progeny, enhances offspring immune competence and buffers them against impacts of pathogens formerly experienced by their parents (Sadd *et al.*, 2005; Grindstaff *et al.*, 2006; Hasselquist & Nilsson, 2009).

The maternal transfer of immunological active components like antibodies via blood, milk, or egg is of major importance during early life stages (Ehrlich, 1892; Brambell, 1970b; Brambell, 1970a; Boulinier & Staszewski, 2008), when the adaptive immune system is still subject to maturation (Rossiter, 1996; Lindholm *et al.*, 2006). A mechanism that enables parents to pass on their immune components to the progeny, and in this way protect their young during this crucial period, should therefore be adaptive (Marshall & Uller, 2007). Particularly, if the prevailing parasite assemblage is similar across generations, persistent or even multigenerational TGIP might be favoured (Lemke *et al.*, 1994; Reid *et al.*, 2006). TGIP has evolved in vertebrates (Ehrlich, 1892; Grindstaff *et al.*, 2003; Hasselquist & Nilsson, 2009; Jiménez de Oya *et al.*, 2011), but can also be found in invertebrates, where it is achieved by other mechanisms than antibody-mediated immunity (Little *et al.*, 2003; Sadd *et al.*, 2005; Moret, 2006; Freitak *et al.*, 2009; Roth *et al.*, 2010; Freitak *et al.*, 2014; Salmela *et al.*, 2015). TGIP was determined as a maternal trait, because non-genetic immunological experience was supposed to be exclusively passed on via the egg or placenta, while sperm cells were considered too small to carry those (Bonduriansky & Day, 2009). However, lately the potential of parental transfer of epigenetic traits influencing the offspring phenotype was discovered (Jiang *et al.*, 2013; Jablonka & Lamb, 2014; 2015). Epigenetics concern stimuli-triggered changes in gene expression that do not involve an alteration of the nucleotide sequence, but are heritable across generations (Berger *et al.*, 2009; Gómez-Díaz *et al.*, 2012). Subsequently, factors that affect the gene expression but not the DNA sequence can be passed on to the next generation and contribute to the phenotypic plasticity of the offspring (Youngson & Whitelaw, 2008; Jablonka & Lamb, 2014; 2015; Szyf, 2015). Here, RNA molecules (Rassoulzadegan *et al.*, 2006; Rassoulzadegan & Cuzin, 2015), DNA methylation (Jiang *et al.*, 2013; Ci & Liu, 2015) and histone modifications (Campos *et al.*, 2014; Gaydos *et al.*, 2014; Jones, 2015; Ragnathan *et al.*, 2015), factors associated with regulation of gene expression, can be trans-generationally inherited and manifest as stable

epigenetic marks in the following generation (Kappeler & Meaney, 2010; Jablonka & Lamb, 2015). Environmental stressors such as parasites and pathogens manipulate the host's immune system while interfering with histone acetylation/deacetylation processes (Mukherjee *et al.*, 2012; Mukherjee *et al.*, 2015) or DNA-methylation patterns (Marr *et al.*, 2014). To date, epigenetic inheritance of immune priming upon pathogen exposure across generations based on DNA methylation changes was only demonstrated to occur in plants (Dorantes-Acosta *et al.*, 2012; Luna *et al.*, 2012), whereas histone modifications might be associated with inheritable immune memory upon infection in eukaryotes (Youngblood *et al.*, 2010; Ragunathan *et al.*, 2015). However, it is still unresolved, which mechanisms are responsible for mediating immune priming effects that base on epigenetic changes in vertebrates.

If epigenetic factors are involved or biparental if males overcome the mechanistic barrier of limited sperm size, TGIP must not be limited to females. In a beetle biparental TGIP was discovered first (Roth *et al.*, 2010; Zanchi *et al.*, 2011). If both parents transfer their pathogen experience to the progeny, offspring benefits could be more than additive (Roth *et al.*, 2010) resulting in an induced phenotypic plasticity (Jokela, 2010). This could imply an enhanced adaptive potential to handle parasitic infections (Marshall & Uller, 2007). However, investment in immunity is costly and traded-off against other life-history traits like fecundity and/or reproduction (Contreras-Garduño *et al.*, 2014). As specific costs are associated to maternal and paternal TGIP, an asymmetric investment of the parents may have evolved (Zanchi *et al.*, 2011).

Biparental investment in offspring immunity also exists in the sex-role reversed pipefish *Syngnathus typhle* (Roth *et al.*, 2012). Here, females deposit their eggs into a protective brood-pouch of the males, in which they are fertilized and nursed until being released as independent juveniles (Berglund *et al.*, 1986; Kvarnemo *et al.*, 2011). The evolution of sex-role reversal coupled with an internal brooding structure may mechanistically enable a paternal transfer of non-genetic factors. Our previous study (Roth *et al.*, 2012) suggested that sex-role reversal may open the door for biparental influences on offspring immunity.

We here aimed to investigate the connection of TGIP and parental investment by unravelling the strength and differences between maternal and paternal impact and their interactive biparental investment into offspring immune protection in the sex-role reversed pipefish *S. typhle*. We measured the effects of maternal, paternal and biparental TGIP on offspring immunity and life-history by utilizing an extended set of 29 immune genes and 15 genes related to epigenetic modification processes as well as immune cell activity and life-history traits.

Chapter I

We hypothesized (I.) that the combination of maternal and paternal immune priming, *i.e.* biparental immune priming, results in a synergistic effect. By including two different allopatric bacteria species for maternal and paternal exposure (*Vibrio* spp. and *Tenacibaculum maritimum*) in a fully reciprocal design, we explored whether bacteria specific biparental immune priming may harbour additive immune priming effects.

We further hypothesized (II.) that parental immune priming in *S. typhle* is asymmetric and directly connected to the reversed mating system with a stronger long-lasting paternal immune priming effect. To assess the maintenance of parental immune priming we compared one-week old and four-month-old juveniles. We expected (III.) that the parental immune priming effect ceased upon maturation, with maternal effects ceasing earlier than paternal effects.

We assessed genes associated to epigenetic regulation processes like DNA methylation and histone acetylation/methylation to explore the connection between epigenetic modification and regulation of immune gene expression and its non-genetic transfer over generations. We predicted (IV.) that changes in epigenetic traces in the offspring generation show parental sex-based differences.

Finally, we addressed costs and benefits of TGIP by monitoring key life-history traits (size, mass, condition factor, hepatosomatic index) of the juveniles and evaluated the time to reach sexual maturity. We hypothesized (V.) that strength of maternal, paternal and biparental immune priming predicts the severity of a resource allocation trade-off and expected paternal and biparental immune priming to be associated with greater energetic costs.

Material and Methods

Parental generation (F0-treatment)

Broad-nosed pipefish *Syngnathus typhle* were caught in the south-western Baltic Sea (54°44'N; 9°53'E, Germany) in spring 2013 and hosted under Baltic summer conditions (15 psu, 18°C, and 14-16 h light), while being fed twice a day with mysids. For the experimental setup mature pipefish were either left naïve or injected with 50 µl of 10⁸ cells/mL heat-killed bacteria solution according to Roth *et al.* (2012). Two novel bacteria species *Vibrio* spp. (Italian isolate, I2K3) (Roth *et al.*, 2012a) and *Tenacibaculum maritimum* (DSM No.: 17995) (Suzuki *et al.*, 2001) were applied to exclude any confounding effects with previous pathogen encounter in the wild. Both bacteria types are influencing the immune system of fish in a specific way by causing two different types of diseases: 'Flexibacteriosis' (Bernardet *et al.*, 1990; Kolygas *et al.*, 2012) and 'Vibriosis' (Alcaide *et al.*, 2001). This allowed us to analyse the transmission of bacteria specific immune memory by mothers and fathers respectively, and potential additive immune priming effects.

As to do so, mating pairs of immune challenged individuals were formed in 36x80 L aquaria connected to a semi-flow through system with maternal, paternal or biparental immune challenge in a fully reciprocal mating design: 1.(MatV+ x PatT+), 2.(MatT+ x PatV+), 3.(MatN- x PatT+), 4.(MatN- x PatV+), 5.(MatT+ x PatN-), 6.(MatV+ x PatN-), 7.(MatN- x PatN-) with (V+) for *Vibrio*, (T+) for *Tenacibaculum* and (N-) for naïve (seven parental treatments were replicated eight times, resulting in 56 breeding pairs (families) in separate breeding tanks) (Figure S1). A sham exposure with PBS was not implemented as former studies suggest that TGIP is not confounded by the sterile needle injection (Roth *et al.*, 2012) and sham injection with PBS does not induce immune gene expression (Birrner *et al.*, 2012).

Filial generation 1 (F1-treatment)

All 56 couples mated successfully after the immune challenge and about five weeks later (July 2013), one-week-old offspring (eight days post birth) were exposed to homologous and heterologous heat-killed bacteria (*Vibrio* (V+) and *Tenacibaculum* (T+)), or stayed naïve as control (N-). Therefore, a needle was dipped into a solution of 10⁹ cells/ml heat-killed bacteria solution and the juveniles were pricked intraperitoneally into the body cavity and kept for 20 hours in separate tanks. After incubation time, juvenile's body standard length was measured, and whole body was sampled for RNA extraction. Since we were only using families with a minimum clutch size of 15 juveniles, 28 families each with 4 replicates per parental treatment were chosen. For each of the 28 families, 15 offspring were used and

Chapter I

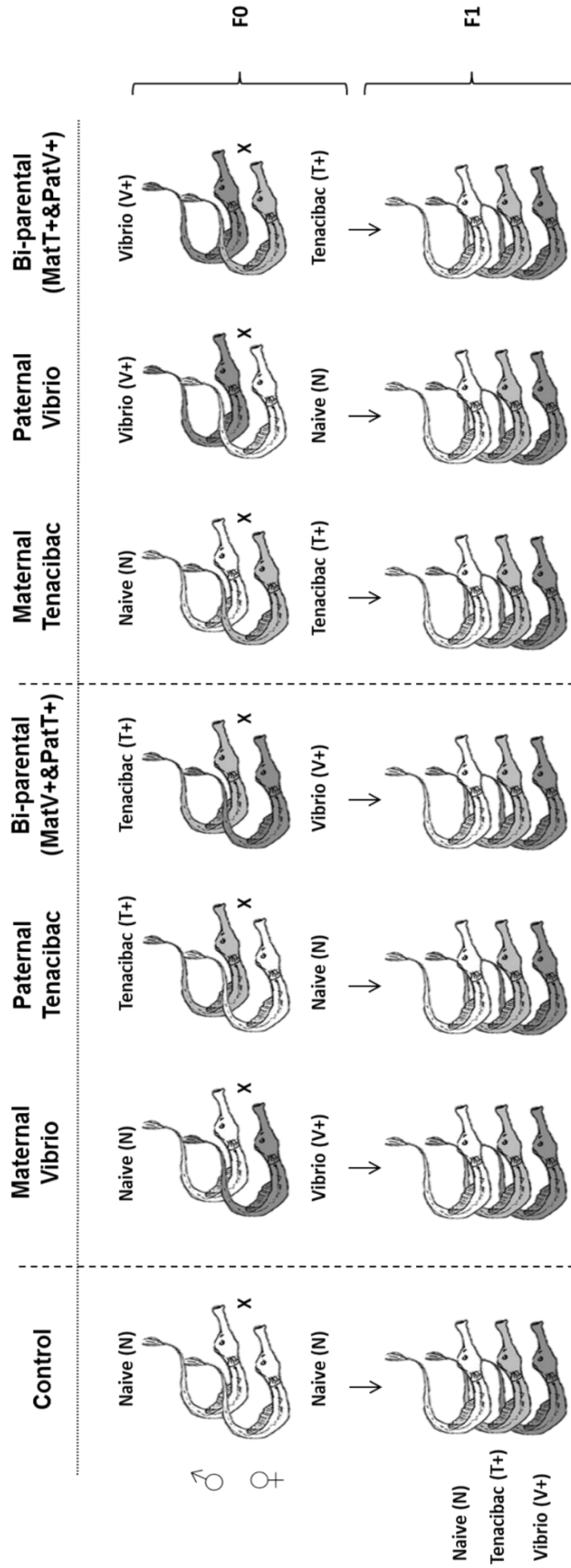
distributed over the three treatments resulting in 5 samples per offspring treatment and a total number of 420 sampled juveniles.

Remaining F1-offspring (approx. 420 pipefish) were pooled according to their 7 parental treatment groups and transferred into 36x80 L aquaria connected to a semi-flow through circulation system using three tank replicates per parental treatment and a density of 20 pipefish per tank. Four-month-old juveniles (not fully sexually mature) were applied to the same procedure as described above to compare TGIP between different developmental stages. Consequently, the F1-juveniles from the 7 different parental treatment groups were injected with 20 μ l 10^8 cells/mL heat-killed bacteria species *Vibrio* (V+) and *Tenacibaculum* (T+) or stayed naïve (N), using 3 replicates per offspring treatment. In total, 9 individuals of the 7 parental treatment groups at two consecutive sampling events were randomly collected out of the tanks, resulting in a total number of 126 sampled juveniles. After incubation time (20 hours), juvenile body standard length and body mass was measured before they were sacrificed. The liver was weighed and a Hepatosomatic index (HSI), ratio of liver mass relative to body mass ($HSI = \text{liver mass [g]} / \text{body mass [g]}$), calculated to evaluate the energy status of the fish (Chellappa *et al.*, 1995). Furthermore, a fish body Condition Factor (CF) was determined after the method of Frischknecht (1993).

For characterizing the immune response, and to reach information about the activity of the adaptive and innate immune system, we measured the absolute number of lymphocytes and monocytes in the head kidney and blood according to the protocol of Roth *et al.* (2011).

The RNA extraction of 420 whole body samples of early stage juvenile pipefish (one-week post birth) and 126 gill tissue samples of late stage juvenile pipefish (four-month post birth) was performed and gene expression of 44 target genes and 4 housekeeping genes was measured for all 546 samples using a Fluidigm BioMarkTM based on 96.96 dynamic arrays according to Beemelmanns & Roth (2016c in review).

Remaining F1-offspring (approx. 280 pipefish) were left immunologically untreated and for maintaining an equal density ratio (20 pipefish per tank), they were pooled within the maternal, paternal and biparental treatment groups and randomly distributed into three tank replicates. The time for the F1-offspring to reach sexual maturity was recorded.



Supplement Figure S1 (in publication Beemelmanns & Roth 2016 this figure is placed in the supplement): Experimental design to explore maternal, paternal and biparental immune priming effects and parental bacteria specificity in the pipefish *Syngnathus typhle* over one generation. In total seven parental treatment groups were applied whereby each sex of the parent was exposed either to a different bacteria strain or stayed without any treatment: **1.** Control (both parents naïve (N)), **2.** only maternal *Vibrio* (MatV+) or **3.** *Tenacibaculum* (MatT+), **4.** only paternal *Vibrio* (PatV+) or **5.** *Tenacibaculum* (PatT+), both parents received different bacteria exposures **6.** Biparental (MatV+PatT+) and **7.** Biparental (MatT+PatV+). F1 offspring were one-week and four-month post birth applied to the homologous or heterologous bacteria challenges as their parents or received no challenge as control. To test for uni-parental immune priming effects the parental treatment groups were pooled into following four: **1. Control:** Naïve (control); **2. Maternal:** Maternal *Vibrio* (Mat V+) & Maternal *Tenacibaculum* (MatT+); **3. Paternal** *Vibrio* (PatV+) & *Tenacibaculum* (PatT+) **4. Biparental** Bi-(MatV+PatT+) & Bi-(MatT+PatV+).

Data Analysis and statistics

The gene expression data was processed and analysed according to Beemelmanns & Roth (2016c in review) with subsequent modifications. All plots and statistical tests were performed in R (v3.2.2 R Core Team, 2015) and PRIMERv6 (Clarke & Gorley, 2006). Multivariate statistics were used to infer differences in the entire expression pattern of 29 immune genes and 15 genes associated to epigenetic regulation (§1.3 Introduction Table 1; Table S2 of Chapter 3).

Data analysis was conducted on three different levels to address our hypotheses. First, we evaluated maternal, paternal and biparental immune priming effects in combination with the two pathogen species (*Vibrio* and *Tenacibaculum*). Consequently, we assessed the effect of seven parental treatment combinations on gene expression (29 immune genes), life-history parameters (body size, body mass, body condition factor (CF), and hepatosomatic index (HSI)) of one-week-old juveniles and four-month-old juveniles and immune cell count measurements (lymphocyte/monocyte ratio of blood and head kidney) for the latter. On the second level, we explored the impact of maternal and paternal effects in the biparental treatment combination by examining the F0-parental x F1-offspring interaction terms. We elucidated whether maternal and paternal effects in a biparental combination had additive immune priming effects.

On the third level, we analysed maternal, paternal and biparental effects on genes associated to epigenetic regulation processes (DNA methylation and histone acetylation/deacetylation and methylation/demethylation). Parental bacteria-specific groups were combined merging the parental *Vibrio* and *Tenacibaculum* treatments into four parental treatments.

The PERMANOVA model (`vegan` package - `adonis` function in R) was based on a Bray-Curtis Matrix of non-transformed $-\Delta\text{Ct}$ -values, applying `‘F0-parents’` and `‘F1-offspring’` treatment as fixed factors and including `‘Family’` as nested factor within the `‘F0-parents’` treatments and `‘Size’` as covariate to correct for size relevant influences. The analysis of similarity (ANOSIM) was performed with the software PRIMERv6 (Clarke, 1993; Clarke & Gorley, 2006) according to Beemelmanns and Roth (2016c in review) based on a Bray-Curtis distance matrix and 4th-root transformation, which allowed a pairwise comparison between the levels of parental and offspring treatment. We applied a principle component analysis (PCA) for the evaluation of differential gene expression profiles caused by the parental treatments. Further, we used a between-class analysis (BCA) to illustrate the interaction between F0-parental and F1-offspring treatment (Dolédec & Chessel, 1987; Thioulouse *et al.*, 1995; Chessel *et al.*, 2004). In addition, a corresponding scatterplot was added to demonstrate the contribution of each single gene. Here, the direction of the arrows

determines the correlation between variables (genes) and principle components. The length of the arrow is directionally proportional to the contribution of each gene to the total variability (Oksanen *et al.*, 2007).

Statistical univariate approaches were applied for life-history parameters and immune cell counts. Assumptions for variance normality for each response variable were graphically examined and tested with Shapiro-Wilk-test and Levine test after Box-Cox transformation using Jmp9 (SAS Institute Inc., USA). A linear model was fitted using the fixed factors 'F0-parents' and 'F1-offspring', the non-independent factor 'Family' or respective 'Sampling day' or 'Tank' nested into 'F0-parents' while taking 'Size' as covariate. For the body size analysis the same model without covariate was used. All significant ANOVAS for F0-parental and F1-offspring effects were followed by post-hoc Tukey's HSD test.

Results

I.) Maternal, paternal and biparental immune priming effects in one-week vs four-month-old juveniles

One-week-old F1-juveniles - Immune gene expression

29 candidate genes connected to pathways of the innate, the adaptive immune system and complement component system were significantly affected upon the parental bacteria treatments (PERMANOVA-*immune*: $F_{6,377}=12.44$, $p<0.001$, Table S1, Figure 1A). The principle component analysis (PCA) revealed both a difference between the two applied parental bacteria treatments (*Vibrio* and *Tenacibaculum*) and a parental sex-based effect (Table S2, Figure 1A). F1-offspring of mothers and fathers challenged with *Vibrio* or *Tenacibaculum* displayed a significantly differential expression profile along the second principle component (18.9 % of total variation) clustering according to the specific bacteria species (ANOSIM-*immune*: MatV+ vs MatT+ $p=0.001$; PatV+ vs PatT+ $p=0.001$, Table S2, Figure 1A). However, both biparental treatment groups clustered together with the maternal-*Tenacibaculum* as well as paternal-*Tenacibaculum* treatments, which included their respective single parental *Tenacibaculum* treatment (Figure 1A). Hence, the biparental treatments were more similar to the maternal and paternal *Tenacibaculum* in comparison to the parental *Vibrio* treatment. Particularly, the paternal *Tenacibaculum* treatment was not significantly different from the biparental treatment containing the respective paternal *Tenacibaculum* treatment (ANOSIM-*immune*: PatT+ vs Bi-(MatV+PatT+) $p>0.05$, Table S2, Figure 1A).

Four-month-old F1-juveniles - Immune gene expression

In four-month-old juveniles the identical 29 immune genes were significantly influenced by the parental bacteria treatments (PERMANOVA-*immune*: $F_{6,98}=3.21$, $p<0.001$, Table S1, Figure 2A). In the multivariate PCA analysis a difference between the two parental bacteria treatments (*Vibrio* and *Tenacibaculum*) independent of the parental sex effect clustered in opposed directions along the first principle component (27.8 % of total variation) (ANOSIM-*immune*: MatV+ vs MatT+ $p=0.001$; PatV+ vs PatT+ $p<0.028$, Table S3, Figure 2A). Both paternal treatments were not significantly different from their respective biparental treatments (ANOSIM-*immune*: PatT+ vs Bi-MatV+PatT+ $p>0.05$ and PatV+ vs Bi-MatT+PatV+ $p>0.05$, Table S3, Figure 2A) indicating a long-lasting paternal immune priming effect.

Four-month-old F1-juveniles - Immune cell counts

The parental immune challenge of four-month-old F1-offspring significantly affected the number of lymphocytes and monocytes in the head kidney (PERMANOVA-*cells.hk*: $F_{6,97}=7.29$, $p<0.001$, Table S1) and blood (PERMANOVA-*cells blood*: $F_{6,97}=4.66$, $p<0.001$, Table S1). The lymphocyte/monocyte-ratio of F1-offspring in the head kidney was strongly elevated by the maternal as well as paternal *Vibrio* treatment (ANOVA-LM-*ratio.hk*: $F_{6,73}=6.4$, $p<0.001$, *TukeyHSD*: C<MatV+, C<PatV+, Table S5, Figure 4). In turn, solely the paternal *Tenacibaculum* treatment was inducing the L/M-ratio in F1-offspring (*TukeyHSD*: C=MatT+, C<PatT+, Table S5, Figure 4). When parents received maternal-*Vibrio* and paternal-*Tenacibaculum* treatment in a biparental combination the LM-ratio was significantly raised in contrast to the control group (*TukeyHSD*: C<Bi-(MatV+PatT+), Table S5, Figure 4) but showed a similar pattern as the maternal or paternal combinations. In contrast to that, the L/M-ratio in the blood of F1-offspring was solely positively influenced upon the paternal *Vibrio* treatment (ANOVA-LM-*ratio blood/F0-parents*: $F_{6,73}=5.15$, $p<0.001$, *TukeyHSD*: C<PatV+, Table S5, Figure 4) and further revealed a *Vibrio*-specific offspring treatment effect (ANOVA-LM-*ratio blood/F1-offspring*: $F_{2,73}=7.89$, $p<0.001$, *TukeyHSD*: F1-N < F1-V+, Table S5)

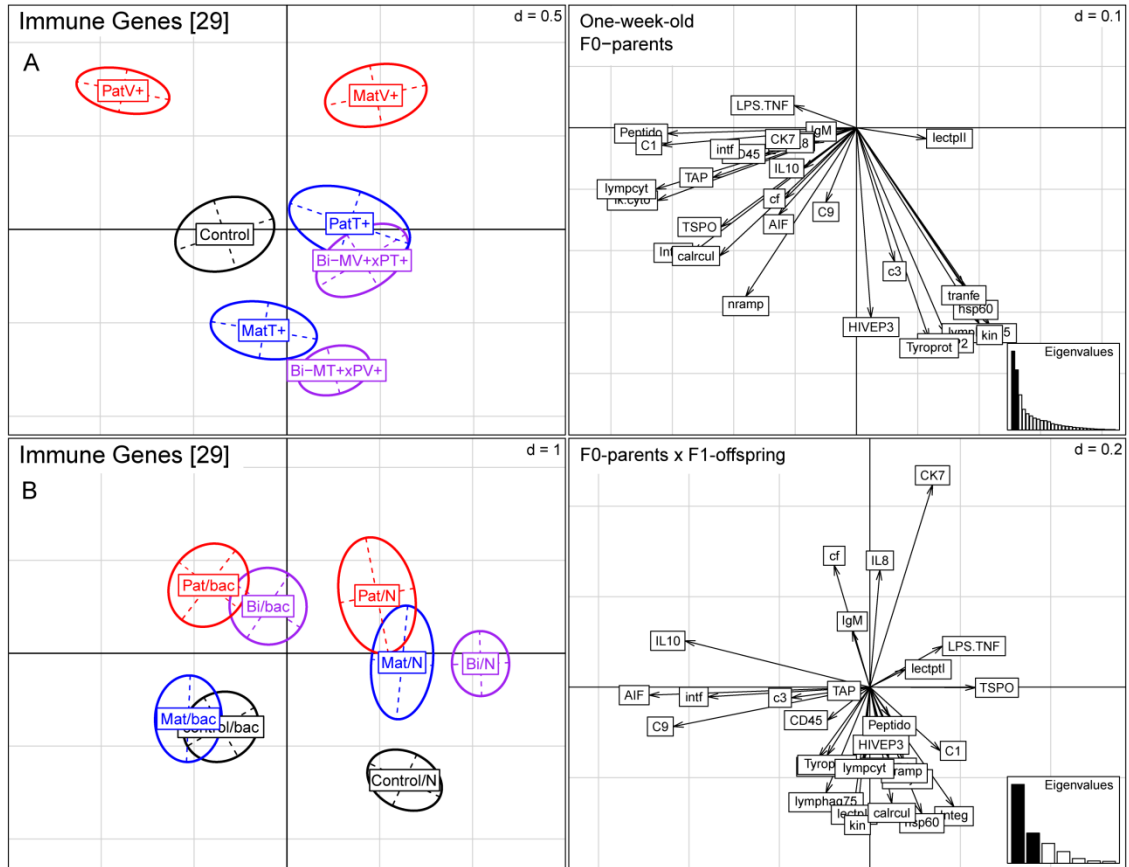


Figure 1: Immune gene expression of one-week-old F1-juveniles (N=420). **Panel 1A:** PCA plot based on 29 immune genes visualizing expression profiles of seven F0-parental treatment groups (parental control (Control), paternal *Vibrio* (PatV+) and *Tenacibaculum* (PatT+), maternal *Vibrio* (MatV+) and *Tenacibaculum* (MatT+), and respective biparental (Bi-(MatV+PatT+)/Bi-(MatT+PatV+)) treatment groups. **Panel 1B:** BCA-plot based on 29 immune genes displaying combined four F0-parental treatment groups (parental control (Cont), paternal (Pat), maternal (Mat), and biparental (Bi)) x F1-offspring treatment (*Vibrio* (F1-V+), *Tenacibaculum* (F1-T+), and Naïve (F1-N)) interaction. Colours represent different parental bacteria species challenges with *Vibrio* in red, *Tenacibaculum* in blue and the joint biparental groups in purple. On the right side for each PCA and BCA plot a corresponding scatterplot is illustrated and represents the contribution of each variable (immune genes) to the total variability. The Eigenvalues are represented by a bar chart in the upper left corner, with the two bars corresponding to the two axes used to draw the PCA or BCA plot (**1A:** PC1: 24.7%, PC2: 18.9%; **1B:** PC1: 52.8%, PC2: 20.1%)

Chapter I

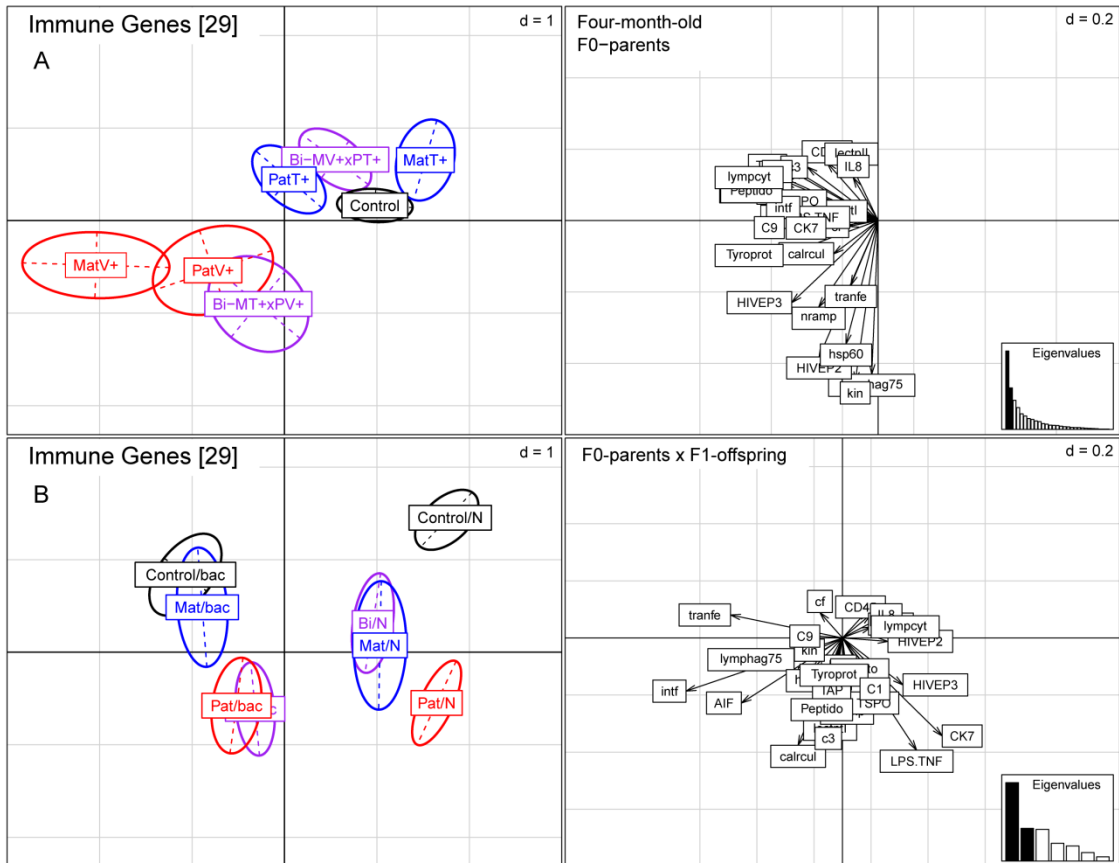


Figure 2: Gene expression four-month old juveniles (N=126). Panel 2A: PCA plot based on 29 immune genes visualizing expression profiles of seven F0-parental treatment groups (parental control (Control), paternal *Vibrio* (PatV+) and *Tenacibaculum* (PatT+), maternal *Vibrio* (MatV+) and *Tenacibaculum* (MatT+), and respective biparental (Bi-(MatV+PatT+)/Bi-(MatT+PatV+)) treatment groups. **Panel 2B:** BCA-plot based on 29 immune genes displaying combined four F0-parental treatment groups (parental control (Cont), paternal (Pat), maternal (Mat), and biparental (Bi)) x F1-offspring treatment (*Vibrio* (F1-V+), *Tenacibaculum* (F1-T+), and Naïve (F1-N)) interaction. Colours represent different parental bacteria species challenges with *Vibrio* in red, *Tenacibaculum* in blue and the joint biparental groups in purple. On the right side for each PCA and BCA plot a corresponding scatterplot is illustrated and represents the contribution of each variable (immune genes) to the total variability. The Eigenvalues are represented by a bar chart in the upper left corner, with the two bars corresponding to the two axes used to draw the PCA or BCA plot (**2A:** PC1: 27.8%, PC2: 14.7%; **2B:** PC1: 41.6%, PC2: 17.3%).

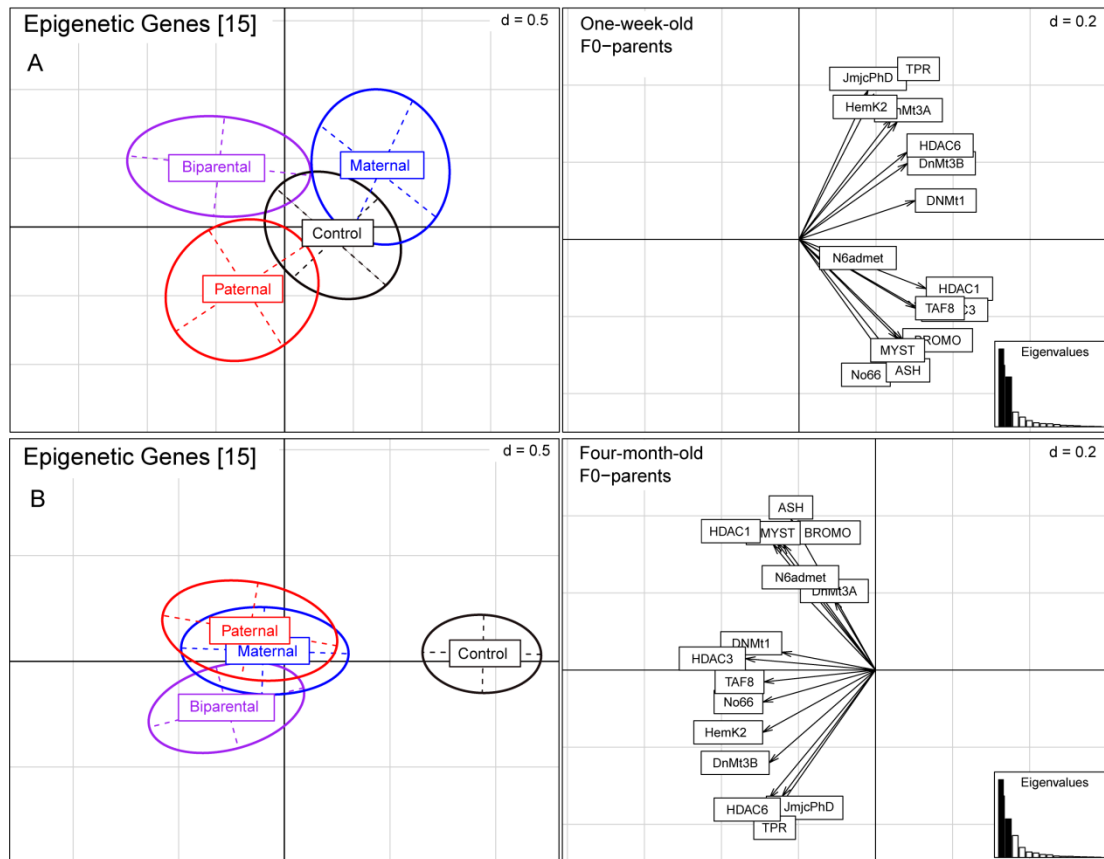


Figure 3: Gene expression of 15 epigenetic genes for one-week old (3A) and four-month old juveniles (3B). PCA plots are based on 15 genes associated to epigenetic regulation processes (DNA methylation and histone modifications) displaying expression profiles of combined four F0-parental treatment groups (parental control (black), paternal (red), maternal (blue), and biparental (purple)) On the right side for each PCA and BCA plot a corresponding scatterplot is illustrated and represents the contribution of each variable (epigenetic genes) to the total variability. The contribution of each gene is thereby symbolized by the length of an arrow which is directional proportional with the contribution of each variable. The scale of the graph is given by a grid size in the upper right corner The Eigenvalues are represented by a bar chart in the upper left corner, with the two bars corresponding to the two axes used to draw the PCA or BCA plot (**3A**: PC1: 44.2%, PC2: 28.3%; **3B**: PC1: 45.8%, PC2: 22.7%).

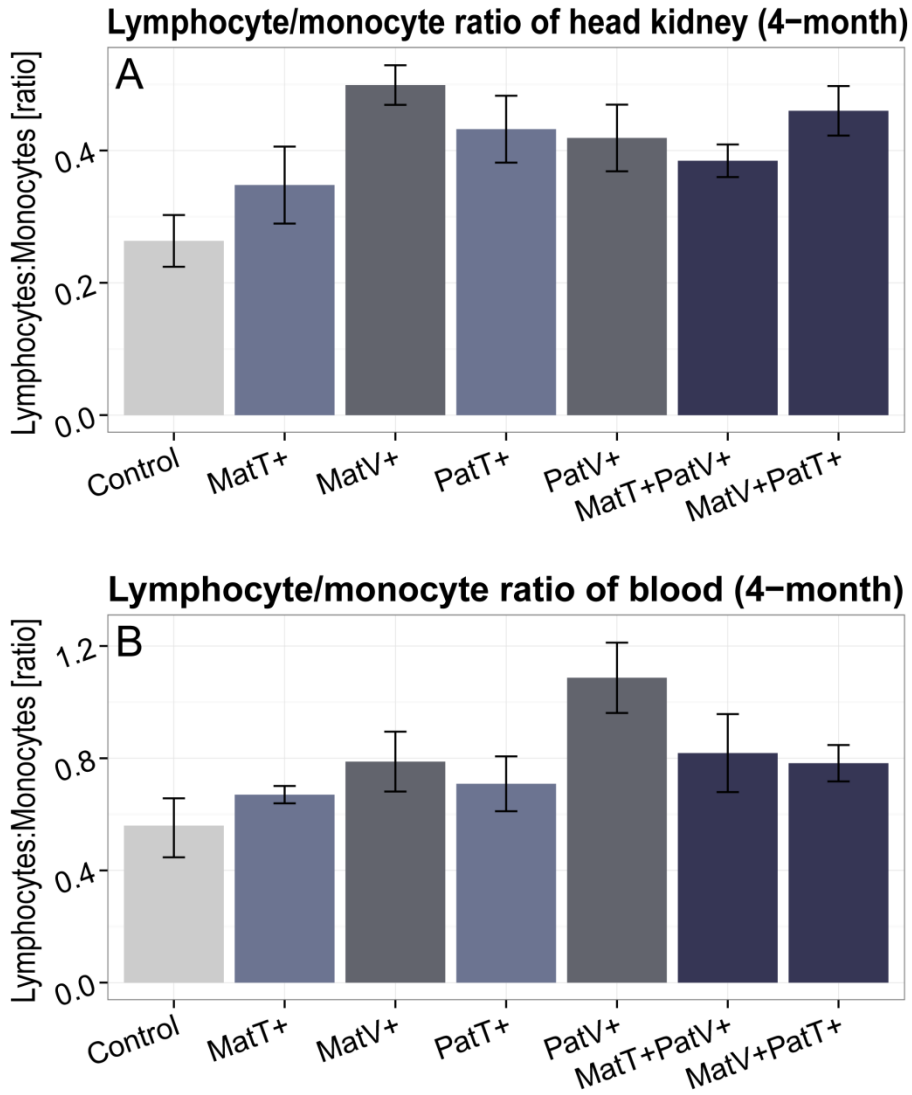


Figure 4: Parental treatment effects based on immune cell count measurements of four-month-old juveniles (N=126) Panel 4A: lymphocyte/monocyte ratio of head kidney, **Panel 4B:** lymphocyte/monocyte ratio of blood. Barplots are grouped according to F0-parental treatments (parental control (Control), maternal *Vibrio* (MatV+) and *Tenacibaculum* (MatT+), paternal *Vibrio* (PatV+) and *Tenacibaculum* (PatT+), and biparental (Bi-(MatV+PatT+)/Bi-(MatT+PatV+)). Respective error bars represent the standard error of the mean (+/-sem).

II.) Interaction of maternal, paternal and biparental immune priming effects and bacteria specificity

One-week-old F1-juveniles - Immune gene expression

Based on the 29 immune genes a significant interaction between the F0-parental and F1-offspring treatment in the one-week-old juveniles was detected (PERMANOVA-*immune* $F_{12,377}=1.75$, $p<0.001$, Table S1, Figure 1B). The applied pairwise comparison of interaction terms showed that the biparental immune priming effect was significantly influenced upon the paternal *Tenacibaculum* immune challenge. *Tenacibaculum* exposed F1-offspring of the biparental treatment (Bi-MatV+PatT+) were not significantly different from *Tenacibaculum* challenged offspring of the single paternal *Tenacibaculum* treatment (ANOSIM-*immune*: Bi-(MatV+PatT+)/F1-T+ vs PatT+/F1-T+ $p>0.05$, Table S2). With paternal *Vibrio* treatment the two groups revealed a significantly different immune gene expression pattern (ANOSIM-*immune*: Bi-(MatT+PatV+) /F1-V+ vs PatV+/F1-V+ $p=0.004$, Table S2). On the contrary, both maternal treatment interaction terms were significantly different from both biparental treatment interactions (ANOSIM-*immune*: Bi-(MatV+PatT+)/F1-V+ vs MatV+/F1-V+ $p=0.023$; Bi-(MatT+PatV+)/F1-T+ vs MatT+/F1-T+ $p=0.001$, Table S2).

To simplify the visualization of the complex interaction terms, the two respective parental and offspring bacteria treatment groups (*Vibrio* and *Tenacibaculum*) were combined into one single bacteria treatment group (*bac*) to focus on parental effects only. As demonstrated in the between-class analysis (BCA), a robust F1-offspring treatment effect is explained by the first principle component (52.8 % of total variation) displaying a clustering of the parental bacteria treatments apart from the parental control (PERMANOVA-*immune*: $F_{2,377}=18.36$, $p<0.001$, Table S1, Figure 1B). Moreover, F1-offspring of the biparental bacteria treatment, which were challenged with bacteria (*F0-biparental/F1-bac*) assembled together with the F1-offspring of the corresponding paternal bacteria treatment (*F0-paternal/F1-bac*), whereas F1-offspring of the maternal treatment exposed to bacteria (*F0-maternal/F1-bac*) were similar to F1-offspring of the control (*F0-cont/F1-bac*) (Figure 1B).

Four-month-old F1-juveniles - Gene expression & Immune cell counts

In four-month-old offspring there was no interaction between F1-parental treatment and F0-offspring treatment on the 29 immune genes (PERMANOVA-*immune* $F_{12,97}=0.83$, $p>0.05$, Table S1, Figure 2B). The lymphocyte/monocyte ratio in the head kidney of four-month-old juveniles revealed a significant F1-parental treatment and F0-offspring treatment interaction

(PERMANOVA-*cells.hk*: $F_{12,97}=2.75$, $p<0.005$, Table S1). The LM-ratio in the head kidney of F1-offspring with paternal *Vibrio* challenge was more pronounced upon homologous *Vibrio* exposure compared to the heterologous *Tenacibaculum* treatment, indicating a paternal *Vibrio* specificity effect on the cellular level (ANOVA-LM-ratio.*hk*: $F_{12,73}=2.9$, $p<0.002$, TukeyHSD: F0-PatV+ x F1-T+ < F0-PatV+ x F1-T+, Table S5, Figure S2).

III.) Maternal, paternal and biparental immune priming effects connected to epigenetic regulation mechanisms

One-week-old F1-juveniles - gene expression of epigenetic regulation genes

In one-week-old F1-offspring 15 genes responsible for epigenetic regulation mechanisms like DNA methylation and histone-modifications displayed a significant parental treatment effect (PERMANOVA-*epigen*: $F_{6,377}=6.6$, $p=0.001$, Table S1, Figure 3A). To reduce complexity and focus on parental treatment effects, we applied an ANOSIM for pairwise comparisons between combined maternal, paternal and biparental effects independent of the two parental bacteria treatments (Table S4). The ANOSIM indicated that the effect was not driven by the parental treatment, since there was no differential expression compared to the control group (ANOSIM-*epigen*: Pat vs C $p>0.05$; Mat vs C $p>0.05$, Bi vs C $p>0.05$, Figure 3A, Table S4). It was rather due to differences within the maternal, paternal and biparental treatments (ANOSIM-*epigen*: Pat vs Mat $p=0.019$; Bi vs Mat $p=0.002$, Bi vs Pat $p=0.004$, Figure 3A, Table S4).

Four-month-old F1-juveniles - gene expression of epigenetic regulation genes

Opposed to the effects we found for one-week old juveniles, epigenetic regulation genes of four-month-old juveniles were affected by the parental bacteria challenges in comparison to the control group (PERMANOVA-*epigen* $F_{6,93}=6.6$, $p=0.001$, Table S1; ANOSIM-*epigen* Pat vs Cont $p=0.001$; Mat vs Cont $p=0.006$, Bi vs Cont $p=0.001$, Figure 3B, Table S4) demonstrated by an opposed clustering on the first principle component of the PCA (45.8 % of total variation). No differences between maternal, paternal and biparental treatment could be identified (Figure 3B, Table S4).

IV.) Costs of immune priming - Life-history traits

One-week-old F1-juveniles - Life-history (size)

One-week-old F1-offspring with parental bacteria treatments had a larger body size than the control group (ANOVA-*size.one-week*: $F_{2,378}=30.04$, $p<0.001$, C<MatV+, C<MatT+, C<PatV+, C<PatT+, Table S5, Figure S3A). F1-offspring from mothers challenged with bacteria even revealed an elevated body-size than offspring with paternal treatment (*TukeyHSD*: PatV+<MatV+, PatT+<MatT+, Table 7, Figure S3A). In addition, F1-offspring of mothers with *Vibrio* exposure were bigger in size than offspring of mothers with *Tenacibaculum* treatment (*TukeyHSD*: MatT+<MatV+, Table S5, Figure S3A).

Four-month-old F1-juveniles - Life-history

In a multivariate approach body size, mass, condition factor (CF) and hepatosomatic index of four-month-old juvenile pipefish were significantly influenced by the parental immune challenge (PERMANOVA-*life-size/mass/CF/HSI*: $F_{6,98}=2.56$, $p<0.004^{**}$, Table S1). However, body length and mass of four-month-old juveniles were only elevated if exposed to a maternal *Vibrio* immune challenge. Offspring with maternal *Vibrio* exposure were 1.35 +/- 0.32 [cm] larger and 0.27 +/- 0.071 [g] heavier as the control group (ANOVA-*size*: $F_{6,98}= 2.86$, $p=0.013$, *TukeyHSD*: C<MatV+, Table S5, Figure S3B; ANOVA-*mass*: $F_{6,98}= 4.43$, $p=0.001$, *TukeyHSD*: C<MatV+, PatT+<MatV+, C<Bi-(MatV+PatT+), Table S5, Figure S3C). Further, the paternal *Tenacibaculum* treatment positively influenced the hepatosomatic index of F1-offspring, which had a larger liver (1.24 +/- 0.24 [mg]) than offspring of the parental control treatment (ANOVA-*HSI*: $F_{6,74}= 2.39$, $p=0.034$, *TukeyHSD*: C<T+, Table S5, Table S1, Figure S3D).

Six-month old F1-offspring - Life-history

Upon a parental immune challenge, juvenile males developed a brood pouch for sexual reproduction one month (average 38.6 +/- 2 days) later than offspring of unexposed parents (ANOVA-*maturity.males*: $F_{3,168}= 158.8$, $p<0.001$, *TukeyHSD*: C<Mat, C<Pat, C<Bi, Table S5, Figure S4). If both parents received the bacterial, males reached sexual maturity even later (6 +/- 1 days) (*TukeyHSD*: Mat<Bi, Pat<Bi, Table S5, Figure S4).

Discussion

I.) Does combined maternal and paternal immune priming cause a more than additive effect?

We explored the synergistic effects of maternal and paternal immune priming applying two different bacteria species (*Vibrio* spp. and *Tenacibaculum maritimum*). Despite our expectations, the biparental effects rather compensated each other and did not pile up to an additive impact. Opposed to this, biparental immune priming with the same bacteria phlotypes for both parents induced synergistic beneficial effects (Roth *et al.*, 2012). This discrepancy may arise due to strong bacteria specific immune priming effects (Beemelmans & Roth 2016b in submission). The impact of biparental treatment in one-week-old juveniles was driven by *Tenacibaculum* bacteria, as offspring receiving biparental treatment combinations revealed a similar immune gene expression profile as their respective single maternal and single paternal *Tenacibaculum* treatments. On the contrary, that offspring treatment group with single parental *Vibrio* treatments clustered far apart from the control group and in opposite direction of the parental *Tenacibaculum* groups points to an antagonistic *Vibrio*-mediated parental effect. Our results thus suggest that the bacteria type determines the strength and degree of biparental immune priming.

This bacteria-specific effect was also found in four-month-old juveniles as indicated by similar immune gene expression profiles. Older juveniles of biparental treatment groups were solely influenced by the paternal bacteria treatment, now independent of the applied bacteria species. Hence, fathers who received a *Vibrio* or *Tenacibaculum* bacteria challenge showed a similar immune gene expression profile as their corresponding biparental treatment combinations, while the maternal groups were significantly different from the respective biparental groups. Thus, also in four-month old juveniles no synergistic parental immune priming effect was detected, but a dominating paternal immune priming effect. In line with this, the immune cell activity in the head kidney and blood of four-month-old juveniles was upregulated upon the paternal treatment compared to the control.

II.) Do we find asymmetric parental immune priming?

Consistent with our hypothesis (II.) we identified differences between maternal and paternal immune priming. Our data indicate asymmetric parental investment into offspring immunity with a stronger paternal than maternal contribution. One-week-old F1-offspring of fathers exposed to *Tenacibaculum* revealed an undifferentiable immune gene expression profile from F1-offspring of parents exposed to biparental treatment including also the paternal *Tenacibaculum* challenge (Bi-(MatV+PatT+) identical to PatT+). In this combination, we further identified a significant interaction term indicating a paternal *Tenacibaculum* specificity effect. This suggests that fathers mediated the biparental immune priming effect. Four-month-old juveniles even revealed stronger paternal immune priming effects independent of the applied bacteria species. When focussing only on the maternal, paternal and biparental interaction term, we found a stable and stronger paternal impact on immune gene expression than the maternal treatment pattern for one-week old and four-month-old juveniles.

F1-offspring of fathers exposed to *Vibrio* or *Tenacibaculum* bacteria induced their immune cell proliferation earlier in the head kidney, the major immune organ of teleosts, in comparison to the control group. In addition, a homologous paternal *Vibrio* exposure increased the cellular immune defense compared to the heterologous combination, which indicates a strong paternal *Vibrio* specificity effect. That the lymphocyte/monocyte-ratio in the blood of F1-offspring was increased only upon the paternal *Vibrio* treatment, suggests that the paternal *Vibrio* treatment caused a fast induction of cellular immunity in the next generation. In case of maternal treatment, only an exposure to *Vibrio* induced cell proliferation in the head-kidney of F1-offspring, which highlights that mothers only provided cellular protection against potentially familiar *Vibrio* bacteria. The parental generation was wild-caught in the Kiel Fjord where pipefish are in constant contact to various *Vibrio* phylotypes (Roth *et al.*, 2012a). During this study, we used a *Vibrio* phylotype isolated from an Italian pipefish (Roth *et al.*, 2012a), which we supposed to be allopatric and most likely novel to the immune system of Baltic pipefish. However, potentially the epitope structure of Italian and Baltic *Vibrio* phylotypes of their natural habitat was not differentiable for *S. typhle* either due to high similarities or due to immunological cross-reactivity. On the contrary, the *Tenacibaculum* bacteria were isolates from a different fish species in the Pacific (Suzuki *et al.*, 2001). Most likely, the short maternal contact to *Tenacibaculum* bacteria was not sufficient to provide immunological long-term protection on the cellular level via the egg. The close connection to the fathers during male pregnancy was long enough to boost the cellular immune defense against both experienced bacteria.

These results support our hypothesis (II.) that in this sex-role reversed mating system challenged fathers have a stronger influence on offspring immunity. Probably as here the fathers are closely connected to the progeny during male pregnancy they transferred immunity for a longer time period. In teleosts, females produce costly eggs, in which they deposit nutrition proteins and immune components over the egg yolk (Bly *et al.*, 1986; Avtalion & Mor, 1992; Swain *et al.*, 2006; Bobe & Labbé, 2010). In the sex-role reversed pipefish *S. typhle* females oviposit their eggs into the male's brood-pouch where they are fertilized and remain during pregnancy (4 weeks) (Berglund *et al.*, 1986). Freshly-hatched juveniles consume proteins of the maternal yolk sac, but are also supplied with additional nutrition and oxygen via the paternal brood-pouch (Azzarello, 1991; Carcupino *et al.*, 1997; 2002; Ripley & Foran, 2006; 2009; Kvarnemo *et al.*, 2011). Consequently, males and females have different possibilities to transfer non-genetic components to stimulate offspring's immunity. In females, a deposition into the egg yolk is most likely, while males may transfer substances through the sperm-fluid and placenta-like structure in the brood pouch. Males and females seem to have evolved different mechanisms to invest into the immune defense of their progeny.

III.) Are there differences in terms of stability between maternal, paternal and biparental immune priming?

Parental effects are acknowledged to be of short duration (Lindholm *et al.*, 2006). Yet, in line with previous findings (Roth *et al.*, 2012) our data indicate that the impact of parental TGIP persisted to four-month-old offspring. The TGIP effects did not cease after the maturation of the adaptive immune system, which takes approximately 4-8 weeks in fish (Magnadóttir *et al.*, 2005). The continuity of TGIP was influenced by the sex of the parent. In accordance with our hypothesis (III.), we validated paternal effects to be longer lasting than maternal effects. In four-month-old juveniles, the impact of paternal immune priming on immune gene expression could not be differentiated from the biparental treatment combinations. This implies that the biparental impact was driven by the paternal treatment. Moreover, immune cell activity in the head kidney came with specificity in case of paternal *Vibrio* exposure, and the paternal immune priming effect was even preserved in the blood. Our results thus indicate that paternal immune priming was driving the biparental effects in four-month-old juveniles.

IV.) Are changes in epigenetic regulation genes attributed to parental sex-based differences?

In one-week-old F1-juveniles genes involved in epigenetic regulation processes like DNA methylation and histone acetylation/methylation revealed no differential expression upon the parental treatment. Yet, we found paternal effects on histone acetylation/deacetylation genes. Through an altered transcriptional regulation, paternal immune priming may influence offspring immune gene expression, potentially facilitated over inheritable immune memory carried across generations by histones (Ragunathan *et al.*, 2015).

In four-month-old juveniles, the same genes were affected by maternal, paternal and biparental bacteria challenge. During development and maturation DNA methylation pattern and epigenetic marks are under constant change (Monk *et al.*, 1987; Razin & Shemer, 1995) and highly flexible in fish embryos (Lee *et al.*, 2015). A consistent pattern of epigenetic traces can thus likely only establish after maturation when programming of parental DNA methylome is complete (Ci & Liu, 2015). Our recent data even suggest that long-lasting parental immune priming effects can be maintained via epigenetic marks and are preserved until the second generation (Beemelmans & Roth 2016c in review). Opposed to the opinion that TGIP ceases after the maturation of the adaptive immune system, long-lasting TGIP in four-month-old juveniles and even in the second generation (Beemelmans & Roth 2016c in review) are mediated through inherited maternal and paternal epigenetic marks.

V.) Are there allocation trade-offs and costs associated to maternal, paternal and biparental immune priming?

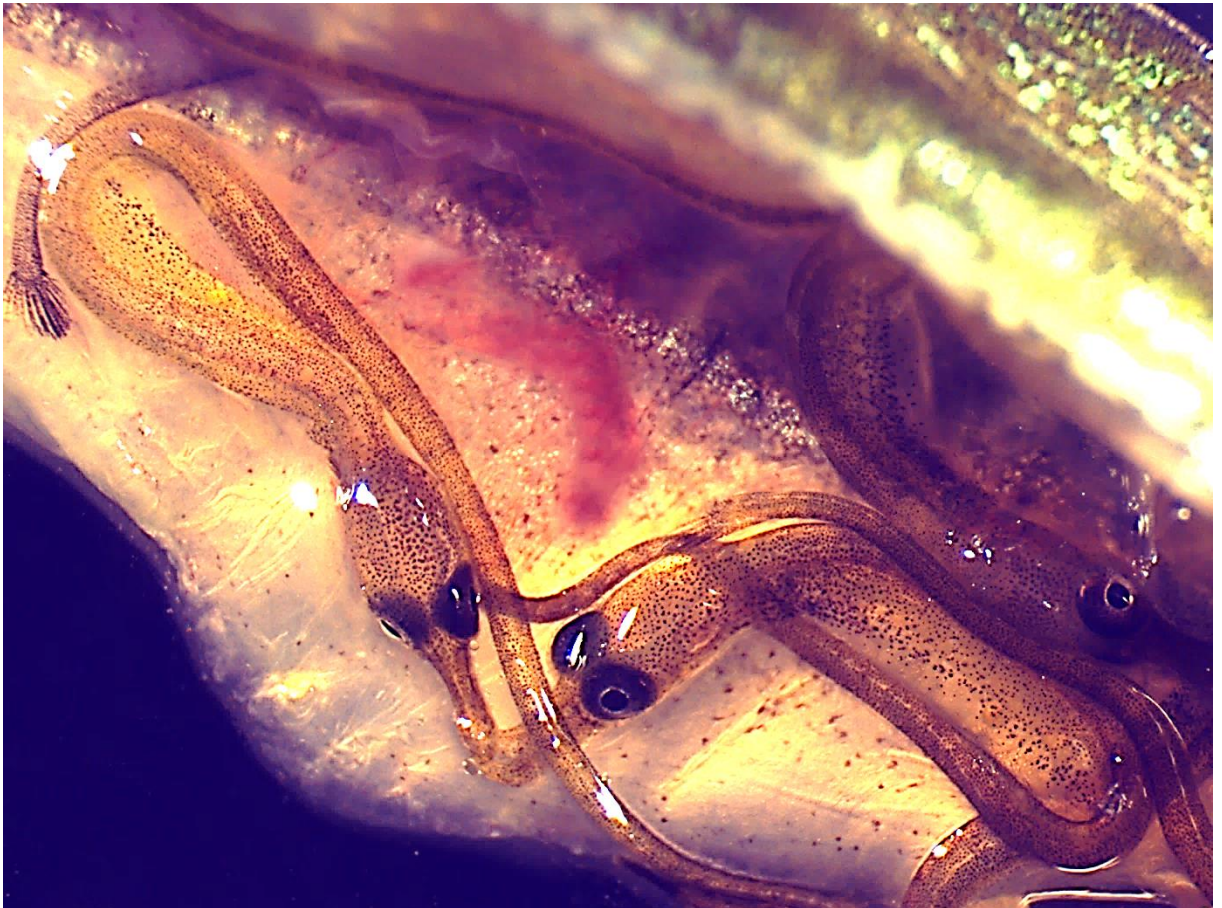
The maintenance of induced immunity is energetically costly and therefore, enhanced levels of immune defense in primed offspring imply trade-offs with other fitness-related traits (Lochmiller & Deerenberg, 2000; Schmid-Hempel, 2011; Zanchi *et al.*, 2011). In line with this, TGIP exhibited costs in terms of prolonged time of reproduction. F1-males of the biparental treatment needed significantly longer to produce a brood-pouch tissue and, hence, reach sexual maturity than individuals of uni-maternal or uni-paternal treatments. This implies high costs of biparental immune priming. As long-lasting paternal immune priming bears equal costs as maternal immune priming in terms of delayed maturity, it suggests that the paternal mechanism of immune transfer is more effective and less costly. However, maternally primed offspring revealed additional benefits through an improved body condition, as reflected by larger offspring size, indicating that exposed mothers invest more into current clutch due to the risk to not survive the next reproductive event (Landis *et al.*, 2015).

Conclusion

Our study highlights the difference between maternal and paternal immune priming. It supports our predictions of asymmetric parental investment into offspring immunity and dominating long-lasting paternal immune priming. Enhancement of immunity upon paternal immune challenge altered gene expression and improved pathogen specific immune cell activity. As offspring are born in their father's environment and most likely experience a similar pathogen assembly, transfer of a solid long-term protection about current pathogens should be favoured. While maternal TGIP had a strong impact on young juveniles, it diminished over offspring maturation and came with low specificity. Even though maternal effects were weaker and of decreased stability, prolonged reproduction time was equally affected by both parents. However, these detrimental effects may be compensated by positive maternal impact on offspring size and body mass.

It seems that parental pathogen experience induced differential and sometimes even antagonistic gene expression patterns. Hence, in a biparental combination with two different bacteria species, the specific bacteria type determined the strength and degree of biparental immune priming. This led to a compensated biparental impact instead of synergistic additive biparental effects as it was shown in a previous study that used identical pathogens for both parents (Roth *et al.*, 2012). As the biparental treatment did not lead to an additive long-term effect and also caused a severe delay in offspring maturation, biparental immune priming seems a costly strategy (Contreras-Garduño *et al.*, 2014). Our results indicate that TGIP reveals important consequences on the evolution of life-history, host/pathogen coevolution and parental investment strategies. We found first evidence for the involvement of epigenetic regulation processes like DNA methylation and histone acetylation, which were increasingly affected by both parents with the age of their offspring. We suppose that maternal and paternal epigenetic marks might mediate long-term protection. Further research should focus on parental DNA methylation and histone acetylation pattern upon pathogen exposure to unravel the mechanisms behind TGIP. To study whether it holds true that TGIP correlates with parental investment, mating systems with varying maternal and paternal contribution should be examined.

Chapter II



Chapter II

Title picture of Chapter II: Close-up picture of two developing juveniles post yolk sac stage in the paternal brood pouch of *Syngnathus typhle*; approximately 5 weeks post hatch (Copyright: Anne Beemelmans).

Bacteria specific biparental immune priming in the pipefish *Syngnathus typhle*

Anne Beemelmans¹ and Olivia Roth¹

¹*Helmholtz-Centre for Ocean Research Kiel (GEOMAR), Evolutionary Ecology of Marine Fishes, Düsternbrooker Weg 20, 24105 Kiel, Germany*

In submission for Journal of Evolutionary Biology, January 2016

Cited in this thesis as Beemelmans & Roth 2016b

Abstract

The transfer of acquired and specific immunity against previously encountered bacteria from mothers to offspring (bacteria specific immune priming) yields in an induced offspring resistance. While commonly this transfer of immunity is limited to females, in the sex-role reversed pipefish *Syngnathus typhle* males nurse the embryos over a placenta-like structure, which opens the door for additional paternal immune priming. This phenomenon provides an exclusive opportunity to examine parental investment beyond the provisioning of the DNA.

In our study, we experimentally investigated the potential of bacteria specific biparental immune priming in the pipefish *S. typhle*. Using a fully reciprocal design with two different bacteria species (*Vibrio* spp. and *Tenacibaculum maritimum*) we could not only disentangle the severity of maternal and paternal immune priming, but also address the impact of bacteria specific parental immune priming and its prevalence during maturation.

Our results suggest that parental immune priming effects were bacteria specific. Whereas in one-week old juveniles both parental bacteria challenges induced a differently altered gene expression profile, in four-month-old offspring protection was only maintained against familiar *Vibrio* spp. bacteria. Additionally, a significantly altered expression pattern of 15 epigenetic genes connected to DNA methylation and histone-modifications indicates that these genes might be crucial players in the regulation of immune gene expression and its non-genetic inheritance. Individual investment into immunity is energetically costly leading to resource allocation trade-offs. Here, reduced fecundity and delayed maturation time of adult F1-offspring in case of parental bacteria exposure is pointing to a trade-off associated with parental immune priming and fecundity.

Key words: Specificity, Host-parasite interaction, Epigenetic inheritance, Biparental immune priming

Introduction

Parasites and pathogens are ubiquitous and impose strong selection on their hosts (Hamilton *et al.*, 2008), which resulted in the evolution of highly specific immune defense mechanisms in hosts (Schmid-Hempel & Ebert, 2003; Boots & Bowers, 2004). These are initiated by discriminating non-self, potentially dangerous invaders by the recognition of broadly conserved pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway, 1997; Kurtz, 2004), followed by an activation of an immediate innate immune response. As the pathogen recognition repertoire of the innate immune system is limited, it exhibits only weak specificity (Medzhitov & Janeway, 1997; Kurtz, 2005). Immune specificity is the ability to differentiate between two distinct pathogens without cross-reactivity, leading to a difference in strength of the immune reaction towards particular pathogens (Frank, 2002; Kurtz, 2005). An essential improvement that has evolved with jawed vertebrates (*Gnathostomes*) is the adaptive immune system, which allows, based on somatic diversification of immunoglobulin receptors, the recognition of foreign peptides (epitopes) and further, the storage of long-term immune memory (Cooper & Alder, 2006; Flajnik & Kasahara, 2010). Immune 'memory' facilitates the recognition of previously encountered pathogens inducing a faster and more powerful immune response upon a secondary exposure with homologous pathogens (Kurtz, 2005). This beneficial ability of jawed vertebrates is reached by antigen-specific antibody-producing B- and T- cells, which are characterized by a high degree of specificity and long-lasting immunological memory (Burnet, 1976; Kurtz, 2004; 2005; Janeway *et al.*, 2008).

Mothers can prime the immune system of their offspring by transmitting their specific immune memory trans-generationally, enabling their offspring to cope better with pathogens of the maternal environment (Grindstaff *et al.*, 2006). More importantly, as in all vertebrates the development of lymphoid organs starts after birth and juveniles possess only immature adaptive immunity, maternal immune priming was found to be an essential defense strategy (Grindstaff *et al.*, 2006; Boulinier & Staszewski, 2008; Hasselquist & Nilsson, 2009; Zhang *et al.*, 2013). During that time period of high vulnerability young juveniles mainly rely on maternally induced immune compounds until they have developed own immune-competence (Swain *et al.*, 2002; Grindstaff *et al.*, 2006; Swain *et al.*, 2006; Zapata *et al.*, 2006; Hasselquist & Nilsson, 2009; Zhang *et al.*, 2013). In mammals, maternal exposure with particular pathogens induces antibody production in the mother, which can transfer their acquired immunity to the offspring over the placenta or milk, providing pathogen-specific protection (Patterson *et al.*, 1962; Brambell, 1970a; Reuman *et al.*, 1983). This phenomenon is known as trans-generational immune priming (TGIP) and not only found in vertebrates with antibody based adaptive immune system (Grindstaff *et al.*, 2003; Grindstaff *et al.*, 2006; Swain *et al.*, 2006; Hasselquist & Nilsson, 2009; Jiménez de Oya *et al.*, 2011; Ramos *et al.*,

2014), but also exists in invertebrates lacking a mechanistic homologue to the vertebrate adaptive immune system (Little *et al.*, 2003; Sadd *et al.*, 2005; Freitak *et al.*, 2009; Roth *et al.*, 2010; Freitak *et al.*, 2014; Salmela *et al.*, 2015). Although maternal effects are considered to be more important early in life and decline over developmental time (Lindholm *et al.*, 2006), recent studies provide evidence that these effects might also have long-lasting consequences over several generations (Beemelmans & Roth 2016c in review, Ramos *et al.* 2014).

TGIP was considered to be limited to the mothers as the physiological connection between father and offspring in conventional sex-roles is rather restricted. Yet, recent studies support growing evidence that immune priming can also be mediated via fathers (Roth *et al.*, 2010; Zanchi *et al.*, 2011). Hence, phenotypic variation might be induced by paternal effects to a higher extent than initially assumed (Crean & Bonduriansky, 2014; Kaufmann *et al.*, 2014). Exceptional cases like sex-role-reversed fish species (e.g. *Syngnathids*) where males nurse their embryos over a placenta-like structure or fish with strong paternal care (e.g., *Cichlidae*) provide unique opportunity for a paternal transfer of immune components (Pizzolon *et al.*, 2010), and thus, allows biparental immune priming (Roth *et al.*, 2012). Despite the physical connection that mechanistically facilitates a paternal transfer of soluble immune factors, both parents also pass on epigenetic marks to the next generation such as DNA methylation pattern and histone modifications (Jablonka & Lamb, 2014; Ci & Liu, 2015; Szyf, 2015). Epigenetic changes caused by environmental stressors can lead to altered gene expression independent from mutations in the DNA, which are, however, heritable across generations (Berger *et al.*, 2009; Kappeler & Meaney, 2010; Jablonka & Lamb, 2015; Szyf, 2015). Biotic environmental stressors like pathogens may cause epigenetic changes in the host, leading to direct effects on epigenetic variation of the host's phenotype (Gómez-Díaz *et al.*, 2012; Mukherjee *et al.*, 2015). If inherited epigenetic changes minimize the severity of an infection, selection could favour specific DNA methylation patterns or histone modifications (Poulin & Thomas, 2008). It would advance our knowledge to address whether pathogen exposure of the parents can directly induce stable epigenetic changes in the offspring, which would facilitate the transfer of specific immune memory (Youngblood *et al.*, 2010; Gómez-Díaz *et al.*, 2012).

When trans-generational immune priming comes with immunological specificity, pathogen specific parental (and/or biparental) immune priming is achieved. Consequently, offspring are better protected against homologous pathogen challenges experienced by the parental generation as compared to heterologous combinations (Schmid-Hempel, 2011). When parents and offspring share the same environment a transfer of immunological relevant cues

about the future pathogen exposure would allow a plastic adaptation to the prevailing pathogen environment (Moret, 2006). Although, over their life time, hosts face a range of different pathogens and parasites in their natural habitat, encountering the same pathogens repeatedly within and across generations remains likely due to the pathogens' short generation time and their clumped distribution (Dybdahl & Lively, 1998; Lively & Dybdahl, 2000). Consequently, the probability of future exposure to similar pathogens is influencing the type, specificity and length of immunological defense (Tidbury *et al.*, 2011). An induced immunity carries energetic costs, implying a trade-off between immune response and other life-history traits like development, maturation, reproduction, and growth through resource allocation trade-off or physiological constraints (Lochmiller & Deerenberg, 2000; Schmid-Hempel, 2005b; Ardia *et al.*, 2011; Ardia *et al.*, 2012; Contreras-Garduño *et al.*, 2014). Hence, if immune priming is costly, a trade-off with other costly life-history traits may shape the outcome of immune priming across generations (Contreras-Garduño *et al.*, 2014). Previous research mainly focused on the benefits of immune priming, but the analysis of complex interactions between costs and benefits is required to understand the strategy and the evolutionary origin of immune priming.

The immune system of bony fishes (teleosts) characterizes a transition point between species relying only on innate immunity and species using adaptive immunity (Flajnik & Kasahara, 2010; Workenhe *et al.*, 2010). Due to their limited repertoire of antibodies and slow maturation of their lymphocytes teleosts fundamentally rely on their innate immune defense (Uribe *et al.*, 2011). To date, it is still puzzling to which degree fishes are able to create a specific immune memory and whether immune specificity can be transferred across generation. So far, studies have shown that freshly hatched free-living juveniles rely on maternally derived immune components supplied during oogenesis (Johnson *et al.*, 1982; Bly *et al.*, 1986; Sin *et al.*, 1994; Swain *et al.*, 2006; Swain & Nayak, 2009). Although, certain studies have identified maternal derived antibodies in the egg yolk of fish embryos (Bly *et al.*, 1986; Hanif *et al.*, 2004; Swain *et al.*, 2006; Swain & Nayak, 2009), limited knowledge exists on their ability to trigger a bacteria specific immune response in the fry.

Syngnathidae (seahorses and pipefish) do neither possess a spleen nor a gut-associated lymphatic tissue, in which cells of the adaptive immune system assemble and proliferate (Matsunaga & Rahman, 1998). The recent discovery of an absence of one of the most important parts of the adaptive immune system, the MHCII pathway, in the pipefish *Syngnathus typhle*, is representing a potential secondary reduction of the adaptive immune system (Haase *et al.*, 2013). Here we aimed to investigate to what extent this fish species is able to transfer bacteria specific immunity (specificity) to the next generation. By using S.

typhle as a model system, and two phylogenetic different marine bacteria species, we tested for trans-generational bacteria-specific immune priming and maternal versus paternal specificity effects on offspring immune resistance. Hereby, the parental generation was exposed to two different allopatric and heat-killed bacterial epitopes (*Vibrio* spp. and *Tenacibaculum maritimum*) in a fully reciprocal mating design. Gene expression patterns of 29 immune genes as well as immune cell activity of F1-offspring (one-week and four-month-old juveniles), likewise challenged with homologous or heterologous parental pathogens, were assessed. This approach facilitated the (I.) disentangling of the severity of parental bacteria-specific immune priming (specificity) over juvenile development. We additionally evaluated maternal and paternal specificity on innate and adaptive immune genes as we (II.) expected that mothers and fathers differ in their investment into pathways of the offspring immune system to reach an optimal immune priming effect. To further address possible epigenetic mechanisms of inheritance, we tested (III.) whether genes associated to epigenetic regulation processes are affected upon the parental bacteria treatment. Finally, we investigated (IV.) whether the channelling of energy resources to immune priming bears costs in terms of disadvantages in other life-history traits

Material and Methods

Parental generation (F0-treatment)

The parental pipefish generation was sampled, hosted and treated as described in Beemelmans & Roth (2016a in press). As immune challenge we used an injection with heat-killed pathogen epitopes and therefore build on a previous study where biparental immune priming has been found in response to heat-killed immune elicitors (Roth *et al.*, 2012). In our experimental design, each sex of one mating pair was vaccinated with a different novel (allopatric) bacteria-species, either *Vibrio* spp. (Italy species, I2K3) (Roth *et al.*, 2012a) or *Tenacibaculum maritimum* (Suzuki *et al.*, 2001). After immune challenge, the parental generation was hosted in the following five final mating combinations (Figure 1): 1. (♀Naive x ♂Tenacibaculum), 2. (♀Naive x ♂Vibrio), 3. (♀Tenacibaculum x ♂Naive), 4. (♀Vibrio x ♂Naive), 5. (♀Naive x ♂Naive). The five parental treatment groups were replicated eight times, resulting in 40 breeding pairs (families). All couples mated successfully within 1-3 days after the immune challenge and juveniles hatched after 4 weeks of male pregnancy.

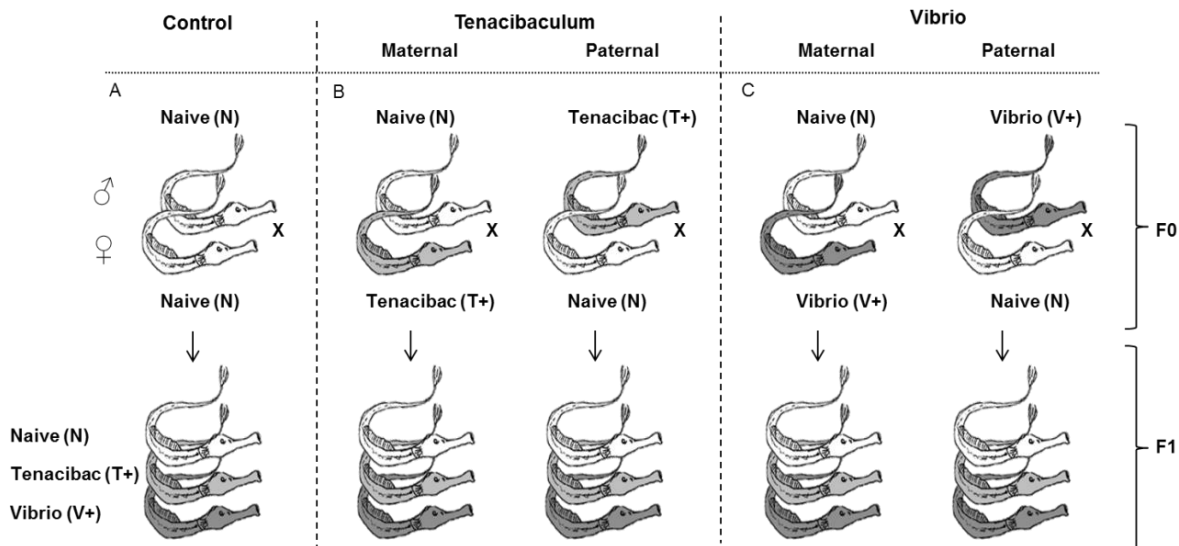


Figure 1: Experimental design to explore bacteria-species specific immune priming in the pipefish *S. typhle* over one generation. In total five parental treatment groups 1. **Control:** Naïve; 2. **Maternal** *Tenacibaculum* (Mat T+); 3. **Paternal** *Tenacibaculum* (Pat T+); 4. **Maternal** *Vibrio* (Mat V+); 5. **Paternal** *Vibrio* (Pat V+). We conducted two consecutive sampling events of the F1-generation (one-week post birth and four-month post birth). For the two main statistical approaches treatment groups were differently combined. To test bacteria specific immune priming parental treatment groups belonging either to *Tenacibaculum* (B) or *Vibrio* (C) were pooled into following three: **A. Control:** Naïve (control); **B. F1-*Tenacibaculum*:** Maternal *Tenacibaculum* (Mat T+) & Paternal *Tenacibaculum* (Pat T+); **C. F1-*Vibrio*:** Maternal *Vibrio* (Mat V+) & Paternal *Vibrio* (Pat V+). For maternal and/or paternal specificity analysis only the single parental *Tenacibaculum* or *Vibrio* groups were chosen.

Filial generation 1 (F1-treatment)

One-week-old offspring (eight days post birth) were exposed to the same heat-killed *Vibrio* (V+) and *Tenacibaculum* (T+) bacteria-species used for the parental generation or stayed without any treatment as control (N) as described in Beemelmans & Roth (2016a in press). We were able to use 20 families with an equal distribution of four times replicate per five parental treatment groups. For each of the 20 families, 15 offspring were used and distributed over the three different treatment groups resulting in a total number of 300 juveniles. Four-month old juveniles were exposed to the same procedure like the one-week-old juveniles as explained in Beemelmans & Roth (2016a in press), for comparing immune priming effects between different developmental stages. Consequently, four-month-old juveniles of five different parental treatment groups were applied to the treatment by using three replicates per offspring treatment and two sampling events, resulting in a total number of 90 sampled individuals. Life-history parameters (body size, body mass and liver weight) were collected and hepatosomatic Index (HSI) and fish body condition factor (CF) calculated as defined in Beemelmans & Roth (2016a in press). For characterizing the humoral innate

and adaptive immune response, we measured the absolute number of lymphocytes and monocytes in the blood and head kidney according to the protocol of Roth *et al.* (2011).

Finally, the RNA was extracted of 300 whole body samples of early stage juvenile pipefish (one-week post birth) and 90 gill tissue samples of late stage juvenile pipefish (four-month post birth). The expression of 44 target genes and 4 housekeeping genes was measured simultaneously for all 390 samples using a Fluidigm BioMark™ based on 96.96 dynamic arrays according to Beemelmans & Roth (2016c in review)

Remaining F1-offspring were left immunologically untreated until they reached adulthood and individuals were crossed within former parental treatment groups as described in Beemelmans & Roth (2016a in press). Thereby, time point of maturity and clutch size of approx. six month old F1-offspring were recorded.

Data analysis and statistics

We evaluated whether our response variables (gene expression, immune cell counts and life-history traits) of juvenile pipefish from two consecutive age classes (one-week and four-month old) showed bacteria-specific immune responses upon the acute offspring exposure (F1-treatment) and the parental challenge (F0-treatment). Therefore, the five parental treatment groups (Figure 1) were combined into following three treatment groups: 1. Control: Naïve (control); 2. F1-Tenacibaculum: Maternal Tenacibaculum (Mat T+) & Paternal Tenacibaculum (Pat T+); 3. F1-Vibrio: Maternal Vibrio (Mat V+) & Paternal Vibrio (Pat V+).

Secondly, we explored whether offspring that received the same bacterial strain as the parents (homologous) showed an enhanced immune response (specificity) compared to those that experienced different bacteria exposures (heterologous) as their parents. As to do so, we examined statistically and graphically the (F0)-parental priming x (F1)-offspring challenge interaction.

Thirdly, we analysed parental sex-specific immune priming differences to investigate whether mothers and/or fathers equally provide specific bacteria protection. Therefore, the final dataset was reduced into sole maternal and sole paternal treatment groups of one-week-old (120 each) and four-month-old juveniles (36 each) (Figure1).

The data analysis was performed in R (v3.2.2) and PRIMERV6 (Clarke & Gorley, 2006) according to Beemelmans & Roth (2016a in press) and Beemelmans & Roth (2016c in review) with minor modifications. A permutational multivariate analysis of variance (PERMANOVA) was applied for immune gene expression (29 target genes) as well as epigenetic regulation genes (15 target genes) of one-week-old juveniles (300 samples) and four-month-old F1-juveniles (90 samples). For the latter, we further assessed life-history

parameters (body size, body mass, body condition factor (CF), and hepatosomatic index (HSI)) and immune cell counts.

The PERMANOVA model (`vegan` package - `adonis` function in R) was based on a Bray-Curtis Matrix of non-transformed $-\Delta\text{Ct}$ -values. We applied 'F0-parents' and 'F1-offspring' treatment as fixed factors, included 'Family' as fixed nested term within the 'F0-parents' treatment and incorporated 'Size' as covariate to account for size relevant influences. The analysis of similarity (ANOSIM) was performed with the software PRIMERv6 (Clarke, 1993; Clarke & Gorley, 2006) based on a Bray-Curtis distance matrix and 4th-root transformation to disentangle differences between parental and offspring treatment groups using a pairwise comparison (Brazma & Vilo, 2000). Further, we applied a between-class analysis (BCA), which is a particular case of principal component analysis (`ade4` package - `bca` function in R) to investigate graphical clustering according to the respective treatment group of interest (Dolédec & Chessel, 1987; Thioulouse *et al.*, 1995; Chessel *et al.*, 2004). The BCA was used to identify the principal components based on the center of gravity of each parental treatment group to highlight differences between them (Arumugam *et al.*, 2011).

Statistical univariate approaches were applied for life-history parameters and immune cell count measurements as described in Beemelmans & Roth (2016a in press). Basically, a linear model was fitted for each response variable using the fixed factors 'F0-parents' and 'F1-offspring', the non-independent factor 'Family' or 'Tank' nested with 'F0-parents' and 'Size' as covariate. In addition, body size was assessed separately as phenotypic trait using the same model without covariate. All significant ANOVAs were followed by post-hoc Tukey's HSD test to include single interaction terms.

To assess life-history trait measurements of six month old F1-offspring (time point of maturity and clutch size) a linear mixed effect model (`nlme` package - `lme` function in R) was applied including the fixed factor 'F0-parents' and the random-term 'Tank' in the model.

Finally, a correlation analysis was applied to connect the biological relevance of gene expression patterns and immune parameters (`PerformanceAnalytics` package in R). By using a Pearson correlation matrix, we correlated each single gene ($-\Delta\text{Ct}$ -values) with each immune cell measurement in order to determine whether or not particular immune genes can be used as indicators for direct immune performance (Birrer *et al.*, 2012).

Results

I.) Immune response against two different pathogens (F1-offspring treatment effect)

One-week-old F1-juveniles - Gene expression

The acute immune challenge of one-week-old F1-offspring (F1-offspring treatment) significantly affected the expression of 44 target genes (PERMANOVA-*all* $F_{2,273}=9.91$, $p<0.001$; Table 1, Figure S1). The main F1-offspring treatment effect was identified for all immune genes (PERMANOVA-*immune* $F_{2,273}=11.55$, $p<0.001$; Table 1, Figure 2A). Here, the Between-Class Analysis (BCA) visualizes, that *Vibrio* (V+) and *Tenacibaculum* (T+) treatment groups cluster with overlapping centers of gravity opposed to the Naïve (N) control group along the first axis, which explains 93% of the total variation (Figure 2A). We did not find evidence for bacteria specific immune response as both treatment groups revealed an identical immune gene expression pattern (ANOSIM-*immune* V+ vs T+ $p=0.94$; Table S1, Figure 2A). Overall, innate immune genes (PERMANOVA-*innate* $F_{2,273}=16.44$, $p<0.001$; Table 1) and complement immune genes (PERMANOVA-*complement* $F_{2,273}=30.59$, $p<0.001$; Table 1) displayed a more prevalent reaction than adaptive immune genes (PERMANOVA-*adaptive* $F_{2,273}=2.099$, $p=0.040$; Table 1, Figure S1). Likewise epigenetic genes revealed an effect upon the acute immune treatment (PERMANOVA-*epigen* $F_{2,273}=3.83$, $p=0.001$; Table 1, Figure 4A).

Four-month-old F1-juveniles - Gene expression

The acute immune challenge of four-month-old F1-offspring significantly affected the expression of 44 target genes (PERMANOVA-*all* $F_{2,73}=2.456$, $p=0.005$; Table 1, Figure 3A, Figure S2). In the Between-Class Analysis (BCA) for all immune genes *Vibrio* and *Tenacibaculum* treatment groups clustered without overlapping centers of gravities opposed to the naïve control group along the first axis, which explains 88% of total variation. However, as both F1-treatment groups were statistically similar (ANOSIM-*immune* V+ vs T+ $p=0.24$; Table S2, Figure 3A), we could exclude a bacteria specific immune response. The offspring effect was predominantly driven by innate immune genes (PERMANOVA-*innate* $F_{2,73}=3.29$, $p<0.001$; Table 1, Figure S2) whereas adaptive immune genes, complement component genes and epigenetic genes were not affected (Table 1, Figure S2).

Table 1: Results from PERMANOVA analysis of gene expression, immune cell counts and life-history traits of one-week and four-month old F1-juveniles. Multivariate PERMANOVA analysis to assess the effect and interaction of the two fixed factors F0-parents and F1-offspring, including the nested factor nested in F0-parents ('family' (for one-week-old) and 'sampling day' (for four-month-old)) and size as covariate on relative gene expression data (-ΔCt-values), proportion of immune cells (%) and life-history parameters. Each analysis was based on a Bray-Curtis distance matrix with p-values obtained by 999 permutations. Significant p-values are marked in bold letters (significance code: <0.001***, 0.001**, 0.01*, 0.1>p-value≥0.05 trend •).

Age	Response Variables	Residuals DF	F0-parents		F1-offspring		F0:F1		Size-covariate		F0-parents : Nested Factor											
			Df	F.Model	Pr(>F)	Sig	Df	F.Model	Pr(>F)	Sig	Df	F.Model	Pr(>F)	Sig								
1	All genes (44)	273	2	14.353	<0.001	***	2	9.912	<0.001	***	4	1.479	0.052	•	1	6.273	<0.001	***	17	15.124	<0.001	***
	Immune genes (29)	273	2	19.936	<0.001	***	2	11.554	0.001	***	4	1.361	0.084	•	1	6.319	0.001	***	17	13.878	<0.001	***
	Innate genes (13)	273	2	20.792	<0.001	***	2	16.446	0.001	***	4	1.379	0.119	•	1	9.270	0.001	***	17	14.658	<0.001	***
	Innate & Adaptive (5)	273	2	17.845	<0.001	***	2	10.358	0.001	***	4	1.591	0.08	•	1	3.458	0.02	*	17	10.502	<0.001	***
	Adaptive (8)	273	2	13.413	<0.001	***	2	2.099	0.040	*	4	1.181	0.296	•	1	2.315	0.059	•	17	15.547	<0.001	***
	Complement (3)	273	2	16.844	<0.001	***	2	30.594	<0.001	***	4	2.002	0.043	*	1	7.596	<0.001	***	17	11.337	<0.001	***
	Epigenetic (15)	273	2	3.335	0.002	**	2	3.833	0.002	**	4	1.722	0.055	•	1	9.718	<0.001	***	17	17.291	<0.001	***
	Methylation (9)	273	2	2.566	0.025	*	2	2.589	0.028	*	4	1.331	0.192	•	1	12.073	<0.001	***	17	16.250	<0.001	***
	Acetylation (5)	273	2	5.874	<0.001	***	2	6.052	0.002	**	4	2.768	0.007	**	1	3.052	0.051	•	17	20.347	<0.001	***
4	All genes (44)	73	2	4.547	<0.001	***	2	2.456	0.006	**	4	0.629	0.915	•	1	1.1204	0.348	•	7	2.207	0.002	**
	Immune genes (29)	73	2	4.193	0.002	**	2	2.842	<0.001	***	4	0.625	0.920	•	1	1.1424	0.221	•	7	2.187	<0.001	***
	Innate genes (13)	73	2	2.348	0.015	*	2	3.296	<0.001	***	4	0.757	0.22	•	1	1.409	0.76	•	7	2.467	<0.001	***
	Innate&Adaptive (5)	73	2	4.504	0.004	**	2	4.295	0.002	**	4	0.419	0.93	•	1	2.4076	0.08	•	7	2.585	<0.001	***
	Adaptive (8)	73	2	2.897	0.011	*	2	1.606	0.130	•	4	0.602	0.855	•	1	0.5071	0.707	•	7	2.165	0.002	**
	Complement (3)	73	2	9.781	0.002	**	2	0.244	0.869	•	4	0.553	0.740	•	1	0.5357	0.527	•	7	1.598	0.121	•
	Epigenetic (15)	73	2	5.392	<0.001	***	2	0.898	0.489	•	4	0.579	0.846	•	1	0.7672	0.504	•	7	2.223	0.006	**
	Methylation (9)	73	2	6.019	<0.001	***	2	1.026	0.379	•	4	0.625	0.767	•	1	0.8479	0.455	•	7	2.137	0.012	*
	Acetylation (5)	73	2	4.803	0.002	**	2	0.928	0.485	•	4	0.642	0.737	•	1	0.6618	0.494	•	7	2.878	0.002	**
	Immune cell counts (6)	73	2	21.157	<0.001	***	2	7.381	<0.001	***	4	1.454	0.16	•	1	2.2194	0.109	**	7	10.786	<0.001	***
	Cells head kidney (3)	73	2	11.492	<0.001	***	2	8.093	<0.001	***	4	1.213	0.28	•	1	6.0392	0.007	**	7	3.728	<0.001	***
	Cells blood (3)	73	2	20.832	<0.001	***	2	7.375	<0.001	***	4	1.827	0.11	•	1	-0.3058	0.995	•	7	15.345	<0.001	***
	Size/mass/CF/HSI	74	2	5.187	<0.001	***	2	0.447	0.771	•	4	0.846	0.52	•	1	1.3681	0.19	•	7	1.3681	0.19	•

Four-month-old F1-juveniles - Immune cell count

The humoral immune response measured through the absolute amount of immune cells in the head kidney (PERMANOVA-*cells.hk* $F_{2,73}=8.09$, $p<0.001$; Table 1) and blood (PERMANOVA-*cells.blood* $F_{2,73}=7.37$, $p<0.001$; Table 1) was activated upon the acute treatment in four-month-old F1-offspring, but there was no difference between the two bacteria treatments detectable (Table S2). More precisely, the amount of monocytes in the head kidney was significantly lower than in the naïve control group (ANOVA-*mono.hk* $F_{2,55}=17.8$, $p<0.001$; *TukeyHSD*: $N>V+$ and $N>T+$; Table S3, Figure 7A) but, in turn significantly higher in the blood (ANOVA-*mono.blood* $F_{2,55}=6.98$, $p=0.002$; *TukeyHSD*: $N<V+$ and $N<T+$; Table S3, Figure 7B).

II.) Bacteria-specific immune priming effect (F0-parental treatment effect)*One-week-old F1-juveniles - Gene expression*

One-week old F1-offspring of the three different parental treatment groups (Naïve, *Vibrio*, *Tenacibaculum*) revealed significantly different immune gene expression profiles (PERMANOVA-*immune* $F_{2,273}=19.93$, $p<0.001$; Table 1, Figure 2B). On opposed direction along the BCA axis 1 (66% variation), the two parental bacteria treatment groups clustered apart from the control group, demonstrating a strong parental treatment effect (ANOSIM-*immune* $F0V+$ vs control $p=0.001$; $F0T+$ vs control $p=0.003$; Table S1). Moreover, the two parental *Vibrio* and *Tenacibaculum* bacteria treatment groups significantly clustered opposed to each other (ANOSIM-*immune* $F0V+$ vs $F0T+$ $p=0.001$; Table S1, Figure 2B), leading to a triangle shape, which is representing a bacteria-species specific immune priming effect. A similar pattern was identified for innate (PERMANOVA-*innate* $F_{2,273}=20.791$, $p<0.001$; Table 1), adaptive immune genes (PERMANOVA-*adaptive* $F_{2,273}=13.41$, $p<0.001$; Table 1), and complement component genes (PERMANOVA-*complement* $F_{2,273}=16.84$, $p<0.001$; Table 1) (Figure S3). For the latter two gene categories, it is remarkable that the parental *Vibrio* treatment revealed a more dominant effect than the parental *Tenacibaculum* treatment (ANOSIM-*adaptive* $F0V+$ vs control $p=0.004$, $F0T+$ vs control $p=0.06$; ANOSIM-*complement* $F0V+$ vs control $p=0.006$; $F0T+$ vs control $p=0.238$; Table S1, Figure S3). Epigenetic genes displayed a parental treatment effect (PERMANOVA-*epigen* $F_{2,273}=3.33$, $p=0.001$; Table 1, Figure 4B), which was predominantly driven by acetylation genes (PERMANOVA-*acetyl* $F_{2,273}=5.87$, $p<0.001$; Table 1).

Four-month-old F1-juveniles - Gene expression

In four-month-old juveniles we found significantly altered expression profiles among the three parental treatment groups (PERMANOVA-*immune* $F_{2,73}=4.54$, $p=0.002$; Table 1, Figure 3B) that differed from previously described patterns in one-week-old juveniles. Now the symmetrical triangle structure as depicted for one-week-old juveniles in the BCA was sidewise shifted and only the parental *Vibrio* treatment group significantly clustered along the first axis (91% variation) opposed to the parental control group (ANOSIM-*immune* F0V+ vs control $p<0.001$; Table S2, Figure 3B). In turn, the parental *Tenacibaculum* treatment did not influence the gene expression of four-month-old juveniles significantly (ANOSIM-*immune* F0T+ vs control $p=0.256$; Table S2, Figure 3B). The same *Vibrio* specific parental immune priming effect was consistent to different extents for innate immune genes (PERMANOVA-*innate* $F_{2,73}=2.34$, $p<0.015$; Table 1; ANOSIM-*innate* F0V+ vs control $p<0.005$; Table S2), complement component genes (PERMANOVA-*complement* $F_{2,73}=9.78$, $p=0.002$; Table 1, ANOSIM-*complement* F0V+ vs control $p=0.001$; Table S2) as well as adaptive immune genes (PERMANOVA-*adaptive* $F_{2,73}=2.89$, $p=0.011$; Table 1; ANOSIM-*adaptive* F0V+ vs control $p<0.001$; Table S2) (Figure S4). In addition, gene by gene analysis displayed that upon parental *Vibrio* treatment key immune genes were mainly up-regulated in comparison to control group (Figure S5). In comparison to the results of one-week-old juveniles, epigenetic genes displayed the same pattern as described for immune genes (PERMANOVA-*epigen* $F_{2,73}=5.39$, $p<0.001$; Table 1, ANOSIM-*epigen* F0V+ vs control $p<0.001$; Table S2, Figure 5B). Methylation genes played a slightly more important role (PERMANOVA-*methyl* $F_{2,73}=6.02$, $p<0.001$; Table 1) than acetylation genes (PERMANOVA-*acetyl* $F_{2,73}=4.80$, $p=0.002$; Table 1) (Figure S4).

Four-month-old F1-juveniles - Immune cell counts

The parental immune challenge of four-month-old F1-offspring significantly affected the number of immune cells (lymphocytes and monocytes) in head kidney and blood (PERMANOVA-*cell.counts* $F_{2,73}=21.15$, $p<0.001$; Table 1, Figure 6A). As demonstrated in the BCA the two parental *Vibrio* and *Tenacibaculum* bacteria treatment groups were significantly clustering apart from the parental control group (ANOSIM-*counts* F0T+ vs control $p<0.001$; F0V+ vs control $p<0.001$; Table S2, Figure 6A) along the first axis (89% variation). The observed clustering pattern resembles a triangle shape, demonstrating a bacteria-specific immune priming effect based on immune cell production (ANOSIM- *cell.counts* F0T+ vs F0V+ $p<0.001$; Table S2, Figure 6A).

Chapter II

By using a statistical univariate approach, each cell count variable was analysed separately (Table S3, Figure 7 A-D). Particularly, we found an increased lymphocyte/monocyte-ratio in the head kidney upon parental bacteria challenge (ANOVA-*LM-ratio.hk* $F_{2,55}=11.5$, $p<0.001$; *TukeyHSD*: C<T+, C<V+; Table S3, Figure 7C). The significantly higher proportion of lymphocytes in the blood compared to the control group (ANOVA-*L/M-ratio.blood* $F_{2,55}=33.6$, $p<0.001$; *TukeyHSD*: C<T+, C<V+; Table S3, Figure 7D) indicates a higher humoral adaptive immune response of offspring with parental bacteria exposure. Moreover, parental *Vibrio* challenge induced a significantly higher lymphocyte/monocyte-ratio in the blood of four-month-old juveniles in comparison to offspring with parental *Tenacibaculum* treatment (*TukeyHSD*: C<T+<V+, Table S3, Figure 7D).

To connect the biological relevance of gene expression and cellular measurements, a correlation analysis was conducted for four-month-old F1-individuals ($-\Delta\text{CT}$ values were correlated with cellular immune parameters). Following genes connected to pathways of the innate system presented a positive correlation with the number of monocytes in the head kidney: *Lectin protein II* ($R^2=0.26$, $p=0.014$), *Interferon* ($R^2=0.25$, $p=0.019$), *Peptidoglycan* ($R^2=0.30$, $p=0.004$), *Tyrosine kinase* ($R^2=0.23$, $p=0.032$), *Complement component 3* ($R^2=0.35$, $p<0.001$) (Table S6). Additionally, following immune genes displayed a positive correlation with amount of monocytes in the blood: *Lectin protein I* ($R^2=0.28$, $p=0.038$), *Interleukin 1* ($R^2=0.23$, $p=0.029$), *Complement component 3* ($R^2=0.23$, $p=0.01$), *Lymphocyte antigen 75* ($R^2=0.22$, $p=0.038$), and *Complement sub-component 1q* ($R^2=0.34$, $p<0.001$) (Table S6). Furthermore, there was a significant negative correlation between lymphocytes and the expression of the adaptive immune genes *HIVEP3* ($R^2= -0.23$, $p=0.031$) and *Complement sub-component 1q* (*hk*: $R^2= -0.25$, $p=0.016$; *blood*: $R^2=-0.28$, $p=0.007$) (Table S6).

Chapter II

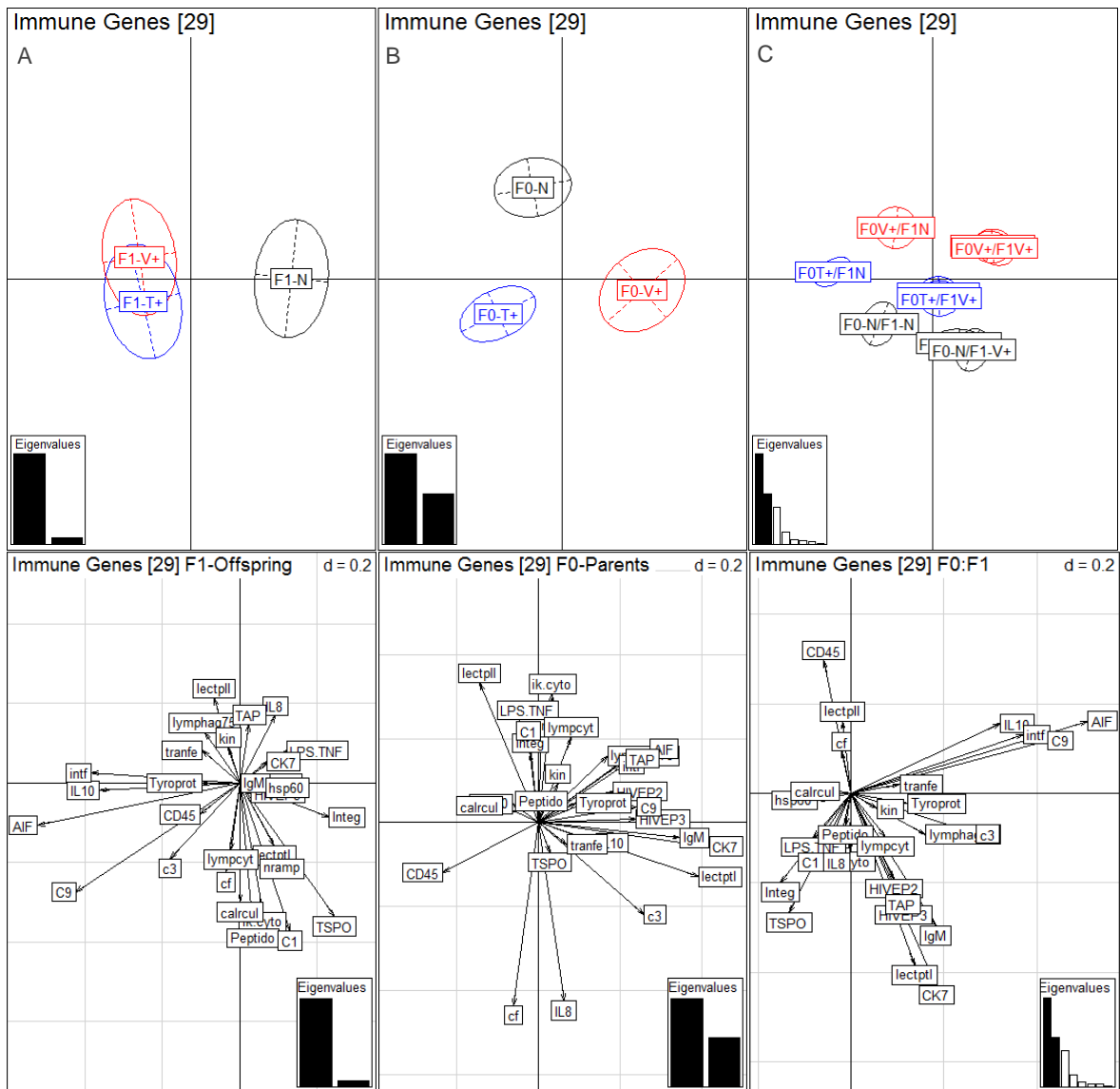


Figure 2: Between Component Analysis (BCA) based on 29 immune genes of one-week-old juveniles (N=300). Different levels of factors were included in the between component analysis **A: Factor F1-offspring treatment** (F1-Vibrio (F1-V+) in red, F1-Tenacibaculum (F1-T+) in blue, F1-Naïve (F1-N) in black); **B: Factor F0-parental treatment** (F0-Vibrio (F0-V+) in red; F0-Tenacibaculum (F0-T+) in blue; F0-Naïve (F0-N) in black); **C: Factor F1:F0-treatment interaction**. In the underlying scatterplot the response variables (immune genes) are symbolized by arrows whereby the direction and the length of the arrows show the quality of the correlation between variables and principle components. The length of the arrow is directional proportional with the contribution of each variable to the total variability. The scale of the graph is given by a grid, which size is given in the upper right corner. The eigenvalues bar chart is drawn in the upper left corner, with the two black bars corresponding to the two axes used to draw the BCA plot.

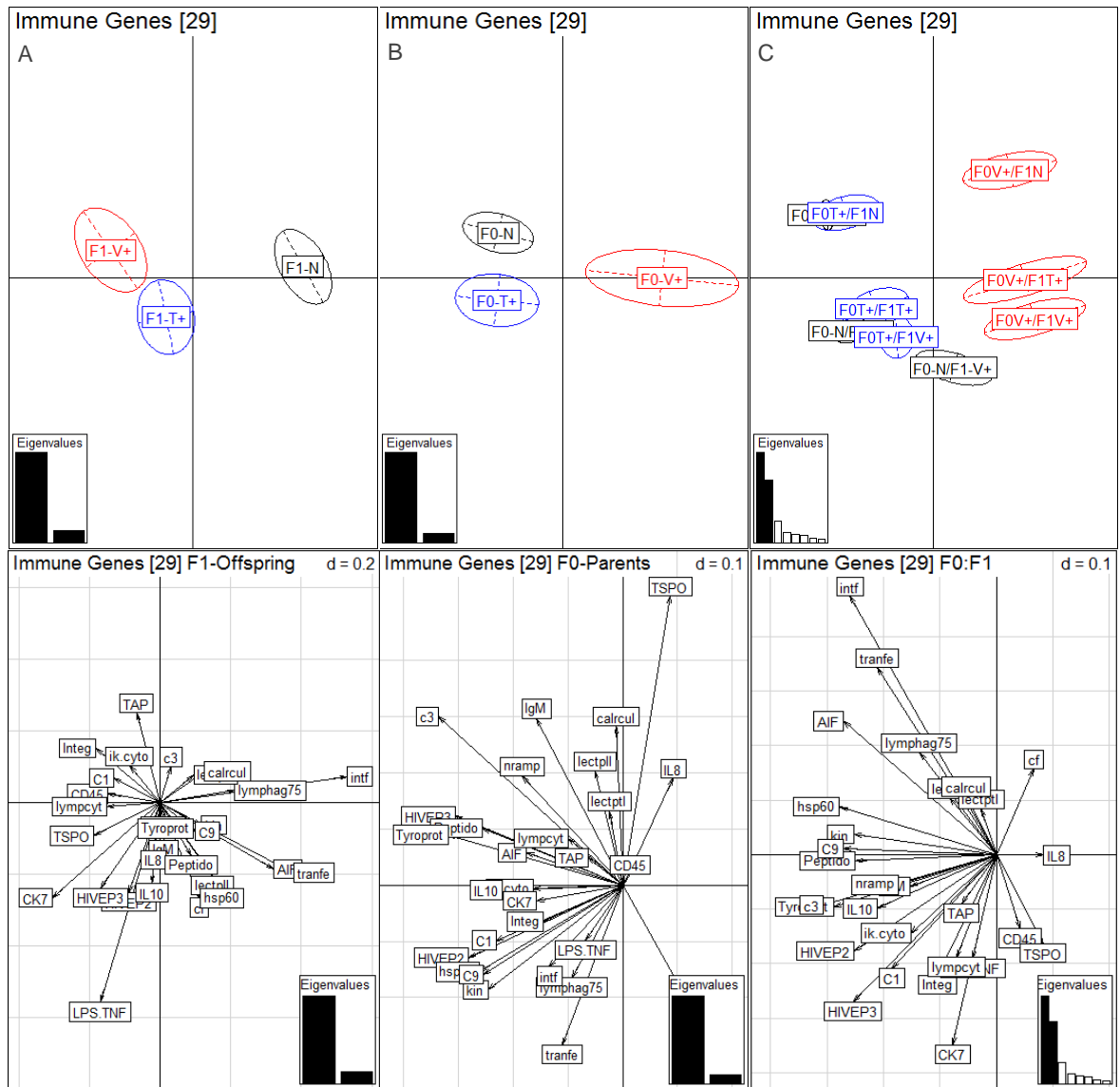


Figure 3: Between Component Analysis (BCA) based on 29 immune genes of four-month-old juveniles (N=90). Different levels of factors were included in the between component analysis **A: Factor F1-offspring treatment** (F1-Vibrio (F1-V+) in red, F1-Tenacibaculum (F1-T+) in blue, F1-Naïve (F1-N) in black); **B: Factor F0-parental treatment** (F0-Vibrio (F0-V+) in red; F0-Tenacibaculum (F0-T+) in blue; F0-Naïve (F0-N) in black); **C: Factor F1:F0-treatment interaction**. In the underlying scatterplot the response variables (immune genes) are symbolized by arrows whereby the direction and the length of the arrows show the quality of the correlation between variables and principle components. The length of the arrow is directional proportional with the contribution of each variable to the total variability. The scale of the graph is given by a grid, which size is given in the upper right corner. The eigenvalues bar chart is drawn in the upper left corner, with the two black bars corresponding to the two axes used to draw the BCA plot.

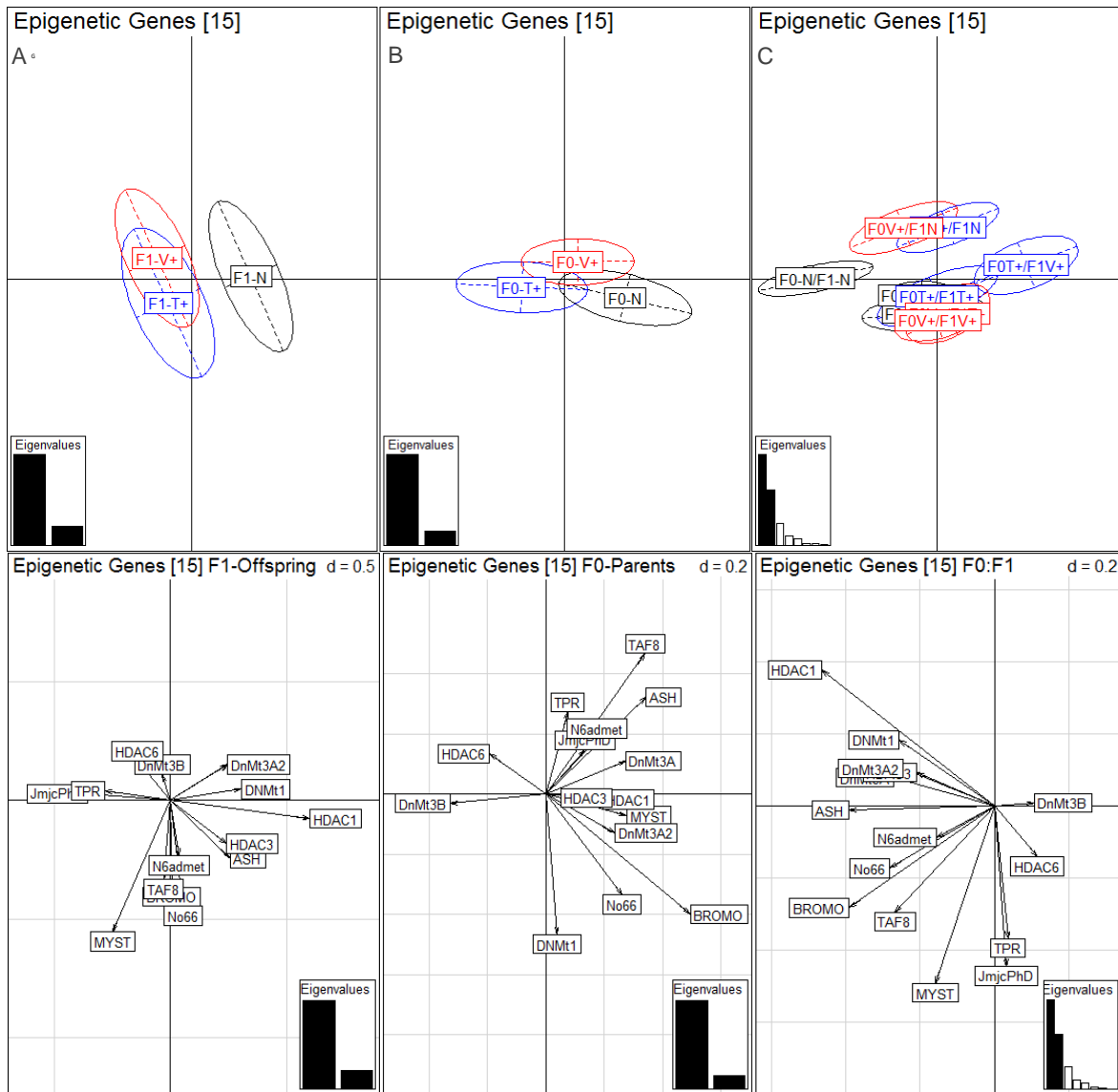


Figure 4: Between Component Analysis (BCA) based on 15 epigenetic genes of one-week-old juveniles (N=300). Different levels of factors were included in the between component analysis **A: Factor F1-offspring treatment** (F1-Vibrio (F1-V+) in red, F1-Tenacibaculum (F1-T+) in blue, F1-Naïve (F1-N) in black); **B: Factor F0-parental treatment** (F0-Vibrio (F0-V+) in red; F0-Tenacibaculum (F0-T+) in blue; F0-Naïve (F0-N) in black); **C: Factor F1:F0-treatment interaction.** In the underlying scatterplot the response variables (epigenetic genes) are symbolized by arrows whereby the direction and the length of the arrows show the quality of the correlation between variables and principle components. The length of the arrow is directional proportional with the contribution of each variable to the total variability. The scale of the graph is given by a grid, which size is given in the upper right corner. The eigenvalues bar chart is drawn in the upper left corner, with the two black bars corresponding to the two axes used to draw the BCA plot.

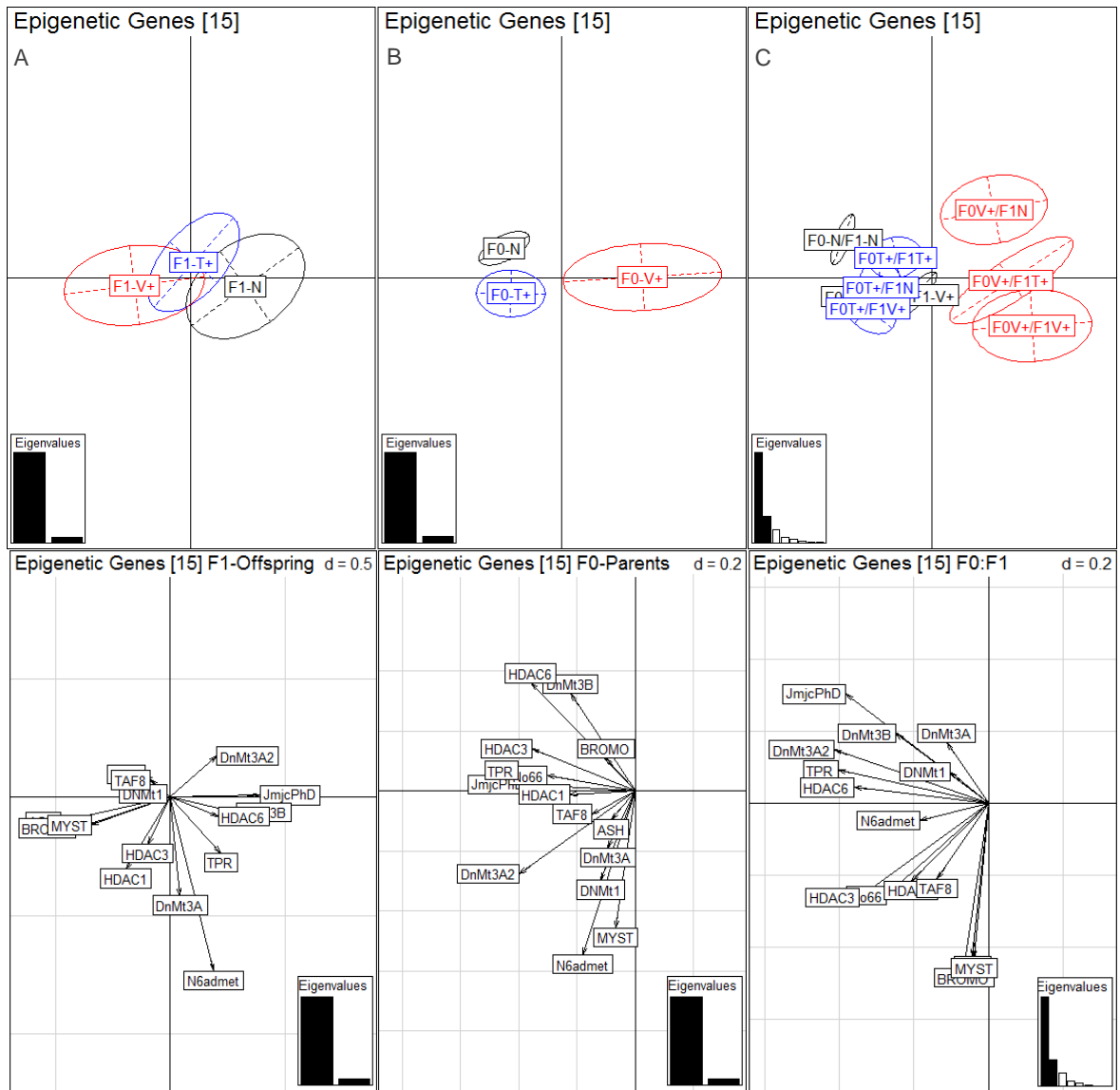


Figure 5: Between Component Analysis (BCA) based on 15 epigenetic genes of four-month-old juveniles (N=300). Different levels of factors were included in the between component analysis **A: Factor F1-offspring treatment** (F1-Vibrio (F1-V+) in red, F1-Tenacibaculum (F1-T+) in blue, F1-Naïve (F1-N) in black); **B: Factor F0-parental treatment** (F0-Vibrio (F0-V+) in red; F0-Tenacibaculum (F0-T+) in blue; F0-Naïve (F0-N) in black); **C: Factor F1:F0-treatment interaction**. In the underlying scatterplot the response variables (epigenetic genes) are symbolized by arrows whereby the direction and the length of the arrows show the quality of the correlation between variables and principle components. The length of the arrow is directional proportional with the contribution of each variable to the total variability. The scale of the graph is given by a grid, which size is given in the upper right corner. The eigenvalues bar chart is drawn in the upper left corner, with the two black bars corresponding to the two axes used to draw the BCA plot.

Chapter II

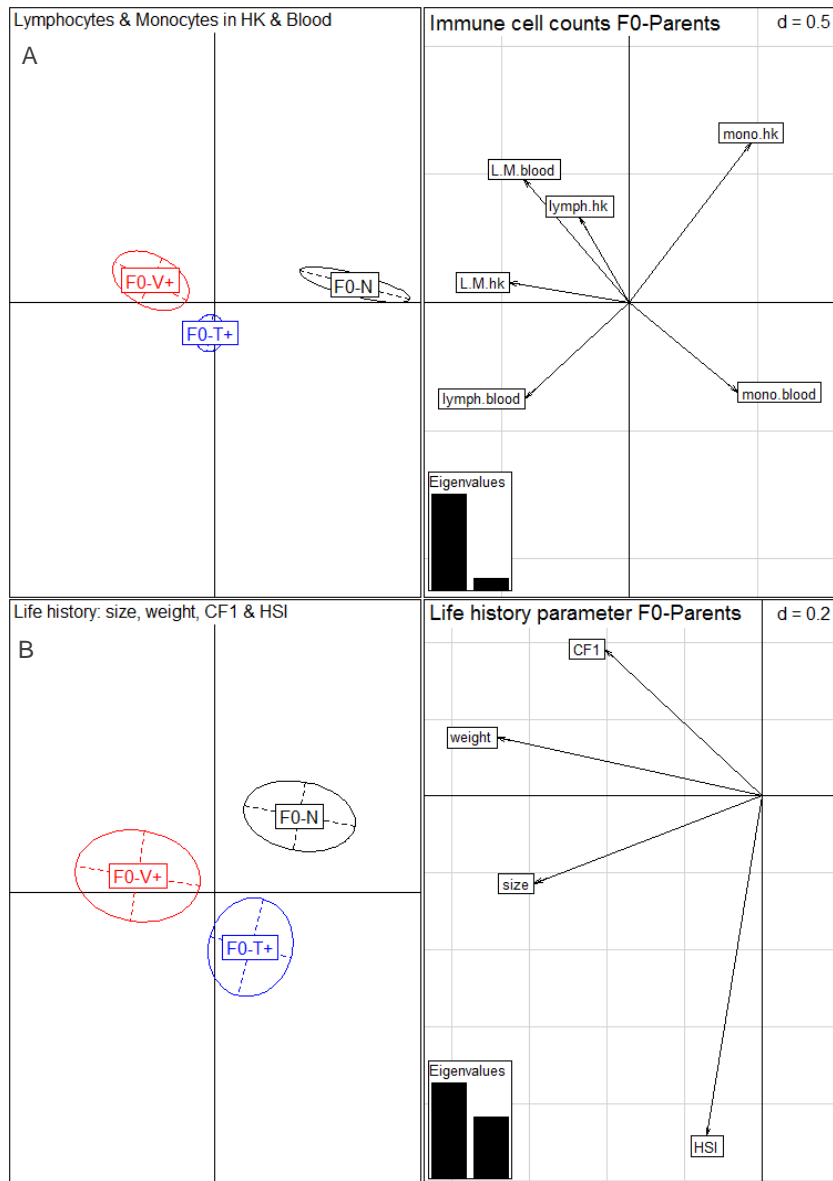


Figure 6: Between Component Analysis (BCA) based on (A) immune cell count measurements (lymphocyte and monocyte counts and ratio of head kidney and blood) and (B) life-history parameter (body size, mass (weight), Condition Factor (CF), and Hepatosomatic Index (HSI)) of four-month-old juveniles (N=90). Between component analysis includes the Factor F0-parental treatment (F0-Vibrio (F0-V+) in red; F0-Tenacibaculum (F0-T+) in blue; F0-Naïve (F0-N) in black). The scatterplot is demonstrating how the each response variable is correlating with the principle components and how much each variable is contributing to the total variability.

Chapter II

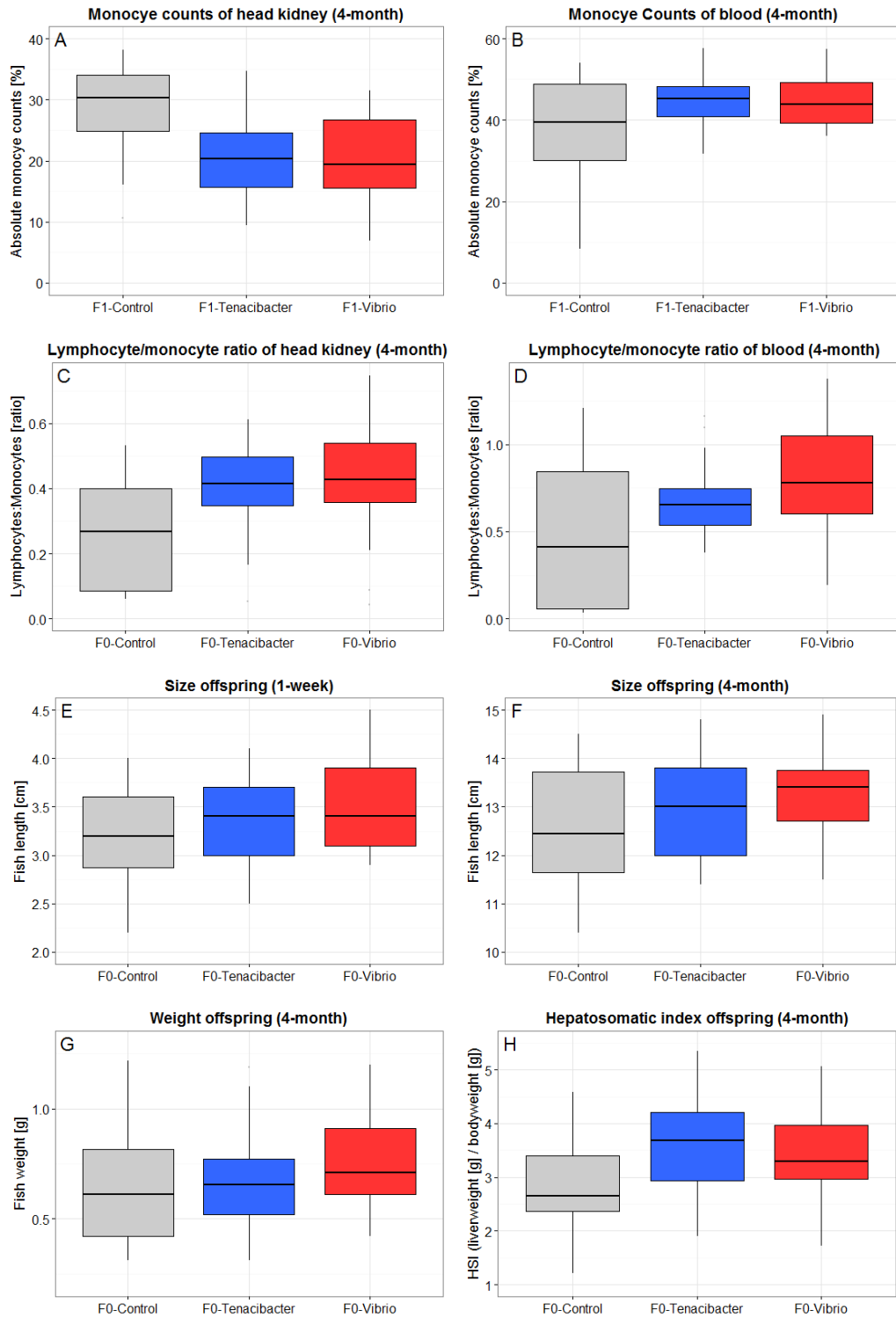


Figure 7: Parental treatment effects visualized by boxplots based on immune cell count measurements and life-history parameter of four-month-old juveniles (N=90) and one-week-old juveniles (N=300). Each boxplot is splitted up according to F0-parental treatment (F0-Vibrio (F0-V+) in red; F0-Tenacibaculum (F0-T+) in blue; F0-Naïve (F0-N) in grey). Different panels are **A:** monocyte counts of head kidney from four-month-old juveniles; **B:** monocyte counts of blood from four-month-old juveniles; **C:** lymphocyte/monocyte ratio of head kidney from four-month-old juveniles, **D:** lymphocyte/monocyte ratio of blood of four-month-old juveniles, **E:** size of one-week-old juveniles, **F:** size of four-month-old juveniles, **G:** Body mass (weight) of four-month-old juveniles, **H:** Hepatosomatic index (HSI) of four-month-old juveniles.

III.) Trans-generational bacteria specificity (F0:F1 treatment interaction)

One-week and four-month-old F1-juveniles – Immune gene expression

We analysed whether the F0-parental treatment x F0 offspring treatment interaction was mainly driven by differences among homologous (parents and offspring same bacteria-species) and heterologous (parents and offspring different bacteria-species) treatment combinations. The BCA interaction plot for all immune genes combines both the F0-parental and F1-offspring treatment effects and their interaction (Figure 2C, 3C). However, the homologous (F0V+/F1V & F0T+/F1T+) and heterologous (F0V+/F1T+ & F0T+/F1V+) treatment combinations neither of the one-week nor of the four-month-old juveniles were significantly different from each other (Figure 2C, 3C, Table 1, S1, S2).

Four-month-old F1-juveniles - Immune cell counts

The F0-parents x F1-offspring interaction of lymphocyte monocyte counts in head kidney and blood illustrate, in line with the gene expression results of one-week and four-month-old juveniles, that homologous (F0V+/F1V & F0T+/F1T+) and heterologous F0V+/F1T+ & F0T+/F1V+ bacteria combinations were not significantly different (Table S2). However, lymphocyte/monocyte ratio of head kidney displayed a trending interaction effect indicating a potential *Vibrio* specificity effect (ANOVA-*L/M-ratio.hk* $F_{4,55}=8.9$, $p=0.074$, Table S3).

IV.) Differences in maternal and/or paternal immune priming and single parental specificity effects

The dataset was divided into single parental groups to focus on differences between maternal and paternal immune priming based on immune gene activity (Table S4, Figure S6). We found that immune genes of the innate immune system of one-week-old juveniles were equally influenced by maternal and paternal bacteria treatment without any hint of specificity upon homologous parental bacteria treatment (PERMANOVA-*innate.paternal* $F_{1,108}=25.75$, $p<0.001$ vs *maternal* $F_{1,108}=11.85$, $p<0.001$; Table S4, Figure S6A vs C). Additionally, the innate immune gene reaction upon acute *Vibrio* and *Tenacibaculum* treatment was for both parental sexes equally strong (PERMANOVA-*innate.paternal* $F_{1,108}=5.04$, $p<0.001$ vs *maternal* $F_{1,108}=6.43$, $p<0.001$; Table S4, Figure S6A vs C). On the contrary, genes of the adaptive immune system only represented a *Vibrio* specific treatment effect whereas there was no specific reaction upon *Tenacibaculum* bacteria treatment

(Figure S6B vs D). Offspring with maternal treatment revealed a more pronounced *Vibrio* specific adaptive immune response than offspring with paternal treatment (Figure S6B vs D). Comparatively, in four-month-old juveniles, with a fully mature immune system, genes of the adaptive immune system were strongly influenced upon the maternal bacteria treatment (PERMANOVA-*adaptive.maternal* $F_{1,108}=8.94$, $p<0.001$; Table S4), revealing a significant interaction term (PERMANOVA-*adaptive.maternal* $F_{2,108}=2.32$, $p=0.036$; Table S4, Figure S6H). Genes of the innate immune system were not affected upon the maternal bacteria treatment (PERMANOVA-*innate.maternal* $F_{1,108}=2.28$, $p=0.09$; Table S4, Figure S6G). Opposed to these findings, the paternal bacteria treatment was significantly influencing innate immune gene expression of offspring (PERMANOVA-*innate.paternal* $F_{1,108}=2.61$, $p=0.022$; Table S4, Figure S6E) and for adaptive immune gene expression (PERMANOVA-*adaptive.paternal* $F_{1,108}=2.61$, $p=0.041$; Table S4, Figure S6F).

V.) Costs of immune priming

One-week-old F1-juveniles - Life-history (size)

F1-offspring with parental bacteria treatment had a larger body size (1.2 +/-0.25 cm) than the control group and offspring with parental *Vibrio* treatment were even significantly bigger than F1-offspring with parental *Tenacibaculum* treatment (ANOVA-*one-week.size* $F_{2,273}=51.54$, $p<0.001$, C<V+, C<T+ and T+<V+; Table S3, Figure 7E).

Four-month-old F1-juveniles - Life-history (size/mass/CF/HSI)

Body size, mass, condition factor (CF) and hepatosomatic index of four-month-old juvenile pipefish were significantly affected by the applied parental bacteria challenge showing a distinct pattern compared to the control group (PERMANOVA-*life-size/mass/CF/HSI* $F_{2,74}=5.18$, $p<0.001$, Table 1; ANOSIM-*life-size/mass/CF/HSI* F0V+ vs control $p=0.005$, F0T+ vs control $p=0.002$; Table S2, Figure 6B). In detail, body length and mass of four-month-old juveniles was significantly influenced exclusively by the parental *Vibrio* immune challenge. Here, offspring with parental *Vibrio* exposure were 1.03 +/- 0.3 cm larger and 0.2 +/- 0.05 g heavier compared to control group (ANOVA-*size* $F_{2,74}=4.65$, $p=0.012$; *TukeyHSD*: C<V+; Figure 7F, ANOVA-*mass* $F_{2,74}=6.25$, $p=0.003$; *TukeyHSD*: C<V+, T+<V+; Table S3, Figure 7G). Moreover, both bacteria treatments of parents also affected the liver size of offspring. Offspring with parental *Tenacibaculum* bacteria treatment had a significantly larger liver (1.069 +/- 0.23 mg) than the control group (ANOVA-*HSI* $F_{2,74}=6.324$, $p=0.003$; *TukeyHSD*: C<T+; Table S3, Figure 7H).

One-week-old F1-juveniles - Maturation

Adult pipefish males (F1) of naïve parents (F0) developed about one month (36.5 +/- 1.5 days) earlier in the season brood pouch tissue for sexual reproduction than offspring of parents with parental *Vibrio* and *Tenacibaculum* treatment (LME-maturity $F_{1,7} = 325,0$ $p < 0.001$; Table S5, Figure S7A). Adult offspring of the parental control group started to reproduce earlier and were having a significant higher number (11.5 +/- 2.6 individuals) of offspring per clutch (LME-clutch.size $F_{1,7} = 7.9$, $p = 0.025$; Table S5, Figure S7B).

Discussion

In our study, we could demonstrate that pipefish parents, if challenged with two different heat-killed bacteria species, are able to prime the immune system of their offspring. Different magnitudes of immune priming are revealed towards *Vibrio* and *Tenacibaculum* bacteria. In young juveniles (one-week-old), resilient immune priming effects, based on immune gene expression of 29 candidate genes, were found against both bacteria species (*Vibrio* and *Tenacibaculum*) exhibiting a distinct bacteria-specific parental priming pattern. On the contrary, immune gene expression and cellular immune response of older juveniles (four-month-old) displayed a strongly preserved immune priming effect solely against *Vibrio* bacteria. The parental *Vibrio* challenge induced a significant higher lymphocyte/monocyte ratio in the blood in comparison to parental *Tenacibaculum* treatment. Moreover, the expression of certain innate and adaptive immune genes (*lymphocyte-antigen 75*, *allograft inflammation factor*, *interferon*, *lectin protein II*, *coagulation factor*, *peptidoglycan recognition protein*, *transferrin*, and *HSP-60*) was upregulated only upon parental *Vibrio* challenge. These findings reveal evidence that specifically for *Vibrio* bacteria a memory was created in the parental generation and trans-generationally transferred. This potentially resulted in a *Vibrio*-specific long-term immune priming effect and enhanced phenotypic plasticity of the offspring. Hence, our study provides, to our knowledge, first evidence for bacteria specific long-term immune priming in teleosts.

The broad-nosed pipefish *S. typhle* is exposed to a variety of *Vibrio* phylotypes in its natural habitat (Roth *et al.*, 2012a). By using an immune challenge with a *Vibrio* isolate of an Italian pipefish (Italy-strain I2K3), we expected this strain to be allopatric and immunological novel for the Baltic pipefish species. However, based on our results of a robust *Vibrio*-specific immune priming effect we might conclude that the adaptive immune system of the pipefish was not specific enough to differentiate between Baltic and Italian *Vibrio* phylotypes. Potentially the epitope structure of the applied *Vibrio*-strain (I2K3) in this experiment was

similar to local *Vibrio* phlotypes of their natural habitat and therefore, more familiar for the immune system of this pipefish population. Even though we filtered the water during the experiment to prevent confounding effects with other bacterial infection (Beemelmans & Roth 2016a), we could not exclude that the parental as well as the F1-generation were in contact with Baltic *Vibrio* bacteria-species throughout the duration of the experiment. Yet, our data suggest that the offspring received non-genetic information about European *Vibrio* bacteria from their parents. That might have been maintained over generations within their population. Further, the juvenile pipefish started to develop a specific immune response against *Vibrio* bacteria within the four month of the experiment.

The *Tenacibaculum maritimum* bacteria used in this experiment were an isolate of a pacific seabream species of Japan (Suzuki *et al.*, 2001). Although we cannot exclude the possibility that the wild parental pipefish population was in touch with any *Tenacibaculum* species within the Baltic sea (Frette *et al.*, 2004), we presumed that this bacteria-strain was immunologically novel for the Baltic pipefish. As the parental long-term immune priming against *Tenacibaculum* bacteria was significantly reduced in four-month-old juveniles, it strongly indicates that immune priming against newly introduced and not prevalent bacteria is decreasing faster during development (Lindholm *et al.*, 2006; Wilson & Réale, 2006). Hence, based on our results, it is tempting to speculate that immune priming against local and prevalent bacteria, with which the parental population was repeatedly in contact before, is more pronounced since the likelihood of a secondary exposure is high. Consequently, the diversity and quantity of specific immune transmission to offspring is reflecting the differences in pathogen environment experienced by their parents as it was shown for vertebrates of higher phylogenetic order *e.g.* specific antibody transmission in birds (Grindstaff *et al.*, 2006).

With respect to the underlying mechanism of this phenomenon, we analysed genes responsible for epigenetic regulation processes and, hence, indirectly could affect the transcriptional regulation of immune gene expression. Our results suggest that epigenetic modification processes might be involved in transmitting this non-genetic information about bacteria specificity trans-generationally. Genes responsible for DNA and histone methylation/demethylation and histone acetylation/deacetylation were predominantly affected by the parental bacteria treatment in four-month-old juveniles. In our study epigenetic regulation genes showed a similar pattern as the immune genes, being significantly affected by the parental *Vibrio* treatment. Hence, our results point to a potential connection of trans-generational immune priming to epigenetic inheritance. The altered expression of genes coding for key players in the epigenetic regulation machinery of immune gene expression supports our hypothesis that epigenetic processes mediate bacteria specific immune priming.

Since DNA methylation and histone modifications are highly dynamic during early development of fish embryos (Lee *et al.*, 2015) most likely consistent epigenetic pattern might take longer to be established. The first dominant epigenetic effect was found in four-month-old juveniles but became significantly more important in the second generation (Beemelmans & Roth 2016c in review).

Since pipefish females invest into the eggs, and males potentially affect the immune system of their offspring via the placenta-like structure during male pregnancy (Roth *et al.*, 2012), different strategies of transferring immunity to offspring may exist. Our results suggest a dissimilar extent of maternal and paternal influences on different immune pathways. In four-month-old juveniles innate immune genes were only affected upon the paternal treatment whereas adaptive immune genes were strongly influenced upon the maternal treatment. These findings are concurrent with results of our previous study in which we found the identical gender-based grandparental immune priming pattern (Beemelmans & Roth 2016c in review). Further, the *Vibrio*-specific effect in the F1-generation based on adaptive immune genes was only pronounced upon maternal treatment whereas paternal influences were stronger on innate immunity. Teleost females prime the adaptive immune system of their offspring by the deposition of immunoglobulins, complement components, and corresponding mRNA transcripts through the yolk into the eggs (Magnadottir *et al.*, 2005; Picchiotti *et al.*, 2006; Swain *et al.*, 2006; Swain & Nayak, 2009; Zhang *et al.*, 2013). It seems that adaptive immunity in a sex-role reversed fish species is still largely driven by the maternal immunological experience. On the contrary, fathers predominantly influenced innate immune gene expression of four-month-old juveniles. Therefore, males may transfer information about immediate protection cues against prevalent pathogens in their environment through the placenta like structure during male pregnancy and/or through epigenetic marks. As immunological pathways are parent-specific affected, maternal and paternal immune priming complement each other and are even maintained until the second generation (Beemelmans & Roth 2016c in revision). These findings have important evolutionary and ecological consequences. As both parents can complementary provide their offspring with immune memory against specific local bacteria species, the protection of offspring may be even more than additive (Roth *et al.*, 2010; Roth *et al.*, 2012). This could result in improved phenotypic plasticity in the immune response and leading to a more specific and stronger reaction upon local and prevalent pathogens. Consequently, maternally and paternally inherited bacteria specific immune priming is thus not only providing specific protection for the young progeny, but it also allows organisms to plastically adapt to the prevailing pathogen environment (Little *et al.*, 2003; Moret, 2006; Roth *et al.*, 2010; Roth *et al.*, 2012).

One-week-old and four-month-old juveniles reacted in a similar way towards the exposure to both bacteria epitopes with an equal up-regulation of immune genes associated with the immediate innate immune system and complement system (Tort *et al.*, 2003). Moreover, our results suggest that in four-month-old juveniles with a fully developed immune system, 20 hours after the immune challenge monocytes already migrated from the head kidney into the bloodstream to the peripheral organs where they most likely differentiated into macrophages and dendritic cells to elicit a non-specific immune response (Janeway *et al.*, 2008; Murphy, 2011). Further, we found a positive correlation between innate immune genes (*Lectin protein II*, *Lectin protein I*, *Complement component 1 & 3*, *Interferon*, *Peptidoglycan recognition protein*, *Tyrosine kinase* and *Ik-cytokine*) and amount of innate immune cells (*monocytes*). This verifies a direct connection between gene activity and immune performance in accordance to a previous study (Birrer *et al.*, 2012). However, lymphocytes, cells of the adaptive immune system responsible for recognizing "non-self" and generating a specific response to eliminate specific pathogens, were not significantly influenced upon the direct treatment. Yet, certain adaptive immune genes displayed a correlation (*HIVEP3* and *Lymphocyte antigen 75*) with lymphocytes in the head kidney and blood indicating that the adaptive immune system started to be activated. Hence, the absence of bacteria specific immune response 20 hours after the immune challenge and the incapacity to create immune specificity upon parental homologous bacteria treatments might be explained either by the non-fully activated adaptive immune system, or even could be ascribed to abnormalities of their adaptive immune system (Haase *et al.*, 2013). *S. typhle* not only lacks a spleen in which antibody producing T-cells and B-cells assemble and proliferate but also a functional MHCII machinery and T-cell related genes like CD8 β / TCR γ , which are key innovations of the adaptive immune system (Matsunaga & Rahman, 1998; Haase *et al.*, 2013). It was supposed that in the *Syngnathidae* family the functional loss of MHCII-mediated adaptive immunity was potentially due to a relatively recent secondary gene loss (Haase *et al.*, 2013). So far, no alternative pathway has been discovered to compensate for the MHCII-mediated adaptive pathway (Buonocore 2015).

Parental *Vibrio* challenge caused an advantage for the F1-offspring not only in terms of increased immunity but also with increased growth, which remained until four-month post birth. Therefore, in this study a more efficient immunity went hand in hand with increased size and mass. But a strong expression of immunity as well as the maintenance of inducible defenses can be costly in terms of energy resources, particularly if the parasitic environment is not met in the next generation and should therefore be traded off against other fitness parameters (Lochmiller & Deerenberg, 2000; Ardia *et al.*, 2012). The liver, an important storage organ of energy reserves, served as estimate about the metabolism and energy

status of the fish (Wootton *et al.*, 1978; Campbell & Love, 1978). Both parental bacteria treatments positively affected the hepatosomatic index, suggesting that immune primed offspring revealed a higher metabolic status. However, costs were found later during sexual maturation of the F1-adults. Prolonged time of males to develop a brood-pouch tissue and reach sexual maturation, delayed reproduction period of about one month, and an overall significant smaller clutch size compared to offspring without parental bacteria challenge. Thus, it appears that immunological costs were compensated by reduced energy investment into reproduction. A significant shift of maturation time and reproduction would have essential ecological consequences for the pipefish. Every summer season (April/May) the pipefish population is migrating into the seagrass meadow of the Baltic coastlines where males can reproduce up to four times per season with several females (Berglund *et al.*, 1986; 1989; Berglund, 1993). Therefore, it is advantageous to mate as early as possible in the season due to predator pressure of a new habitat and also due to their polyandrous mating behaviour (Berglund, 1993). Hence, channelling the resources towards higher immunity and balancing these benefits with reduced reproduction might be a costly strategy, which may shape the outcome of immune priming across generation (Contreras-Garduño *et al.*, 2014).

While bacteria specific biparental immune priming in the pipefish might be beneficial on the individual level, it could have severe ecological and evolutionary consequences on the population level and may alter the dynamics of host/pathogen interaction. Hence, it could increase the likelihood of persistence of a pathogen and therefore enhance the impact of a disease on the population level, leading to a pronounced destabilization effects on host-parasite dynamics (Tidbury *et al.*, 2011; Tate & Rudolf, 2012; Tidbury *et al.*, 2012). Nevertheless, selection for immune priming indicates that there must be an adaptive net profit when the parental and offspring environment match, and that total benefits will outweigh the associated costs (Schmid-Hempel, 2011; Kaufmann *et al.*, 2014). Apart from higher immunity, also other benefits like a larger body size, increased mass and better metabolic condition were identified, which could compensate the costs of reduced reproduction. Thus, producing less offspring in a better shape might be the optimal strategy. In any case, more studies are needed to test for fitness advantages under natural conditions to evaluate definite trade-offs between biparental bacteria specific immune priming and reproduction.

Conclusion

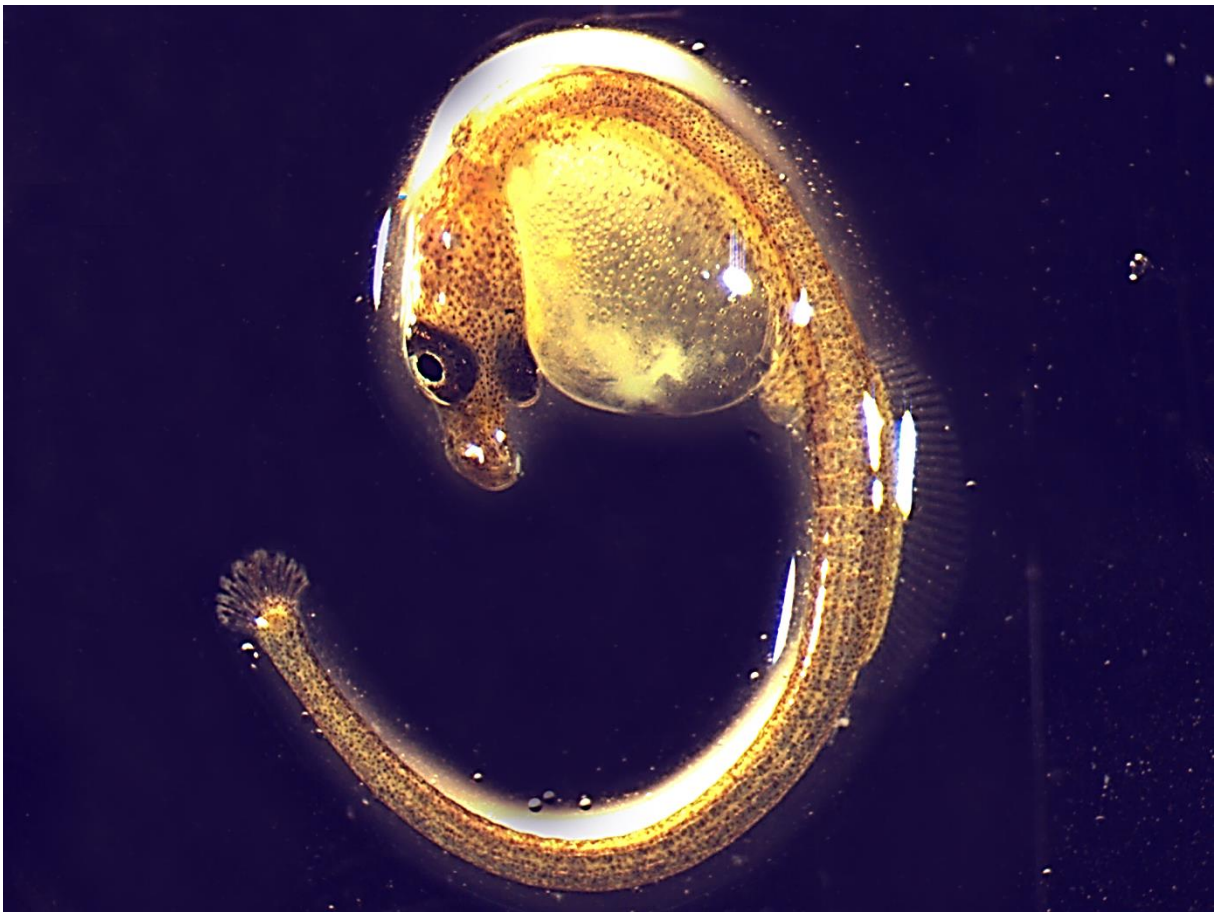
We demonstrated that the broad-nosed pipefish *S. typhle* is able to specifically prime the next generation against local and prevalent but also newly introduced bacteria species. Long-term protection was thereby maintained only against prevailing and familiar *Vibrio* bacteria. We investigated complementing gender-specific contribution to different pathways of the immune system in juveniles of different age classes, indicating differences in the magnitude of maternal and paternal immune priming. Further, epigenetic regulation genes revealed only in four-month-old juveniles a distinct pattern. This indicates that with increasing age of the fish epigenetic regulation processes might be important in transferring immune-related information across generations. Moreover, trade-offs between immune priming and reproduction demonstrated the costs of this immune defense strategy. This might constrain the overall beneficial effects of immune priming.

Future work should focus on subsequent evaluation of the bacterial specificity and physiological mechanisms mediating bacteria specific immune priming effects (e.g. DNA methylation and histone acetylation pattern, antibodies, and microbiota). Further insight into complex interaction between costs and benefits under natural conditions are required for a better understanding of fitness advantages. Finally, theoretical modelling including all necessary observed parameters would permit to predict the impact of pathogen persistence, population structure and could identify the evolutionary origin and maintenance of biparental bacteria specific immune priming in the pipefish.

Acknowledgements

We thank Maude Poirier, Sophia Wagner, Martin Grimm, Verena Klein, Fabian Wendt, Joao Gladeck, Isabel Keller, Susanne Landis, Joanna Miest, Diana Gill, Franziska Brunner and Andrea Franke for support during experimental field and lab work. This study was financed by a grant from the German Research Foundation (DFG) to OR associated to the SPP1399 host-parasite coevolution and a grant from the Volkswagenstiftung to OR. AB was supported by a stipend from the International Max-Planck Research School for Evolutionary Biology (IMPRS).

Chapter III



Chapter III

Title picture of Chapter III: Close-up picture of broad-nosed-pipefish (*Syngnathus typhle*) larvae with yolk sac; approximately 3 weeks post hatch (Copyright: Anne Beemelmanns).

Grandparental immune priming in the pipefish *Syngnathus typhle*

Anne Beemelmans¹ and Olivia Roth¹

¹*Helmholtz-Centre for Ocean Research Kiel (GEOMAR), Evolutionary Ecology of Marine Fishes, Düsternbrooker Weg 20, 24105 Kiel, Germany*

In review for *Molecular Ecology* (Special Issue “Epigenetic Inheritance”), January 2016

Cited in this thesis as Beemelmans & Roth 2016c

Abstract

Phenotypic changes in response to environmental influences can persist from one generation into the next. In many systems, parental parasite experience influences offspring immune responses, known as trans-generational immune priming (TGIP). TGIP in vertebrates is mainly maternal and short-term, supporting the adaptive immune system of the offspring during its maturation. However, if fathers and offspring have a close physical connection, evolution of additional paternal immune priming can be adaptive. Biparental TGIP may result in maximized immunological protection. Here, we investigate multi-generational biparental TGIP in the sex-role reversed pipefish *Syngnathus typhle* by exposing grandparents to an immune challenge with heat-killed bacteria and assessing gene expression and development in the F2-generation. In our study, a grandparental immune challenge induced gene expression of the innate and adaptive immune system in the F2-generation. Similarly, genes mediating epigenetic effects including histone acetylation and DNA methylation were involved in grandparental immune priming. While grand-maternal impact was strong on genes of the adaptive immune system and complement system, grand-paternal exposure changed expression patterns of genes mediating innate immune defense and epigenetic regulation. In a system with male pregnancy, grandparents influenced the immune system of their grand-offspring in a sex-specific manner, demonstrating multigenerational biparental TGIP. The involvement of epigenetic effects suggests that TGIP via the paternal line may not be limited to the pipefish system with its male pregnancy. While the benefits and costs of grandparental TGIP depend on the temporal heterogeneity of environmental conditions, multi-generational TGIP may affect host-parasite coevolution by dampening the amplitude of Red Queen Dynamics.

Key words: Grandparental effects; Immune priming; Epigenetic effects; Immune defense; Host-parasite interaction; pipefish

Introduction

In sexually produced offspring, genotypes are determined by equal maternal and paternal genetic contributions. An offspring's phenotype is also influenced by a plethora of environmental factors experienced during its ontogeny and even factors experienced by its parents (Bonduriansky & Day, 2009; Bonduriansky, 2012; Scheiners, 2014). Such trans-generational plasticity of phenotypes is often adaptive, can promote efficient and rapid acclimation to environmental changes, and even has the potential to modify evolutionary dynamics (Mousseau & Fox, 1998a; Boulinier & Staszewski, 2008; Pigliucci & Müller, 2010). Anti-predator defenses (Agrawal *et al.*, 1999), tolerance of abiotic environmental change (Sunday *et al.*, 2012; Munday *et al.*, 2013; Shama & Wegner, 2014), and induced disease resistance in offspring (Mitchell & Read, 2005; Goellner & Conrath, 2008) are amongst the most studied trans-generational effects that are not inherited via DNA, but through a diversity of alternative mechanisms (Jablonka & Raz, 2009).

The transmission of parental parasite experience that subsequently leads to an enhanced offspring immune defense is known as trans-generational immune priming (TGIP) (Grindstaff *et al.*, 2003; Sadd *et al.*, 2005; Grindstaff *et al.*, 2006; Moret, 2006; Roth *et al.*, 2012). TGIP enables a faster or stronger offspring immune reaction that matches the current parasite environment (Lui, 2000). Environmental variation can result in heterogeneous parasite distributions across environments (Gomulkiewicz *et al.*, 2000), persisting through host generations. Under such circumstances, and where host-dispersal is limited, TGIP is predicted to be evolutionarily adaptive.

In vertebrates, studies of TGIP have mainly focused on the transfer of maternal antibodies (Kowalczyk *et al.*, 1985; Grindstaff *et al.*, 2003; Hasselquist & Nilsson, 2009), while also substances of the innate immune system are involved (Mulero *et al.*, 2007; Swain & Nayak, 2009; Zhang *et al.*, 2013). Usually mothers deposit immune defense components into the eggs, transfer them during development (e.g. via the placenta), or, in mammals, after birth via lactation (Brambell, 1970b; Grindstaff *et al.*, 2003; Hasselquist & Nilsson, 2009). The classical view is that male sperm only contributes to heredity, *i.e.* via DNA, to the offspring. Considering recently discovered hereditary mechanisms that are not based on the pure DNA sequence itself, like DNA methylation or histone acetylation pattern, this view is challenged into question (Danchin *et al.*, 2011; Jablonka & Lamb, 2014; Pigliucci & Finkelman, 2014). Recently, a growing number of examples indicate influences beyond pure transfer of DNA of sperm on offspring and, thus, indicate the fathers having a role beyond the determination of the offspring genotype (Roth *et al.*, 2010; Crean *et al.*, 2012; Jiang *et al.*, 2013; Potok *et al.*,

2013; Crean & Bonduriansky, 2014; Kaufmann *et al.*, 2014). In an invertebrate system with only an ejaculate-based connection between father and offspring, the paternal environment influences offspring immune phenotype (Roth *et al.*, 2010; Eggert *et al.*, 2014). Further, a strong paternal contribution to immune phenotype of progeny was found in vertebrates with intense paternal care or investment (Roth *et al.*, 2012; Kaufmann *et al.*, 2014).

The advantages of TGIP in vertebrates were considered to be strongest during early life stages by strengthening the developing adaptive offspring immune system, with the effect fading upon maturation (Rossiter, 1996; Lindholm *et al.*, 2006). However, some ecological conditions may select for multi-generational TGIP modulating immune responses beyond the F1-generation (Reid *et al.*, 2006). Persistent TGIP should be favoured when the parasite environment is stable over time and, hence, across host generations. So far, our knowledge about TGIP in vertebrates past the early phase of an offspring's life is limited. Only scarce evidence supports the existence of TGIP beyond the maturation of the adaptive immune system of vertebrates (Lemke *et al.*, 2009), while in invertebrates TGIP can apparently cross the borders of more than just one generation (Norouzitallab *et al.*, 2014).

To explore the potential for long-lasting and multi-generational effects on immunity, we experimentally assessed grandparental TGIP in the sex-role reversed pipefish *Syngnathus typhle*. In this fish species, males have evolved a unique placenta-like structure (Ahnesjö & Craig, 2011). Not mothers but fathers are thus the pregnant sex (Jones *et al.*, 1999). During male pregnancy, embryos are provided with nutrients and oxygen over this placenta-like structure (Ripley & Foran, 2009; Kvarnemo *et al.*, 2011), which may mechanistically enable additional paternal investment into offspring immune defense. The pipefish system was chosen as here not only mothers but also fathers can induce offspring immune response over biparental TGIP (Roth *et al.*, 2012). Mature pipefish males and females (F0) were exposed to two heat-killed bacteria (*Vibrio spp.* and *Tenacibaculum maritimum*) or a control prior to mating in a fully reciprocal mating design (Figure S1). By leaving the F1 untreated, we were able to examine grand-maternal, grand-paternal or grand-biparental effects in the F2-offspring upon exposing them to the same bacteria challenge as their grandparents (F0). In the F2-generation, we assessed a life-history trait (body size) along with the gene expression of 44 target genes, including genes functionally associated with innate and adaptive immunity or epigenetic modification through DNA/histone methylation and histone acetylation processes.

We found strong grandparental effects that influenced the immune gene expression of grand-offspring upon bacterial exposure. This grandparental TGIP is sex-specific (grand-father vs. grand-mother) with regard to immune pathway activation and the involvement of epigenetic regulation genes.

Material and Methods

Broad-nosed pipefish *Syngnathus typhle* were caught in the south-western Baltic Sea (54°44'N; 9°53'E, Germany) in spring 2013 and acclimatized to local summer conditions (15psu, 18°C, 14:10h light:dark) within three weeks. We hosted the pipefish in local water out of Kiel Fjord, which was initially cleaned by a sand filter followed by 5, 20 and 50 µm mesh filter, UV-light, surface skimmer and biological filter to reduce the amount of microbes in the aquaria system. The parental generation (F0) was vaccinated as described in Roth *et al.* (2012), using a combination of heat-killed immunological novel *Vibrio* spp. (V+) and *Tenacibaculum maritimum* (T+) bacteria or were left naïve (N) as control. Immune-challenged mature pipefish were used in following mating design: 1. Control: [♀N x ♂N]; 2. Paternal: [♀N x ♂V+/T+]; 3. Maternal: [♀V+/T+ x ♂N] and 4. Biparental: [♀V+/T+ x ♂V+/T+] and kept according to their mating pairs (families) in separate 36x80 L semi-flow through aquaria (16 family replicates per parental bacteria treatment and 8 per control group; 56 families; Figure S1). For the immune challenge, we used a combination of two distinct marine bacteria species to cover a potential wide range of immunological pathways, which could be differentially influenced by TGIP. The *Vibrio* spp. bacteria used in this experiment were an isolate of an Italian pipefish, allopatric and novel for the Baltic pipefish species (Roth *et al.*, 2012a). The *Tenacibaculum maritimum* bacteria were an isolate of a pacific seabream species of Japan (Suzuki *et al.*, 2001), and have, to our knowledge, not been in contact with the Baltic pipefish before. Both, *Vibrio* (s-shaped and flagellated) and *Tenacibaculum* (rod-shaped but non-flagellated), are common gram-negative marine bacteria causing the following diseases in teleost. *Tenacibaculum maritimum* induces 'Flexibacteriosis' also known as 'black patch necrosis' in marine fish (Bernardet *et al.*, 1990; Kolygas *et al.*, 2012). This disease is mainly characterised by haemorrhagic skin lesions, an ulcerative condition leading to important mortalities among marine fish species (Bernardet *et al.*, 1990; Kolygas *et al.*, 2012). *Vibrio* bacteria can trigger 'Vibriosis', a systemic disease of marine fishes (Egidius, 1987), and e.g. *Vibrio harveyi* species are known to cause mass mortalities in captive bred sea horses (Alcaide *et al.*, 2001). The combination of *Vibrio* and *Tenacibaculum* permitted to cover an extended range of bacteria specific TGIP (Beemelmanns & Roth 2016b in submission).

F1-offspring were reared in 36x80 L aquaria according to their parental treatment. Depending on their developmental stage, fish were fed with *Artemia salina* naupliae, copepods (*Acartia spec*) and mysids (*Mysis spec*). F1 individuals were crossed within former parental treatment groups but left immunologically naïve (from each of the 4 parental treatment groups 5 families were chosen to do F1-crosses, resulting in 20 F1 families). In spring 2014, F2-

juveniles were exposed one-week post birth to the same heat-killed *Vibrio* (V+) and *Tenacibaculum* (T+) bacteria used for the F0 generation or left naïve (per F1-crossing 4 families produced F2-offspring resulting in 16 F1-families. Out of each family, 12 individuals were chosen for the direct immune challenge. Per F2-offspring treatment (V+/ T+/ naïve) 4 individual replicates were used, resulting in a total of 192 samples). In addition, this sampling procedure was chosen to conduct a complementary study to our previous work with the pipefish model, in which we addressed maternal, paternal and biparental TGIP to the F1-generation (Roth *et al.*, 2012). Upon immune challenge, F2-juveniles were kept for 20 hours in 10x10 cm tanks at 18°C and 15 psu, using one tank per F2-offspring treatment and family. After the incubation time, juvenile body standard length [cm] was measured and animals were killed with MS 222. The body was transferred into 1 ml RNA-later, kept at 4°C for 24 hrs, and then frozen at -20°C.

Quantification of Gene expression

We quantified the mRNA-level of 44 pre-selected target genes of former studies (Birrer *et al.*, 2012; Roth *et al.*, 2012) and pilot studies using quantitative real time PCR (qPCR). The genes were identified and selected based on an expressed-sequence-tag (EST) library from *S. typhle* exposed to natural *Vibrio* isolates (Haase *et al.*, 2013). Accordingly, immune genes were selected among previously identified genes that were differentially expressed in the pipefish upon infection (Haase *et al.*, 2013) and presumed to be important target genes during our applied immune challenge. The chosen genes were grouped into four functional categories: (i) innate immune system (immediate and non-specific immune defense upon infection i.e. phagocytosis), (ii) adaptive immune system (specific antibody-mediated immune defense), (iii) complement system (complements the antibody and phagocytic cell mediated immune response), and (iv) epigenetic modulators (DNA and histone methylation/demethylation and histone acetylation/deacetylation) (Table S2). Primers were designed and tested according to Birrer *et al.* (2012), whereby all primer pairs attained standard curves with slopes of log quantity vs. threshold cycle (Ct) between -3.6 and -3.1 and efficiencies between 90-100% (Table S8).

The RNA extraction of 192 tissue samples was performed with an RNeasy96 Universal-Tissue Kit (Qiagen) according to manufacturer protocol. Extraction yields were measured by means of a spectrophotometer (NanoDrop ND-1000; peQLab), to allow a reverse transcription into cDNA via a QuantiTect®Reverse-Transcription Kit (Qiagen) of a fixed amount of 800 [ng/µl] per sample. The gene expression of 48 genes was measured using a Fluidigm-BioMark™ system based on 96.96 dynamic arrays (GE-chips). A pre-amplification

step was performed by mixing a 500 [nM] primer pool of all 48 primers with 2.5 mL TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.25 μ L cDNA per sample. The mixture was pre-amplified (10 min at 95°C; 14 cycles: 15 s at 95°C; 4 min at 60°C) and the obtained PCR products diluted 1:10 with low EDTA-TE buffer. For chip loading, a sample mix was prepared by combining 3.5 μ L 2x Ssofast-EvaGreen Supermix low Rox (Bio Rad) with 0.35 μ L 20x DNA-Binding Dye Sample Loading Reagent (Fluidigm) and 3.15 μ L pre-amplified PCR products. An assay mix was prepared by combining 0.7 μ L of 50 [μ M] primer pair mix, 3.5 μ L Assay Loading Reagent (Fluidigm), and 3.15 μ L low EDTA-TE Buffer. Finally, 5 μ L of each sample and assay mix were filled into the GE-chips and measured in the BioMark™ system, applying the GE-fast 96.96 PCR protocol according to Fluidigm instructions. The samples were distributed randomly across chips and each of these included no template controls (NTC), controls for gDNA contamination and two technical replicates per sample and gene.

Data Analysis and Statistics

For each of the two technical replicates per sample, the mean cycle time (Ct), the standard deviation (SD), and the coefficient of variation (CV) were calculated. Samples with a CV larger than 4% were removed, due to potential measurement errors (Bookout & Mangelsdorf, 2003). The combination of the housekeeping genes ubiquitin (Ubi) and ribosome protein (Ribop) showed the highest stability (geNorm $M > 0.85$) (Hellemans *et al.*, 2007) and their geomean was used to quantify relative gene expression of each target gene by calculating $-\Delta$ Ct-values. All plots and statistical tests were performed in R (v3.0.3) and PRIMERv6 (Clarke & Gorley, 2006). Multivariate statistics were used to infer differences in the entire expression pattern of all 44 target genes, but also divided into functional gene categories (Table S2). To evaluate the effects of F0-grandparents and F2-grandoffspring treatment and their interaction, we applied a permutational multivariate analysis of variance (PERMANOVA) using the `adonis` function in R (McArdle & Anderson, 2001). The PERMANOVA model was based on a metric Euclidean distance matrix of non-transformed $-\Delta$ Ct-values, applying `F0-grandparents` and `F2-offspring` treatment as fixed factors, `family` as fixed nested factor within the `F0-grandparents` treatment and `size` as covariate to account for size relevant influences. Initial pre-analysis using a semi-metric (Bray Curtis) and metric (Euclidean) distance matrix as a basis for the PERMANOVA ensured that the latter was the better fit and most of the variation was explained by the input variable. Further, an analysis of similarity (ANOSIM; SIMPER), a permutation-based approach, was performed with the software PRIMERv6. Whereas the ANOSIM approach allowed disentangling differences between groups (Brazma & Vilo, 2000), the SIMPER test was used to quantify the contribution (%) of each gene to the entire differences between treatment groups. In addition, a principle

component analysis (PCA) for graphical visualization was implemented to assess the clustering according to the F0-grandparental treatment, based on differences in expression patterns. Therefore, the relative expression data ($-\Delta\text{Ct}$ -values) were fourth-root transformed to allow a comparison of very low and high values on the same scale (Clarke & Gorley, 2006).

In a second univariate approach, each gene was analysed separately. Thus, assumptions for normality of response variables ($-\Delta\text{Ct}$ -values, size) were graphically examined and tested with the Shapiro-Wilk-test after an appropriate Box-Cox transformation using Jmp9 (SAS Institute Inc., USA). A linear model was fitted for each gene using the fixed factors 'F0-grandparents' and 'F2-offspring', the non-independent factor 'Family' nested into 'F0-grandparents' and 'Size' as covariate. For F2-offspring body size analysis, the same model without covariate was performed. False discovery rate (FDR) corrections of p-values for all follow-up ANOVAs was applied to correct for multiple testing (Benjamini & Hochberg, 1995). All significant ANOVAs for F0-grandparents and F2-offspring effects were followed by post-hoc pairwise t-tests with bonferroni correction to include single interaction effects.

Results

Gene expression

We found that the acute immune challenge of F2-offspring (grand-offspring treatment) significantly affected the multivariate expression of the 44 target genes (PERMANOVA-*all*, $F_{2,162}=4.09$, $p<0.001$, Table 1, Figure 1). A bacterial challenge in the grandparental generation also significantly affected the global expression of the 44 target genes in grand-offspring (PERMANOVA-*all*, $F_{3,162}=9.37$, $p<0.001$; Table 1, Figure 2), an effect that was found in all four functional gene categories of interest. A significant interaction between grandparental and grand-offspring treatment was identified (PERMANOVA-*all*, $F_{2,162}=1.80$, $p=0.003$; Table 1; Figure 1), indicating that grandparental and grand-offspring treatments were not independent. The effect of family as nested term was overall strong and present in all genes (PERMANOVA-*all*, $F_{12,162}=6.47$, $p<0.001$; Table 1). However, the variability explained by family did not influence the grandparental treatment effect (Table S9). Size, included in the model to account for size-related influences on gene expression level, neither revealed a significant effect nor an interaction with the grandparental treatment (Table S9).

Chapter III

Table 1: Results from PERMANOVA analysis of 44 target genes. Multivariate PERMANOVA analysis to assess the effect and interaction of the two fixed factors F0-grandparents and F2-offspring, including the nested factor family (nested in F0-grandparents) and size as covariate on relative gene expression data ($-\Delta\text{Ct}$ -values) based on an Euclidean distance matrix with p-values obtained by 999 permutations (significance code: 0***,0.001**,0.01*). PERMANOVA analysis was conducted for all target genes (44) and divided in following functional gene categories: genes of the innate immune system (13), genes of the adaptive immune system (8), complement component genes (3), methylation/demethylation genes (9), and acetylation/deacetylation genes (5).

PERMANOVA Factors	All target genes [44]			Innate immune genes [13]	
	Df	F Model	Pr (>F)	F Model	Pr (>F)
F0-grandparents	3	9.373	<0.001***	7.613	<0.001***
F2-offspring	2	4.094	<0.001***	2.493	0.016*
F0 * F2	6	1.805	0.003**	2.128	0.008**
Family / F0-grandparents	12	6.469	<0.001***	5.582	<0.001***
Size	1	1.508	ns	1.337	ns
Residuals	162				
Total	186				
	Adaptive immune genes [8]			Complement system [3]	
F0-grandparents	3	17.1	<0.001***	9.443	<0.001***
F2-offspring	2	1.5	ns	6.516	<0.001***
F0 * F2	6	11.0	ns	1.415	ns
Family / F0-grandparents	12	62.2	<0.001***	12.282	<0.001***
Size	1	19.7	ns	1.260	ns
Residuals	162				
Total	186				
	Methylation/Demethylation [9]			Acetylation/Deacetylation [5]	
F0-grandparents	3	7.359	<0.001***	16.180	<0.001***
F2-offspring	2	2.286	0.027*	2.873	0.021*
F0 * F2	6	1.697	0.036*	1.966	0.016*
Family / F0-grandparents	12	6.397	<0.001***	11.339	<0.001***
Size	1	0.821	ns	1.200	ns
Residuals	162				
Total	186				

Grand-offspring treatment and gene expression

We then examined the effects of the acute immune challenge on grand-offspring in the four functional gene categories separately. An induced expression of complement component genes (PERMANOVA-*complement*, $F_{2,162}=6.51$, $p<0.001$, Table 1) and also other genes involved in the innate immune system (PERMANOVA-*innate*, $F_{2,162}=2.49$, $p=0.016$, Table 1) (Figure S3) was found. In contrast, genes of the adaptive immune system were unaffected (Table 1). Expression of genes associated with epigenetic modification was significantly affected (histone acetylation/deacetylation genes [PERMANOVA-*acetyl*, $F_{2,162}=2.87$, $p=0.021$], DNA and histone methylation/demethylation processes [PERMANOVA-*methyl*, $F_{2,162}=2.29$, $p=0.027$], Table 1). DNA methylation genes, responsible for gene silencing, displayed a trend for down-regulation, which implies a general induction of gene expression upon the acute immune challenge (Figure S3).

Grandparental treatment and gene expression

By applying pairwise comparisons in an ANOSIM and visualization via principle component analysis (PCA) (Table 2, Figure 2) based on 44 genes, we found marked and significantly different expression profiles among all four grandparental treatments (Figure 2A). Along the Principle Component (PC) 1, the grandparental control clusters opposed to all other three grandparental treatments, demonstrating a strong grandparental effect on offspring gene expression (Figure 2A). Moreover, the visualized centers of gravity of the grand-paternal and grand-biparental groups overlap, which implies that they are more similar than the grand-maternal group that clusters further apart (Figure 2A). However, all four grandparental treatments were significantly different from each other (ANOSIM-*all*: $p=0.001$; Table 2; Figure 2A). The contribution for each gene ranged between 1-5% (Table S4).

Interaction between grand-offspring and grandparental treatment on grand-offspring gene expression

We applied a PCA to the multivariate gene expression data of all 44 genes for visualization of F0-grandparent x F2-offspring interaction effects (Figure 1, Figure S5). In a two-dimensional PCA depiction, F0 and F2 treatment groups (naïve vs. immune-challenged) are distinct on the first principle component axis (PC1), which explains the largest variance contribution (30% of total variation, Figure 1). The grandparental effect is much stronger than the effects of grand-offspring immune challenge. Upon grandparental immune challenge, grand-offspring revealed significantly different expression profiles in case of own immunological exposure, whereas grand-offspring immunological exposure did not change the expression profile, if grandparents were naïve.

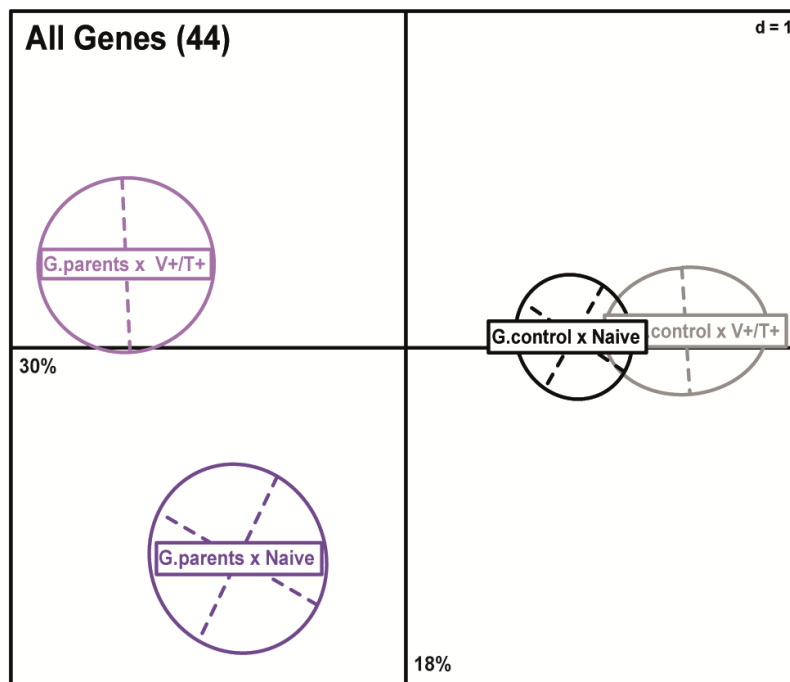


Figure 1: Principle Component Analysis (PCA) of F0-grandparental and F2-grand-offspring interaction effect for all target genes (44). PCA to visualize F0-grandparental x F2-offspring interaction effect on relative gene expression data (forth-root transformed $-\Delta\text{Ct}$ -values) based on an Euclidean distance matrix of all target genes (44). Depicted groups are grandparental control (G.control) x F2-offspring naïve (Naïve), grandparental control (G.control) x F2-offspring bacteria treatment (V+/T+), grandparental treatment (G.parents) x F2-offspring naïve (Naïve), grandparental treatment (G.parents) x F2-offspring treatment (V+/T+).

Grandparental impact on innate immune genes

Grand-offspring expression of the 13 genes involved in the innate immune defense revealed a significant effect upon grandparental bacteria exposure (PERMANOVA-*innate*, $F_{3,162}=7.61$, $p<0.001$; Table 1, Figure 2B). Grand-offspring immune challenge induced innate immune system gene expression (PERMANOVA-*innate*, $F_{2,162}=2.49$, $p=0.016$, Table 1). The F0 x F2 interaction (PERMANOVA-*innate*, $F_{6,162}=2.13$, $p=0.008$; Table 1) shows the same pattern as observed and described for all genes (Figure S5-B).

All grandparental treatment groups displayed different gene expression profiles, no difference was only found in the grand-paternal (GP) vs. grand-biparental (GBi) treatment (ANOSIM-*innate*: GPxGBi: $p=0.960$; Table 2; Figure 2B). This is apparent in a PCA, in which grand-paternal and grand-biparental groups have remarkable overlapping centers of gravities (Figure 2B). This implies that the bacterial environment experienced by the grandfathers drives the grand-biparental impact on genes of the innate immune system.

Table 2: Results from ANOSIM analysis of 44 target genes. Multivariate ANOSIM analysis to assess differences in the gene expression profiles per grandparental (F0) treatment groups applying pairwise comparison on relative gene expression data (fourth-root transformed $-\Delta Ct$ -values) based on an Euclidean distance matrix and 999 permutations. Global significance values over all treatments and for pairwise comparisons between grandparental control (G.control), grand-paternal (G.paternal), grand-maternal (G.maternal), grand-biparental (G.biparental) treatment groups. ANOSIM analysis was conducted for all target genes (44) and divided in following functional gene categories: genes of the innate immune system (13), genes of the adaptive immune system (8), complement component genes (3), methylation/demethylation genes (9), and acetylation/deacetylation genes (5).

ANOSIM	All genes [44]	Innate genes [13]	Adaptive genes [8]	Complement genes [3]	De/Methylation genes [9]	De/Acetylation genes [5]
Global P-value	0.001	0.001	0.001	0.001	0.001	0.001
<i>pairwise comparison</i>						
G.biparental * G.maternal	0.021	0.015	0.149	0.168	0.030	0.012
G.biparental * G.paternal	0.034	0.096	0.002	0.009	0.374	0.505
G.maternal * G.paternal	0.001	0.001	0.001	0.202	0.002	0.001
G.biparental * G.control	0.001	0.001	0.001	0.002	0.001	0.001
G.maternal * G.control	0.001	0.001	0.001	0.018	0.001	0.078
G.paternal * G.control	0.001	0.001	0.001	0.124	0.004	0.001

Grandparental impact on adaptive immune genes

Grandparental effects were found for the eight genes involved in the adaptive immune defense (PERMANOVA-*adaptive*, $F_{3,162}=17,11$, $p<0.001$; Table 1; Figure 2C). Neither did grand-offspring treatment affect the expression of adaptive immune system genes (Table 1), nor could an F0 x F2 interaction be identified (Table 1, Figure S5-C). No statistical differences between grand-maternal (GM) vs. grand-biparental (GBi) groups was discovered (ANOSIM-*adaptive*: GMxGBi: $p=0.149$; Table 2; Figure 2C), also the overlapping gravity centers in the PCA indicate similar gene expression profiles of the two groups. Therefore, the grand-biparental effect on genes of the adaptive immune system is mainly driven by the grand-maternal treatment.

Grandparental impact on complement component genes

The expression of the grand-offspring complement component genes (C3, C9 and C1Q-sco), which mediate between innate and adaptive immune system, changed upon grandparental immune challenge (PERMANOVA-*complement*, $F_{3,162}=9.44$, $p<0.001$; Table 1, Figure 2D). Also, the grand-offspring immune challenge induced an expression of genes of the complement system (PERMANOVA-*complement*, $F_{2,162}=6.52$, $p<0.001$, Table 1), but interaction effects between the F0 x F2 treatments were not identified (Table 1, Figure S5-D). The complement component factors were impacted by both grand-paternal and grand-maternal treatment, displaying similar expression profiles (Figure 2D). As the grand-maternal

(GM) and grand-biparental (GBi) groups are not significantly different and have precisely the same center of gravity in the PCA (Figure 2D), the complement factors are more affected by the grand-maternal treatment (ANOSIM-*complement*: GMxGBi: $p=0.168$; Table 2; Figure 2D).

Grandparental impact on genes involved in epigenetic regulation

The expression profiles of nine genes responsible for the methylation and demethylation of either DNA or histone residues and of five genes responsible for the acetylation and deacetylation of only histone residues in one-week-old F2-juveniles showed a significant change of expression in case of grandparental bacteria exposure (PERMANOVA-*methyI*, $F_{3,162}=7.36$, $p<0.001$; PERMANOVA-*acetyl*, $F_{3,162}=16.18$, $p<0.001$; Table 1, Figure 2E-F). Also, the grand-offspring treatment changed expression of genes associated to epigenetics, i.e. histone acetylation/deacetylation (PERMANOVA-*methyI*, $F_{2,162}=2.29$, $p=0.027$; Table 1), as well as DNA and histone methylation/demethylation processes (PERMANOVA-*acetyl*, $F_{2,162}=2.87$, $p=0.021$; Table 1). Moreover, a F0 x F2-offspring interaction was identified, similar to the interaction described for all genes and innate immune genes (PERMANOVA-*methyI*, $F_{6,162}=1.69$, $p=0.036$; PERMANOVA-*acetyl*, $F_{6,162}=1.97$, $p=0.016$; Table 1, Figure S5E-F). Multivariate pairwise comparisons displayed a higher grand-paternal than grand-maternal effect over the 15 epigenetic regulation genes, since grand-paternal (GP) and grand-biparental (GBi) groups are not significantly different from each other (ANOSIM-*epigenetic*: GPxGBi: $p=0.396$; Table 2). This expression pattern was persistent for methylation as well as for acetylation genes (ANOSIM-*methyI*: GPxGBi: $p=0.374$; ANOSIM-*acetyl*: GPxGBi: $p=0.505$; Table 2, Figure 2E-F). Genes of the acetylation processes of histones showed a pure grand-paternal influence, since the grand-maternal (GM) treatment group was not significantly different from the control (GC) group (ANOSIM-*acetyl*: GMxGC: $p=0.078$, Table 2, Figure 2F).

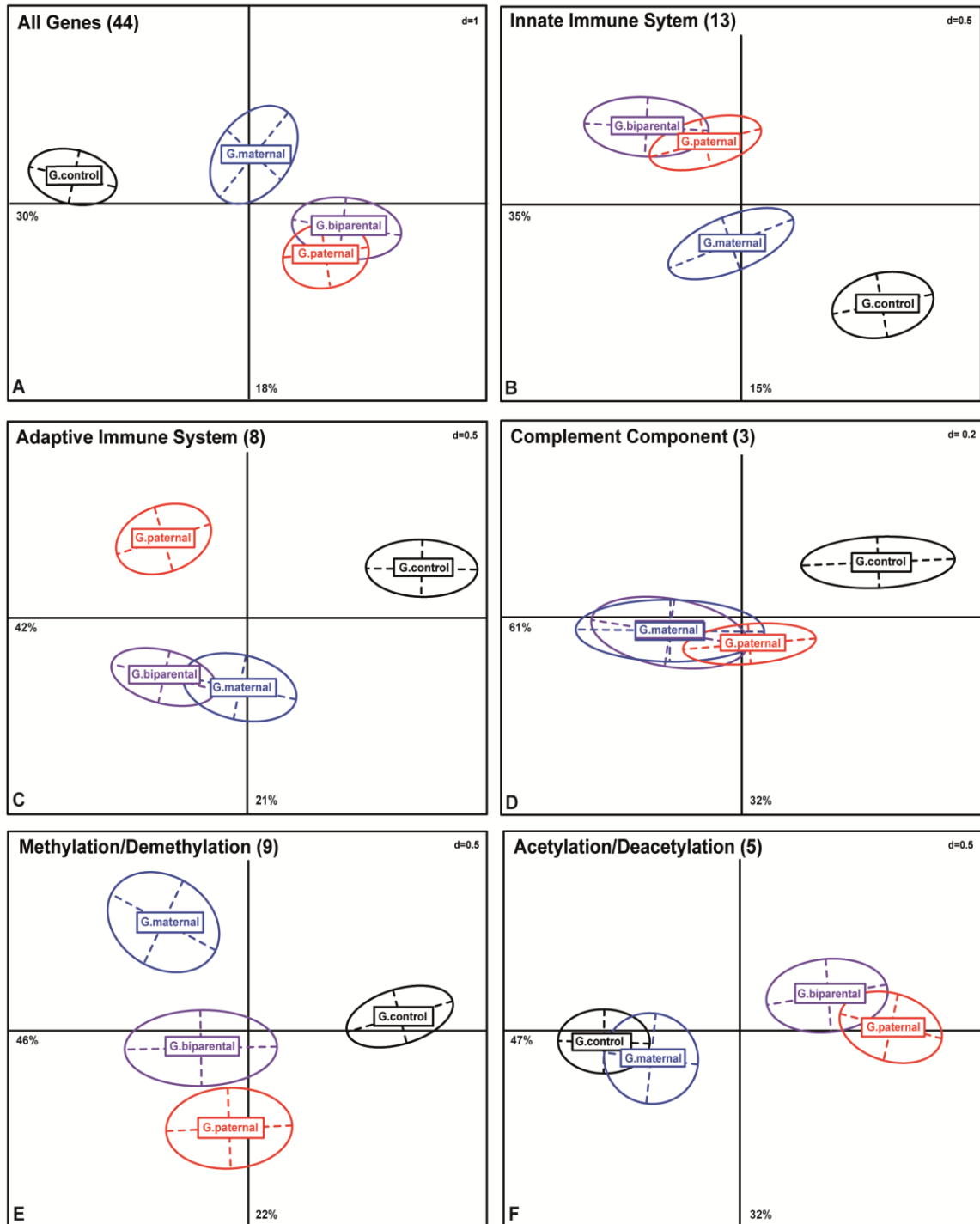


Figure 2: Principle Component Analysis (PCA) of grandparental effect for all gene categories. PCA to visualize differences in the gene expression profiles per grandparental control (G.control), grand-paternal (G.paternal), grand-maternal (G.maternal) and grand-biparental (G.biparental) treatment groups on relative gene expression data (forth-root transformed $-\Delta Ct$ -values) based on an Euclidean distance matrix (N=192). Panel 2A: all target genes (44) and divided in following functional gene categories: Panel 2B: genes of the innate immune system (13), Panel 2C: genes of the adaptive immune system (8), Panel 2D: complement component genes (3), Panel 2E: DNA and histone methylation/demethylation genes (9), and Panel 2F: histone acetylation /deacetylation genes (5). Percentage values of variation explained by principle component 1 and principle component 2 are pictured underneath the corresponding axis.

Table 3: Results from univariate statistical analysis of 44 target genes and size as phenotypic trait. Univariate testing using follow up ANOVAs for each target gene of all categories and size as life-history trait. FDR corrected p-values for F0-grandparents. Significant p-values are marked in bold letters. The abbreviations for grand-paternal (GP), grand-maternal (GM), grand-biparental (GBi) indicate significant difference from the grandparental control group (GC) after pairwise t-test with bonferroni correction, whereas (n.s) means no statistical difference. Grandparental (F0) effects show the differences between the three grandparental bacteria treatment groups.

Genes/ Size Residuals DF=162	Functional Category	F0 Grandparents DF=3		Grand- Paternal vs. Control	Grand- Maternal vs. Control	Grand- Biparental vs. Control	F0 Grandparental Effects
<i>CD45</i>	Adaptive	<0.0001	***	GP	n.s	GBi	GM>GP, GBi
<i>HIVEP2</i>	Adaptive	<0.0001	***	GP	GM	GBi	GM>GP
<i>HIVEP3</i>	Adaptive	0.0075	**	GP	n.s	GBi	GM>GP
<i>IgM-Ic</i>	Adaptive	<0.0001	***	GP	GM	GBi	n.s
<i>Integ-Bt</i>	Adaptive	0.0005	***	GP	GM	GBi	n.s
<i>Lymph75</i>	Adaptive	0.0474	*	GP	GM	GBi	n.s
<i>Lympcyt</i>	Adaptive	<0.0001	***	GP	n.s	GBi	n.s
<i>TAP</i>	Adaptive	<0.0001	***	GP	GM	GBi	n.s.
<i>AIF</i>	Innate	0.0008	***	GP	n.s	GBi	n.s
<i>Calrcul</i>	Innate	0.0578	.	n.s	n.s	n.s	n.s
<i>Cf</i>	Innate	<0.0001	***	GP	GM	GBi	GBi<GP, GM
<i>IL8</i>	Innate	<0.0001	***	GP	GM	GBi	GP>GM, GBi
<i>Intf</i>	Innate	<0.0001	***	GP	GM	GBi	GP<GBi
<i>Kin</i>	Innate	<0.0001	***	GP	n.s	GBi	GM>GBi
<i>LectptI</i>	Innate	0.0193	*	GP	n.s	n.s	n.s
<i>LectpII</i>	Innate	<0.0001	***	GP	GM	GBi	GBi<GM, GP
<i>Nramp</i>	Innate	<0.0001	***	GP	GM	GBi	GBi<GM, GP<GM
<i>Pepdgly</i>	Innate	0.4443		n.s	n.s	n.s	n.s
<i>Tranfe</i>	Innate	0.0022	**	n.s	GM	GBi	GM<GP
<i>Tspo</i>	Innate	0.0001	***	GP	n.s	GBi	GBi<GM, GP<GM
<i>Hsp60</i>	Innate/stress	0.0029	**	n.s	GM	n.s	GM<GP
<i>Ck7</i>	Innate & Adaptive	0.0109	*	GP	n.s	n.s	GP>GM
<i>Ik-cytokine</i>	Innate & Adaptive	0.0086	**	GP	n.s	n.s	GM<GP
<i>IL10</i>	Innate & Adaptive	0.0040	**	n.s	GM	n.s	n.s
<i>LPS-TNF</i>	Innate & Adaptive	<0.0001	***	GP	GM	GBi	GP<GM, GP>GBi
<i>Tyroprot</i>	Innate & Adaptive	0.0005	***	GP	n.s	GBi	GP<GM, GM>GBi
<i>C1Q-sco</i>	Complement	0.0078	**	n.s	GM	GBi	GBi<GM, GP
<i>C3</i>	Complement	0.0353	*	n.s	n.s	GBi	GBi>GP
<i>C9</i>	Complement	0.0072	**	n.s	GM	GBi	n.s
<i>TAF8</i>	Gene activation	0.0033	***	GP	n.s	n.s	GP<GM
<i>ASH2</i>	Gene activation	<0.0001	***	GP	n.s	GBi	GP<GM
<i>TPR</i>	Gene activation	0.0353	*	GP	n.s	n.s	GP>GM
<i>BROMO</i>	Gene activation	<0.0001	***	GP	n.s	GBi	GP>GM,GM>G Bi
<i>MYST1</i>	Gene activation	<0.0001	***	GP	n.s	GBi	GP>GM,GM>G Bi
<i>HDAC1</i>	Gene silencing	0.0039	**	n.s	GM	n.s	n.s
<i>HDAC3</i>	Gene silencing	<0.0001	***	GP	n.s	GBi	GP<GM, GM>GBi
<i>HDAC6</i>	Gene silencing	0.0289	*	n.s	n.s	GBi	n.s
<i>JmjCPHD</i>	Gene silencing	0.3205		n.s	n.s	n.s	n.s
<i>No66</i>	Gene silencing	0.0007	***	n.s	GM	n.s	GP<GM GM>GBi
<i>DnMt1</i>	Gene silencing	0.0173	*	n.s	GM	n.s	n.s
<i>DnMt3a</i>	Gene silencing	0.0279	*	n.s	n.s	n.s	n.s
<i>DnMt3b</i>	Gene silencing	0.0010	**	GP	GM	GBi	n.s
<i>HemK2</i>	Gene silencing	0.0006	***	n.s	GM	GBi	n.s
<i>N6admet</i>	Gene silencing	<0.0001	***	GP	GM	GBi	n.s
<i>Size</i>	Life-history trait	<0.0001	***	GP	GM	GBi	GM<GP, GBi

Gene by gene analysis

We further analysed each gene separately by ANOVAs, using false discovery rate (FDR) and pairwise t-test with bonferroni correction to keep the overall type-1 error rate at $p < 0.05$ (Table 3, Table S6, Figure S7). Forty genes revealed significant grandparental effects (Table 3, Figure 7). Thirteen of those genes were influenced upon all three grandparental bacteria groups (*Hivep2*, *IgM-1c*, *Lymph75*, *TAP*, *Cf*, *Integ-Bt*, *IL8*, *Intf*, *LPS-TNF*, *Lectpl1*, *Nramp*, *DnMt3b*, *N3admet*). Always five genes showed single grand-paternal (*Ik-cytokine*, *Lectpl1*, *CK7*, *TPR*, *TAF8*) and single grand-maternal (*Hsp60*, *IL10*, *HDAC1*, *NO66*, *DnMt1*) treatment effects, whereby two genes were affected upon the grand-biparental (*C3*, *HDAC6*) treatment. Interestingly, eleven genes showed a grand-paternal plus grand-biparental but no grand-maternal effect (*CD45*, *Tspo*, *Hivep3*, *Lymphocyte*, *Tyroprot*, *AIF*, *Kin*, *HDAC3*, *MYST1*, *ASH2*, *BROMO*). Since the grandmothers do not contribute to this effect, we conclude that this observed grand-biparental effect is primarily driven by the grandfathers (Figure S7). Taken together, the expression of more genes examined in our experiment was solely influenced by grandfathers (16 genes) and not by grandmothers (9 genes).

Grand-offspring body size

The body size of one-week-old F2-offspring was negatively influenced by the grandparental immune challenge (ANOVA, $F_{3,162}=15.04$, $p < 0.0001$, Table 3, Figure 3), with F2-offspring from immune-challenged grandparents (GBi) being smaller than F2-offspring from the grandparental control (GC) group ($GBi < GC$; Table 3, Figure 3). In addition, F2-offspring from immune-challenged grandmothers (GM) were smaller than those from immune-challenged grandfathers (GP) ($GM < GP$; Table 3, Figure 3). This indicates a stronger grand-maternal than grand-paternal impact of an immune challenge on offspring size, as already discovered for maternal over paternal effects in the pipefish (Roth *et al.*, 2012).

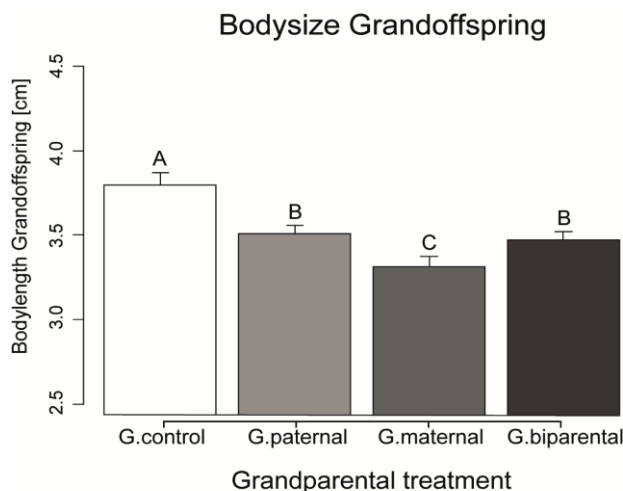


Figure 3: Body size of grand-offspring. Barplot of body size [cm] (+/- SEM) of juvenile F2-grand-offspring (1 week post birth), 20 hrs after immune challenge (N= 192). Mean size with standard error of the mean (SEM) is depicted per grandparental treatment group. The letters A, B, C define significant differences between groups

Discussion

The parental impact on the offspring immune system persisted, once the adaptive immune system reached maturation and even continued beyond the generation boarder, affecting the immune dynamics of the grand-offspring in the pipefish *Syngnathus typhle*. Upon immune challenge in the F2-generation, grand-offspring only induced gene expression, if grandparents had already experienced the bacterial epitopes. While grandparental effects were strong and involved all categories of genes assessed, grand-offspring effects were more specific and depended on the grandparental immunological experience. In the PCA displaying the grandparent x grand-offspring interaction, the distinct gravity centers for grandparental treatments underline their driving effect as opposed to the clustered grand-offspring gravity centers. Grand-offspring effects only affected genes of the innate immune system (*AIF*, *Interferon*, *Hsp60*, *IL10*) and complement component factor *C9*, indicating an activation of the first line of defense in the juveniles. These genes involve immediately available and inducible pathways like bacteria opsonization and lysis over the membrane attack complex (*Complement component 9*), antiviral response (*Interferon*), stress response over molecular chaperone (*Heat shock protein 60*) as well as general inflammatory response (*Allograft inflammation factor*, *Interleukin*). Grand-offspring effects with direct impact on genes of the adaptive immune system were not identified, potentially due to the on-going maturation of the adaptive immune system in one-week-old juveniles (Magnadóttir *et al.*, 2005).

The grandparental immunological treatment altered the gene expression patterns in the grand-offspring, affecting 29 immune genes and 15 epigenetic regulatory genes. When comparing the identified grandparental contributions to TGIP over one generation, we can identify similar patterns (category A to C) as described in (Roth *et al.*, 2012). Category (A) suggests the same extent of differential gene expression when grandfathers, grandmothers, or both grandparents have previously been exposed to a challenge with heat-killed bacteria. In category (B) a parental effect was only found, if both parents were exposed to heat-killed bacteria, leading to an additive biparental immune priming effect. This category could not be identified for grandparental TGIP, indicating that the additive impact of grand-maternal and grand-paternal exposure vanished across two generations. Finally, consistent to category (C), the grand-offspring immune defense was only affected following grand-paternal exposure. In addition to our findings of the former study in 2012, we can add category (D) with pure grand-maternal influences. While TGIP over one generation was either identified for all parental combinations (A), when both parents were immune-challenged (B) or purely

paternal (C), grandparental effects were either consistent for all grandparental bacteria treatment combinations (A), or purely sex-specific (grand-maternal (D) or grand-paternal (C)), but never of additive impact for both grandparents (B). With respect to the gene by gene analysis, a discrepancy between expression pattern of identical genes used for both might be due to (i.) a different time point of sampling (one-week versus five-week old juveniles) and experimental design, (ii) a different combination of housekeeping genes for normalization (2 housekeeping genes versus 1 housekeeping gene), (iii.) a combination of two different bacteria species (*Vibrio* & *Tenacibaculum* versus *Vibrio* cocktail) and more essentially (iv.) a different offspring generation (F2-individuals versus F1-individuals).

The patterns identified are cross-correlated with the targeted functional gene groups, including innate vs. adaptive immune genes, genes of the complement system and epigenetic regulation genes. Genes of the adaptive immune system and the complement component system are influenced by the grand-maternal treatment (category D). Mothers activate the adaptive immune system of their offspring via the deposition of immunoglobulins and complement components into the eggs. However, grand-offspring may rather profit from mRNA transcripts that can be transferred into the egg yolk (Picchiatti *et al.*, 2006; Swain & Nayak, 2009). A grand-maternal priming of the offspring adaptive immune system can result in an earlier usage of acquired immune responses and, hence, a stronger response against pathogens and parasites that persist over several generations, at least from grandparents to their grand-offspring (Norouzitallab *et al.*, 2014).

Genes of the innate immune system were influenced by the grand-paternal treatment (category C). This male-specific effect is consistent with the result of TGIP over one generation (Roth *et al.*, 2012). While females invest into the eggs, males potentially affect the immune system of their offspring via the placenta-like structure during male pregnancy. Our data now suggest that information on prevalent bacterial epitopes is conserved and transferred over more than one generation. Since our experimental protocol used heat-killed bacteria and, thus, excluded parasite-induced selection, the inheritance mechanism is most likely non-DNA sequence based. In contrast to most species with conventional sex roles, in a sex-role reversed pipefish offspring are born into the paternal environment and, thus, share the paternal parasitic experience. This makes the transfer of immunity via the paternal line likely to be adaptive. Hence, both fathers and grandfathers will increase their fitness by altering their phenotype to optimally acclimate offspring to the local parasitic environment (Crean & Bonduriansky, 2014).

While most species boost their offspring's immune response exclusively via maternal TGIP, pipefish rely on both, maternal and paternal TGIP (Roth *et al.*, 2012) that lasts for at least two

generations. This dual developmental plasticity with sex-specific effector pathways gives the next generations an evolutionary advantage in reacting towards potentially virulent parasites. The mechanisms permitting immunological information to be preserved via the paternal line over two generations most likely rely on a combination of small soluble immune components (i) and epigenetic factors (ii) that are transferred via the sperm, the placenta-like structure or the fluid of the paternal brood-pouch tissue. The transfer of immune components (i) is supported by a differential expression upon several genes linked to the innate immune system, such as Allograft inflammation factor (AIF), Interleukin 8 (*IL8*), Lectin protein type I (*lectptI*), Chemokine 7 (*Ck7*), Ik-cytokine (*Ik-cyto*), and Tyroproteinkinase (*Tyroprot*). They all showed a strong grand-paternal effect and, presumably, play a crucial role in the paternal transmission process. DNA methylation and histone modification are responsible for regulating packing and de-packing of the chromatin structure around histone molecules and, consequently, the activation or deactivation of transcription processes for target genes. That such epigenetic modifications of the genome (ii) can be responsible for paternal effects was recently demonstrated in zebrafish displaying paternal methylome transmission (Jiang *et al.*, 2013; Potok *et al.*, 2013). Accordingly, we found strong evidence that DNA and histone modification regulating gene expression is inherited via the paternal line. Genes coding for proteins that are functionally responsible for DNA methylation like DNA-methyltransferase 3b (*DnMt3b*) and N(6)-adenine-specific DNA-methyltransferase2 (*N6admet*) even revealed grandparental effects (category A). However, histone modification processes, such as Histone methyltransferase (ASH2), Lysine-specific demethylase 6A (*TPR*), Histone-acetyltransferase (*KAT2A*) (*BROMO*), Histone-acetyltransferase HAT1 (*MYST1*), and Histone deacetylase 3 (*HDAC 3*), were only affected by the grand-paternal treatment (category C). Our data, thus, propose that environmental stressors like pathogens leave an epigenetic mark on the genome that affect gene expression of genes associated with the innate and adaptive immune system and transcriptional regulation that can be inherited over multiple generations. The fact that grand-paternal TGIP involves epigenetic mechanisms may result in a novel selection scenario for the evolution of TGIP along the paternal line, as the argument that male sperm is too small to transfer any more than just the DNA does not apply any more. A new concept on the role of sperm thus views its role not only as sole messenger of “the other half of the offspring DNA”, but also as important mediator for developmental plasticity and fast phenotypic acclimation to environmental changes (Bonduriansky, 2012; Crean & Bonduriansky, 2014; Szyf, 2015).

The existing interaction between the grandparental and the grand-offspring treatment suggests that the reaction of the F2-generation upon an immune challenge depends on whether or not their grandparents have already received an immunological treatment. Only grand-offspring of immune-challenged grandparents changed their gene expression pattern in case of the bacterial treatment. This implies that the grandparental immune challenge resulted in a preposition of the grand-offspring to react upon a respective immune challenge. This result highlights the importance of multi-generational TGIP, as only animals whose ancestors were exposed to a parasitic assemblage are able to quickly react towards an encounter with parasites or pathogens.

The grandparental TGIP is adaptive, provided that the parental parasitic environment is experienced by the offspring. TGIP, accordingly, gives individuals an advantage whose ancestors successfully defended parasites and transfer this experience to the following generations. However, the maintenance of inducible defenses is energetically costly (Lochmiller & Deerenberg, 2000; Boots & Bowers, 2004), imposing a resource-allocation trade-off on both parents and offspring, due to the costly investment into immune defense. Our former data suggest that the expression of the immune genes used here positively correlates with an activation of the innate (monocytes) and adaptive (lymphocytes) cellular immune system (Birrer *et al.*, 2012; Roth *et al.*, 2012), linking gene expression to a physiological impact of multigenerational TGIP. Here, grandparental immune priming was associated with reduced grand-offspring size. This effect was stronger upon immune priming along the maternal line, indicating that grand-maternal protection results in delayed mating and offspring production. As the mating season of *S. typhle* is short and early offspring profits from higher water temperatures and less competition over the limited food sources, this pattern can have severe consequences. That costs of grand-maternal TGIP were higher than those of grand-paternal TGIP can potentially be assigned to the different mechanistic bases: the induction of adaptive immune responses as transferred via the maternal line are more costly than the activation of innate immune responses via the paternal line that can immediately be induced.

Taken together, TGIP has the potential to change disease dynamics and the spread of epidemics in a population. Under negative-frequency dependent selection, rare parasite alleles may spread quickly in a population, while it takes time for the hosts as a population to counter-adapt under a Red-Queen dynamic (Dybdahl & Lively, 1998). In contrast, as an individual response, TGIP plays out within one generation, because the exposure to a novel parasite will already be met with an amplified immune reaction in the next generation. With

this, the advantage of the novel parasite genotypes vanishes. This dampens the amplitude of predicted frequency dependent selection and may slow down Red Queen dynamics, giving the host an advantage in fast clearance of novel pathogen genotypes (Mostowy & Engelstädter, 2012; Tidbury *et al.*, 2012).

In conclusion, we show that grandparental immune priming effects in pipefish involve a sex-specific contribution to different pathways of the immune system, including an involvement of the epigenetic regulation mechanism. Future work should focus on the evaluation of resistance effects in a survival experiment, bacterial specificity processes, and an in depth analysis of the physiological mechanisms mediating grandparental TGIP.

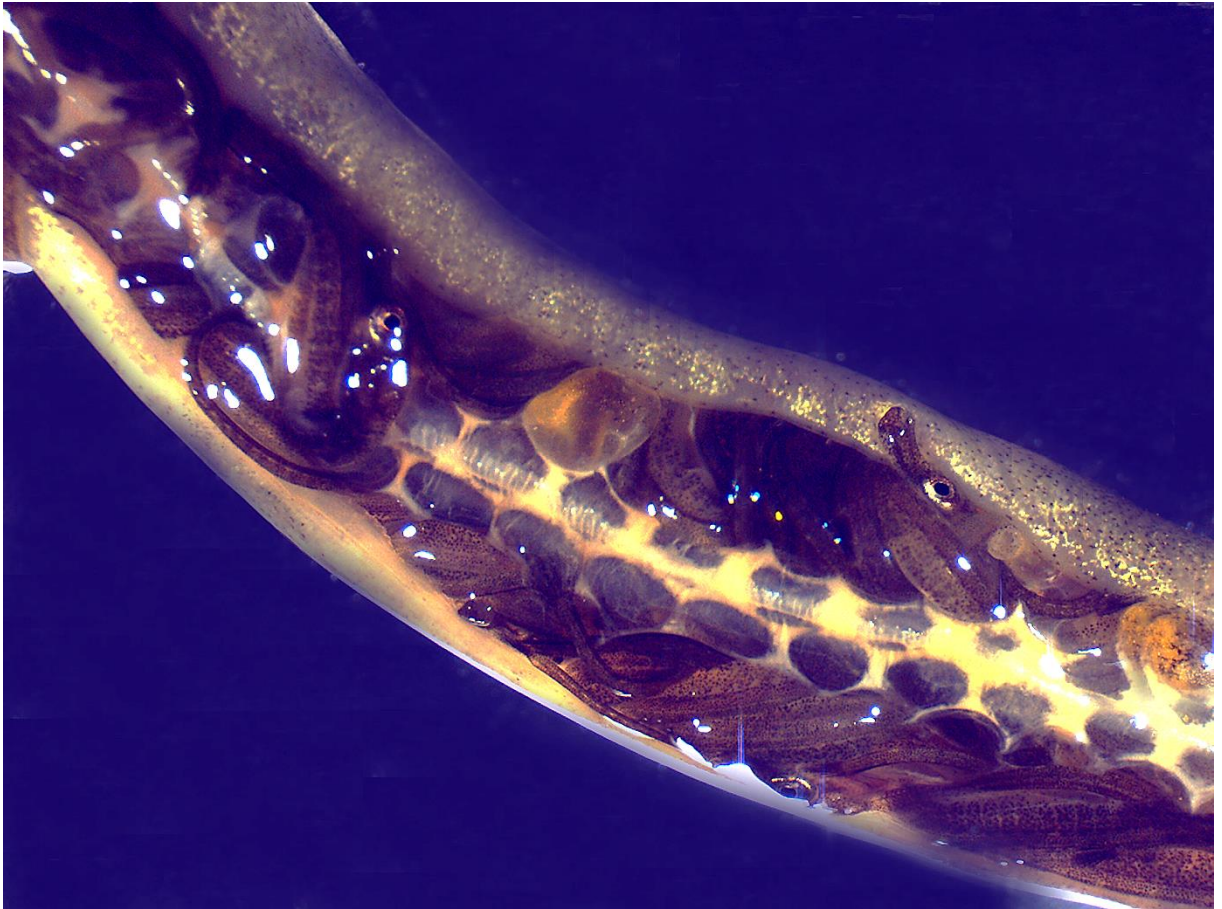
Acknowledgements

We thank Maude Poirier, Martin Grimm, Verena Klein, Sophia Wagner, Fabian Wendt, Isabel Keller and Susie Landis for support in experimental and field-work. Maude Poirier, Diana Gill and Franziska Brunner assisted during gene expression measurements. Input and comments by Thorsten Reusch and Ben Sadd improved the manuscript noticeably. We thank AXIOS and 3 unknown reviewers for their feedback and support. This study was financed by grants from the Volkswagen Foundation and the German Research Foundation (DFG) to OR. AB was supported by a stipend from the International Max-Planck Research School for Evolutionary Biology (IMPRS).

Data accessibility

All data files of the manuscript have been deposited on PANGAEA Server with the Accession Number PDI-9959 (<http://doi.pangaea.de/10.1594/PANGAEA.846114>).

Chapter IV



Chapter IV

Title picture of Chapter IV: Close-up picture of the brood pouch broad-nosed-pipefish (*Syngnathus typhle*) with incorporated larvae; approximately 5 weeks post hatch (Copyright: Anne Beemelmans).

Mechanistic basis of paternal immune priming in the pipefish *Syngnathus typhle*

Anne Beemelmans¹, Olivia Roth¹, Martin Kalbe²

¹*Helmholtz-Centre for Ocean Research Kiel (GEOMAR), Evolutionary Ecology of Marine Fishes, Düsternbrooker Weg 20, 24105 Kiel, Germany*

²*Max-Planck Institute for Evolutionary Biology, 24306 Plön, Germany*

In preparation for Short Communication of Fish & Shellfish Immunology, January 2016

Abstract

Mothers that experienced pathogen infection can prime the immune system of their offspring by transferring immune components such as antibodies over the placenta, milk or egg yolk. In oviparous fish species females deposit immune components to provide protection for developing embryos during their early life stages when their immune system is still naive. In *Syngnatids* (pipefish and seahorses) the evolution of male viviparity allows fathers to nurse the embryos in an enclosed brood pouch, providing not only a higher mechanic protection but also immunological protection. In the sex-role reversed pipefish *Syngnathus typhle* fathers are closely connected to the embryos during male pregnancy over a placenta-like system that mechanistically enables an additional paternal transfer of immune factors. But the mechanistic aspect of paternal immune priming in *S. typhle* remains unexplored.

We analysed the expression of immune and epigenetic regulation genes in the brood pouch tissue of non-pregnant versus pregnant males to evaluate key genes mediating protection and paternal immune priming. Subsequently, an antibody directed against pipefish immunoglobulin light chain was designed to examine the presence of soluble immunoglobulin (IgM) in the pipefish plasma and its potential paternal transfer to the embryos. We found a significant upregulation of lectin, peptidoglycan recognition protein, immunoglobulin light chain, and histone acetyltransferase (KAT2A) transcripts in the brood pouch during male pregnancy indicating their importance in terms of immune protection and potentially paternal priming. We could not confirm the presence of immunoglobulin M in its native tetrameric structure of approx. 800 kDa. These results are pointing to the evolution of a potential different structure or isoform type of IgM which might be connected to the loss of MHC class II mediated antibody response to avoid allograft recognition of 'non-self' eggs.

Key words: Immunoglobulin (IgM), brood pouch, male pregnancy, paternal immune transmission

Introduction

Larvae of oviparous (egg laying) fish species have to develop and survive in a highly unpredictable aquatic environment colonized by a diversity of potentially harmful microbiota (Foey & Picchietti, 2014). Already early in their life fish larvae have to protect themselves against pathogenic microbes (Swain & Nayak, 2009). Partially due to an incomplete established adaptive immune system, embryogenesis and post larvae stages are considered as critical time period accompanied by a high mortality rate (Magnadóttir *et al.*, 2005; Foey & Picchietti, 2014). Early in life fish larvae mostly depend on maternally derived immune molecules (Swain & Nayak, 2009; Zhang *et al.*, 2013). The transfer of immune components from mothers to offspring is known as 'trans-generational immune priming' (TGIP) and is an efficient defense strategy to boost survival of the progeny in case of pathogen exposure (Sadd *et al.*, 2005; Grindstaff *et al.*, 2006; Hasselquist & Nilsson, 2009).

In teleosts the maternally transferred immune factors are stored in form of proteins, peptides, mRNAs, or any other biomolecule in the egg yolk or egg shell (Mulero *et al.*, 2007; Swain & Nayak, 2009; Zhang *et al.*, 2013). Adaptive immune factors like immunoglobulins (Ig) that opsonize bacteria with a high specificity to facilitate their degradation are maternally transferred to the eggs (Bly *et al.*, 1986; Avtalion & Mor, 1992; Lemke & Lange, 1999; Swain *et al.*, 2002; Hanif *et al.*, 2004; Picchietti *et al.*, 2006; Swain *et al.*, 2006). Similarly, immune factors of the innate immune system including lysozymes (Takemura, 1996; Seppola *et al.*, 2009; Wang & Zhang, 2010), antimicrobial peptides (Díaz *et al.*, 1997; Seppola *et al.*, 2009), lectins (Ewart *et al.*, 2001) as well as complement component factors (Løvoll *et al.*, 2006; Wang *et al.*, 2009) are supplied to the offspring by the mothers.

The internal reproduction and provision of developing embryos via placentation in a sterile environment (viviparity) has evolved independently at least 23 times in fish (Guillette Jr, 1987; Wourms & Lombardi, 1992; Blackburn, 2014). Pregnant females can ensure protection and increase survival of their offspring through provisioning of nutrients and immune protection (Guillette Jr, 1987; Wourms & Lombardi, 1992; Blackburn, 2014). *Syngnathids* (seahorses, pipefish) have evolved sex-role reversal and are the only known animals with male pregnancy (Stölting & Wilson, 2007). They are combining the features of maternal provisioning and paternal viviparity, which is most likely maximizing offspring protection (Roth *et al.*, 2012).

In the broad-nosed pipefish *Syngnathus typhle*, a sex-role reversed fish species with male pregnancy, females oviposit their eggs into the paternal brood-pouch in which they get fertilized and nursed over a placenta-like structure (Berglund *et al.*, 1986; Kvarnemo *et al.*, 2011). This brooding structure is a complex vascular network in which cup-like arrangements

of vasculature are surrounding each embryo while providing them with nutrition, ions, and oxygen (Carcupino *et al.*, 1997; 2002; Ripley, 2009). Thus, embryos are protected during their sensitive life stage and can securely develop in this internal brooding structure of their fathers until the vascular structure breaks down and they are released as independent free swimming juveniles (Azzarello, 1991; Carcupino *et al.*, 1997; 2002; Ripley & Foran, 2006; 2009; Kvarnemo *et al.*, 2011). Since fathers are connected to the embryos during male pregnancy over a placenta-like structure, an additional paternal transfer of immune components could potentially occur to enhance offspring survival. Indeed, in our previous studies (Roth *et al.*, 2012; Beemelmanns & Roth 2016a, b) we found strong evidence for biparental immune priming with long-lasting paternal influences on offspring immunity. However, the underlying mechanism of biparental immune priming has remained elusive.

In mammals the trans-placental transmission of Ig includes an Fc receptor-mediated transcytosis of Ig across the syncytiotropho-blast barrier and a transcellular path through the endothelium of fetal capillaries (Simister, 2003; Fuchs & Ellinger, 2004). Even though the antibody diversity in fish is reported to be lower than in mammals (Magnadóttir *et al.*, 2005; Uribe *et al.*, 2011), they are possessing four Ig isotypes, IgM, IgD, IgZ and IgT (Stenvik & Jørgensen, 2000; Flajnik, 2005; Foey & Picchiatti, 2014; Hordvik, 2015) (Box 2). The major fish serum IgM shares structural and functional similarities with mammalian IgM, which are present as a soluble tetrameric form (approx. 700-1000 kDa) or are bound to B-cells as receptor (BCR) (Magnadóttir, 1998; Magnadóttir *et al.*, 2005; Uribe *et al.*, 2011) (Box 2). In teleosts it has also been discovered that maternal soluble IgM molecules are passed on in a monomeric form (Avtalion & Mor, 1992; Magnadóttir *et al.*, 2005). The transfer of IgM molecules into oocytes occurs across follicle cells during the early stage of vitellogenesis using a similar transcytosis process as reported in mammals (Picchiatti *et al.*, 2006; Zhang *et al.*, 2013). In addition, a significant high level of IgM gene transcripts has been recorded in several teleost oocytes, indicating a supplemental transfer of maternal IgM mRNA (Picchiatti *et al.*, 2006; Swain & Nayak, 2009). Furthermore, it has been shown, that maternally derived IgM is consumed by the embryo within the completion of yolk absorption process, and disappears completely during larval stages (Mulero *et al.*, 2007; Zhang *et al.*, 2013).

Apart from the adaptive immune system, fish fundamentally rely on innate immunity (Tort *et al.*, 2003). For instance, lectins are implicated in the innate immunity and responsible for the recognition of carbohydrates of the pathogen cell-surface leading to opsonization, phagocytosis or activation of complement component in order to destroy the pathogen (Fujita, 2002; Zhang *et al.*, 2013). So far, three studies have shown that C-type lectins might play an important role in the defense mechanism of the brood pouch of *Syngnathids* (Melamed *et al.*, 2005; Small *et al.*, 2013; Whittington *et al.*, 2015). Lately, Small *et al.* (2013)

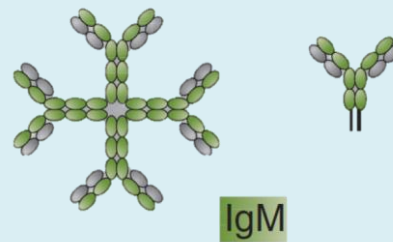
conducted transcriptional profiles of the brood pouch of *S. scovelli* and *S. floridae*. They discovered that multiple C-type lectin paralogs are pregnancy-enriched and pregnancy-depressed. A previous study of Melamed *et al.* (2005) also generated a cDNA library from male brooding tissues of tiger tail seahorses (*Hippocampus comes*) and confirmed the presence of C-type lectins in the fluid of the pouch during pregnancy. The reported data supports that C-type lectins are secreted in significant quantities into the pouch fluid, in particular during early pregnancy, causing erythrocyte agglutination. They concluded that C-type lectins may help to repress bacterial invasion during male pregnancy.

Here, we hypothesize that in the sex-role reversed pipefish TGIP not only occurs in females during oogenesis but also in males during pregnancy. We therefore expect that immune gene transcripts and proteins in the brood pouch tissue help to protect the embryos during early developmental stages and are potentially transmitted over the placenta-like structure of the fathers. Here, we intended to identify IgM of *S. typhle* on transcript and protein level in the plasma of the pipefish and in the brood-pouch tissue. Further, we planned to quantify the level of specific immune genes and genes associated to epigenetic regulation in the brood pouch tissue with progress of male pregnancy (non-pregnant vs pregnant early & late stage). We hypothesize that possible immune gene transcripts are transferred to the fertilized embryos during male pregnancy.

Box 2: Immunoglobulin of fish

Immunoglobulins (Ig) are soluble antibodies or B-cell receptor and involved in eliciting effective specific humoral antibody responses against various antigens in fishes. Igs are composed of two identical heavy and light chains covalently linked to each other which in turn are composed of a constant and variable region. The Fab domain (fragment antigen binding) contains the variable regions of the light chains, which bind with high precision to specific epitopes. Immunoglobulins can (i.) directly neutralize pathogens, (ii.) opsonize ("coating") pathogens for phagocytosis, or (iii.) lyse pathogens via complement system. Teleost possess three major types of immunoglobulins: **IgM**, **IgD** and **IgT/IgZ**, the latter of which is unique for fishes. The isotypes are classified by differences in the composition of the constant region of the heavy chain (μ , δ and τ). All isotypes are fulfilling different functions and are specialized in body compartments such as gut and skin and gill mucus, which is exposed to the environment.

The isotype **IgM** is the most ancient class as it was first found in cartilaginous fish (*i.e.* sharks, rays) and reveal the same function as in all gnathostomes. Specific IgM are secreted by B-cells in the plasma to act as antibody at the site of infection. Predominant IgM in teleost is usually a tetrameric protein complex with native molecular mass varying between 700 and 1000 kDa. The tetramer structure is composed of four monomeric units with two identical heavy chains (ca. 70 kD) and two light chains (ca. 25 kD). Some teleost species and cartilaginous fish also have a monomer of IgM in their serum (200 kD). Transfer of maternal IgM to egg yolk has been documented in several teleostean fishes (plaice, tilapia carp, sea bream, sea bass, salmon, and rainbow trout) and is usually transferred either as a reduced or break-down product or in a monomeric form. Ig supply is almost exhausted at the complementation of yolk absorption until Ig bearing cells firstly appear. In sea bass (*Dicentrarchus labrax*) larvae this time point was documented to be at 49-50 post hatching but is varying with fish species and environmental conditions.



Referred from (Stenvik & Jørgensen, 2000; Flajnik, 2005; Picchiatti *et al.*, 2006; Hordvik, 2015)
Pictures adapted from Wikipedia and Flajnik 2005

Material and Methods

2.1 Pilot study I: Identification of IgM in pipefish plasma

2.1.1 Detection of Immunoglobulin light chain

Transcriptome sequences of *S. typhle*, revealing up to 80% homology to Immunoglobulin light chain (Ig-Lc), were collected from the EST database of Haase *et al.* (2013) and assembled into two main consensus sequences. The sequences were blasted against the non-redundant protein database from NCBI (<http://blast.ncbi.nlm.nih.gov/blast>) and checked in all reading frames against PDB and SwissProt protein databases using Blastx and Blastp. Further, we used ENSEMBL to identify coding regions (CDS) by taking the genome and transcriptome dataset of three-spined stickleback *Gasterosteus aculeatus* as reference (version 67, www.ensembl.org). The potential coding sequence of Ig-Lc was translated into possible peptide sequences and used for pattern searching with the online PROSITE software (<http://us.expasy.org/tools/scanprosite/>) to detect conserved protein domains. In addition, peptide sequences of several teleost species were collected and multiple alignments (CLUSTAL X) with target sequence of *S. typhle* performed to identify conserved region within the domains of the immunoglobulin light chain.

2.1.2 Generation of anti-pipefish-Ig-Lc antibody

We generated a polyclonal antibody against a synthetic peptide of the constant domain of Ig-Lc. For this purpose the basic features and structure of immunoglobulin and its subdomains were analysed with NCBI, Uni-Prot, ExpASy, and PDB programs to choose potential epitopes with a high accessibility and low probability of cross-reactivity with other related domain structures (Figure 1-2; Figures S2-S4). Protein prediction structure software Phyre² (<http://www.sbg.bio.ic.ac.uk/phyre2>) as well as ProtPredict (<https://www.predictprotein.org/>) facilitated a detailed analysis of the three dimensional and secondary protein structure, as well as transmembrane helices and binding sites.

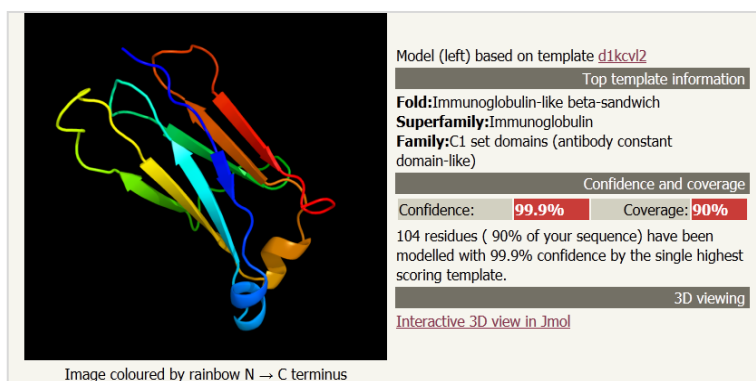


Figure 1: Protein structure prediction of pipefish immunoglobulin constant domain like (C1 set domains) using Phyre² software (online platform version 2/2012).

Finally, we chose a peptide epitope in the protein structure which was hydrophilic, surface orientated, exposed and thus, most likely highly accessible and antigenic (Figure 2, S4). In the following the target peptide of 15 residues ('C-VTLFPPSKEQLQTEK') was synthesized and used to produce highly specific anti-peptide antibody supposed to detect the light chain of pipefish immunoglobulin in a Westernblot approach.

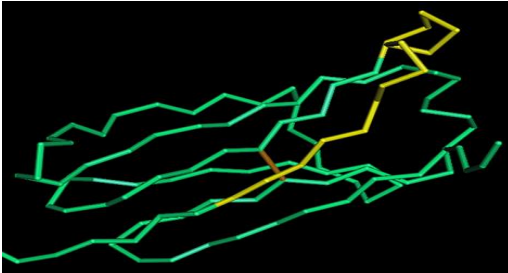


Figure 2: Protein structure prediction of pipefish immunoglobulin light chain. Yellow color indicates the peptide region ('C-VTLFPPSKEQLQTEK'), which was chosen as target (epitope) to generate an anti-peptide antibody.

For the generation of a polyclonal antibody, two rabbits were immunized with 1 ml antigen + 1 ml Freud's Adjuvans at three consecutive time points (Day 0, 14, 35). The peptide synthesis, immunization of rabbits and collection of pre-immune/antisera and high affinity purification was performed by Coring System Diagnostix GmbH (Gernsheim, Germany) following their standard protocol (<http://www.coring.de/>).

2.1.3 Plasma samples

Immune challenged pipefish of previous experiments (30-40 individuals) were killed by an overdose of MS 222, the tip of the tail was cut, and a blood sample collected from the caudal vein using a heparinized microcapillary (80 nm) and stored at 4°C. Blood samples were centrifuged at 3.000 rpm for 7 min and the plasma extracted, pooled, and frozen at -80°C according to Kurtz *et al.* (2007). Plasma pools of wild caught sticklebacks (*Gasterosteus aculeatus*) from 2007 and serum samples of carp (*Cyprinus carpio*) sampled in 2009 were provided by Dr. Martin Kalbe (Parasitology, MPI, Plön).

2.1.4 Gel electrophoresis

Plasma samples were diluted 1:3 with 0.01 M phosphate buffered saline, pH 7.4 (PBS, SIGMA) and 0.5-1 µl were separated by using two different approaches. To analyse the native IgM in its tetrameric structure we firstly separated the proteins by applying them to 1% SDS sample buffer and conducting gel-electrophoresis over a 2.5% agarose gel (stacking gel) at 200 V, 40 mA, and 4 °C for 70 min (native condition). In a second approach, we analysed the subunits (heavy and light chain) of IgM by loading the plasma samples onto a sodium dodecyl sulphate page gel (SDS-PAGE) across a linear 4-20 % gradient using Mini-

PROTEIN TGX (BioRad) acrylamide gels. Gel-electrophoresis of soluble proteins of the pipefish plasma was performed at 200 V, 40 mA for 40 min, after sample treatment with 1 % mercaptoethanol, 1 % SDS and boiling for 5 min at 99 °C (denaturized condition).

The Broad range marker (BioRad) covering 10 kDa to 200 kDa and/or native Bovine IgM (800 kDa) served as molecular size standard. Agarose and SDS gels were stained by a colloidal coomassie staining at Khang (SIGMA) to visualize proteins immobilized in the gel.

1.2.5 Immunoblotting

Following SDS-PAGE separation protocol, gels and membranes were equilibrated for 15 min in 25 mM Tris-Glycine buffer (7.5 pH) and proteins were blotted to a PVDF-membrane (Immobilon-P; 0.45 µm) using a QeqLab tank-transfer system (Biotechnology) at 130 V and 200 mA for 30 min. The membrane was blocked overnight in 3 % BSA/TBST solution before incubation for 2 h with anti-pipefish Ig-Lc antibody (1:300-1:500 dilutions) followed by a secondary peroxidase labelled anti-rabbit IgG antibody (1:160.000 dilution) exposure for 2 h. After antibody incubation ChemiGlow® substrate (Alpha Innotech) was applied for 20-30 min onto the membrane for a chemoluminescence reaction. Membranes were analysed using movie mode of the Alpha Innotech (Biozym) gel imager and Alpha Ease FC (FluorChem) analysis software.

2.2 Pilot study II: Quantification of mRNA transcripts in paternal brood pouch tissue

2.2.1 *Experimental setup and sampling*

Pregnant pipefish males collected from the field (Kiel Fjord, 54°44'N; 9°53'E, July 2013) were hosted under Baltic summer condition (15 PSU; 18 °C) in 100 L tanks separated according to their pregnancy stage: 1. Non-Pregnant (NP), 2. Pregnant/Early (1 week) and 3. Pregnant/Late (3-4 weeks) (Figure 3). Per paternal pregnancy stage eight individual replicates were used. Pregnant males were immune challenged to boost their immune system and trigger a potential paternal transfer of immune components. Therefore, pipefish were first injected with 50 µl 10⁸ cells/mL heat-killed bacteria suspension (*Vibrio* spp. Italy2K3 and *Tenacibaculum maritimum*), followed by a secondary immunization after eight days. 20 hours after the second immune challenge was performed pipefish males were killed by an overdose of the anesthetic (MS-222) and the brood pouch tissue collected. RNA extraction of 24 brood pouch tissue samples was performed according to Beemelmans & Roth (2016c in review) and gene expression of 8 immune genes and 14 genes associated to epigenetic regulation processes (Table S1) were quantified using a Fluidigm BioMark™ based on a 96.96 dynamic array according to Beemelmans & Roth (2016c in review).

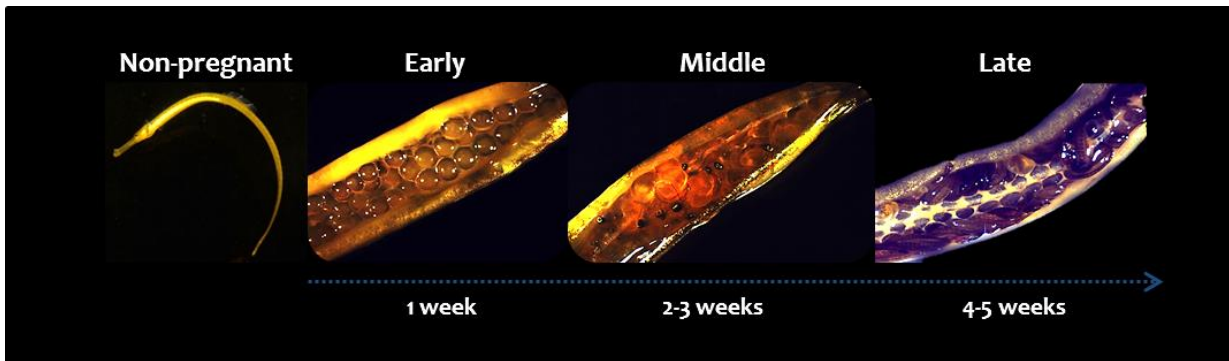


Figure 3: Pregnancy stages of pregnant broad-nosed pipefish (*Syngnathus typhle*) males.

2.2.2 Data analysis and statistics

First, we assessed whether immune gene and epigenetic regulation transcript levels in the brood pouch tissue of non-pregnant pipefish males and pregnant pipefish from two consecutive pregnancy stages were differentially expressed. Therefore, we performed a multivariate data analysis in R (v3.2.2, R Core Team 2015) according to Beemelmans & Roth (2016c in review). We evaluated pregnancy stage effects applying a permutational multivariate analyses of variance (PERMANOVA) for immune gene expression (8 genes) and epigenetic regulation genes (14 genes) of non-pregnant (N=8) and pregnant males (N=16). The PERMANOVA model was based on a Bray-Curtis Matrix of non-transformed $-\Delta\text{Ct}$ -values, applying 'pregnancy stage' as fixed factor. We then applied a Between-Class Analysis (BCA) to investigate graphically a clustering according to the respective pregnancy stages based on differences in expression patterns. Each target gene was then analysed individually using a non-parametric univariate approach to test whether the transcripts were up or down regulated upon male pregnancy. We applied a non-parametric Kruskal-Wallis test using the fixed factor 'pregnancy stage' followed by a Wilcoxon sum and rank test to conduct a pairwise comparison of gene expression between the three different pregnancy stages.

Results & Discussion

3.1 Pilot study I: Identification of IgM in pipefish plasma

3.1.1 Sequence analysis

Ig molecules in teleosts have a tetramer structure of two identical heavy and light chains linked by disulfide bonds (see Box 2, Magnadóttir, 1998). Light and heavy chains are both composed of one variable domain (IgV) with high variability to detect specific antigens, and one constant domain (IgC) (Cheng *et al.*, 2006; Murphy, 2011). We identified a region in the transcriptome of *S. typhle*, which exhibited a 60-70 % homology to the light chain (kappa chain) of immunoglobulin (Figure 1, S2-S4).

Based on Phyre² protein prediction software our target sequence showed high homologies (probability of 99.9 % and coverage of 90%) to the constant domain of the superfamily immunoglobulin (Figure 1, S4). Furthermore, the predicted domain structure was identified as a C1-type classical Ig-like domain with an expected immunoglobulin-like-beta sandwich folding structure (Figure 1, 2, and S2) exclusively found in Ig, B-cell (BCR) and T-cell receptors (TCR). However, based on our sequence analysis, we were not able to disentangle whether *S. typhle* inhibits soluble IgM (antibodies), membrane bound B-cell or T-cell receptors. Therefore, we assumed that soluble antibodies, which are released into the blood, should be detectable using electrophoresis and westernblot. To test the hypothesis, we performed experiments using an anti-pipefish-Ig-Lc-antibody.

3.1.2 Protein analysis

To separate the tetrameric IgM protein of approximately 800 kDa in its native structure, we used a coarse-pored 2.5% agarose gel under non-denaturized conditions. For detection of pipefish IgM we used purified bovine IgM, carp serum and stickleback plasma as a reference. Carp serum and stickleback plasma displayed a dominant band and protein smear in the area between 700-800 kDa close to the purified bovine IgM. These bands represent most likely the native tetrameric IgM of teleost fish with a size of >700 kDa (Magnadóttir 1998). Interestingly, pooled pipefish plasma of 30 individuals did not show any protein band or protein fragments with a size larger than 200 kDa (Figure 4). In addition, we could not detect a native tetrameric IgM molecule in the plasma of *S. typhle* (Figure 4). This result clearly suggests that pipefish possess another isotype or structure of IgM. The most dominant protein band was found at 200 kDa, most likely belonging to monomeric IgM molecules.

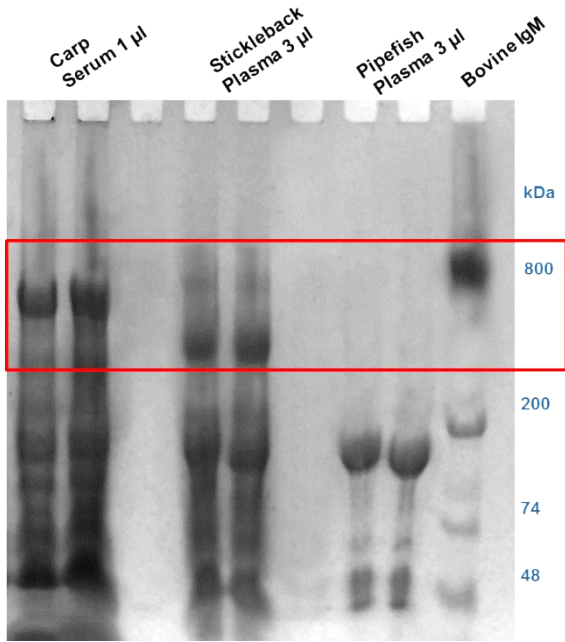


Figure 4: 2.5% Agarose gel. Native plasma proteins were applied on a 2.5 % agarose gel and stained using a colloidal coomassie staining (SIGMA). Per well 3 μ L plasma (1:3 dilution) of wild caught broad-nosed pipefish (*S. typhle*), three-spined stickleback (*G. aculeatus*), 1 μ l (1:3 dilution) carp serum, and 1 μ L broad range marker (BiRad 6-200 kDa) mixed with purified bovine IgM (SIGMA-800 kDa) were loaded on the gel.

To detect the immunoglobulin light chain, we performed a SDS-page of pipefish plasma under denaturated conditions followed by a western blot applying an anti-pipefish-Ig-Lc-antibody. Likewise, we loaded stickleback plasma as reference on the gel. The Western blot clearly determined a band at approximately 23-25 kDa for pipefish plasma and at 20-22 kDa for stickleback plasma (Figure 5). These protein bands most likely belong to Ig light chain subunit molecules, which are estimated to appear at 22 to 27 kDa in different fish species (Magnadóttir, 1998; Cheng *et al.*, 2006; Hordvik, 2015).

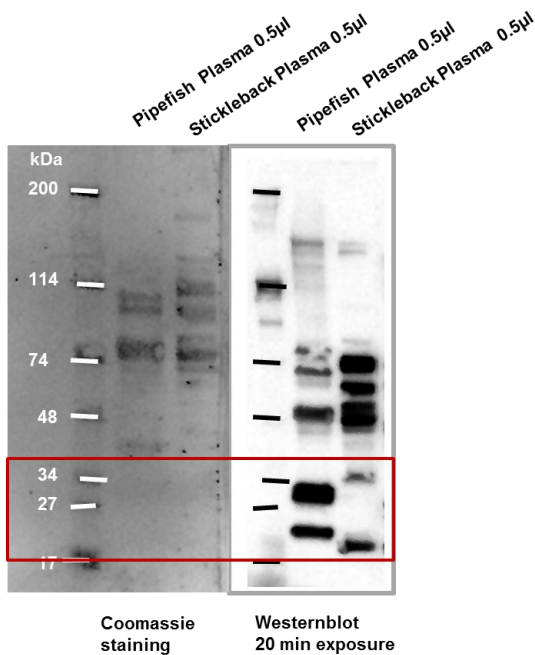


Figure 5: SDS-page and Western blot. **Right side:** Denatured plasma samples were applied on a 4-20 % acrylamide gel (BioRad) and blotted on a PVDF-membrane via tank transfer and proteins stained using a colloidal coomassie staining at Khang. Per well 0.5 μ L plasma (1:3 dilution) of wild caught broad-nosed pipefish (*S. typhle*), three-spined stickleback (*G. aculeatus*), and 1 μ L broad range marker (BioRad) were loaded. **Left side:** Western blot was conducted by using anti-pipefish-IgM-lc-antibody (1:150) as first antibody and peroxidase labelled anti-rabbit-IgG antibody (1:160.000) as secondary antibody. Chemiglow exposure of 30 min allowed visualization of detected bands.

In summary our results indicate that pipefish do not possess tetrameric IgM molecules (800kDa) and possess either monomeric IgM molecules (200 kDa) and/or different isotypes of IgM. In other teleost species it has been shown that monomeric IgM molecules exist and are also maternally transferred (Avtalion & Mor, 1992; Suzuki *et al.*, 1994; Magnadóttir *et al.*, 2005). Our findings of a low molecular IgM structure might be connected with paternal pregnancy or paternal transmission of these molecules. However, so far, we can only speculate whether pipefish own different IgM structure/isotypes or own non-functional IgM due to the high risk of recognizing their incorporated “non-self” eggs as allograft. Our findings of a structural different immunoglobulin molecule of the pipefish could be connected to the loss of MHCII, which is a key component of the antibody mediated adaptive immune response (Haase *et al.*, 2013). To confirm this theory, further studies are needed to shed light on pipefish IgM presence or absence. One possibility to solve this question could be the application of homologous recombinant expression of an IgM subunit to produce anti-pipefish-Ig-antibodies with a higher binding precision.

3.2 Pilot study II: Quantification of mRNA transcripts in paternal brood pouch tissue

In this second pilot study we investigated immune genes and genes responsible for epigenetic regulation processes in the brood pouch tissue of non-pregnant and pregnant males of early and late developmental stages. The brood pouch structure of all three consecutive pregnancy stages revealed a differential immune gene expression (PERMANOVA-*immune* $F_{2,19}=5.98$, $p<0.001^{***}$, Table 1, Figure 6) and epigenetic gene expression (PERMANOVA-*epigen* $F_{2,19}=2.59$, $p<0.0294^*$, Table 1, Figure 6).

Table 1: PERMANOVA results to test for differential gene expression with respect to increasing developmental stages of the brood pouch tissue during male pregnancy (Non-pregnant, Early stage and Late stage) (N=24).

Response Variables DF= 2	Pregnancy stages			
	Residuals DF	R ²	F Model	Pr (F)
Immune genes [8]	19	38%	5.98	<0.001***
Epigenetic genes [14]	19	21%	2.59	0.0294*

Chapter IV

The between class analysis (BCA) indicates for both gene categories (immune genes and epigenetic genes) a similar gene expression pattern (Figure 6). According to the BCA most of the variability was explained by differences between non-pregnant and pregnant males as those groups are clustering on opposed directions along the first principle component (PC) (immune genes: 71%, epigenetic genes 63%). The remaining variability describes differences between early and late stage of male pregnancy demonstrated by directional clustering along the second PC (immune genes: 29%, epigenetic genes 37%).

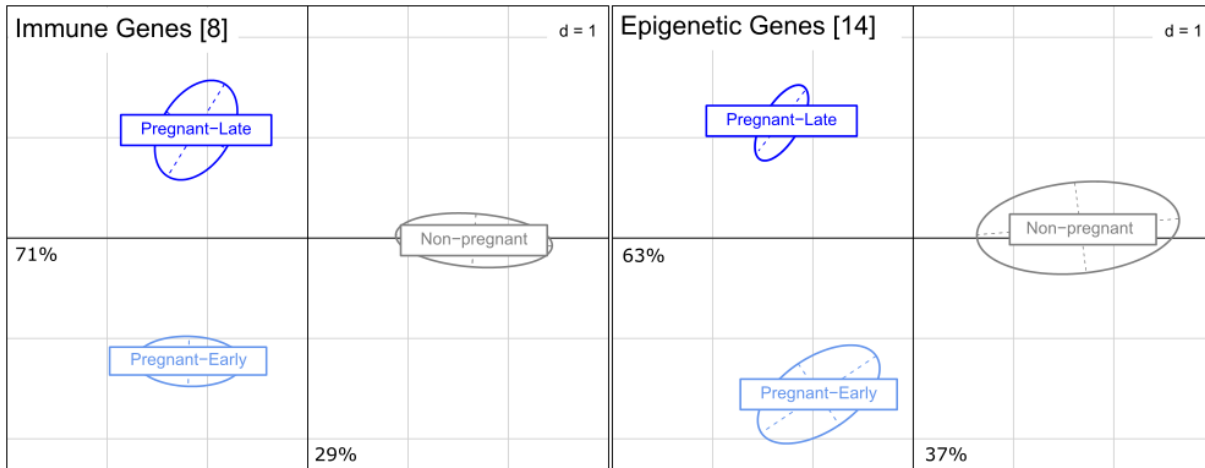


Figure 6: Between Component Analysis (BCA) based on 8 immune genes (A) and 14 genes associated to epigenetic regulation (B) of brood pouch tissue from non-pregnant and pregnant males (N=24). The factor 'pregnancy' stage was included in the between class component analysis to highlight differences between pregnancy stages: 1. Non-Pregnant (NP), 2. Pregnant Early (1 week), and 4. Pregnant late (4/5 weeks).

The clustering pattern demonstrates that valuable differential gene expression in the brood pouch tissue during pregnancy occurred. An antagonistic interplay between upregulated transcriptional activator histone acetyltransferase (KAT2A inside bromo-domain) (ANOVA-*BROMO*: $X^2 = 7.34$, $p=0.026^*$, NP<Early; NP<Late; Table 2, Figure 7), and the downregulated DNA-methyltransferase family member 2 (*HEMK2*) gene responsible for gene silencing (ANOVA-*HEMK2*: $X^2 = 8.71$, $p=0.013^*$, NP>Late; Table 2, Figure 7). It indicates that during pregnancy cells in the brood pouch tissue were highly transcriptionally active. Apart from restructuring processes like thickening and vascularization, also transcription of immune components is mediated as it was shown for other *Syngnathids* (Small *et al.*, 2013; Whittington *et al.*, 2015).

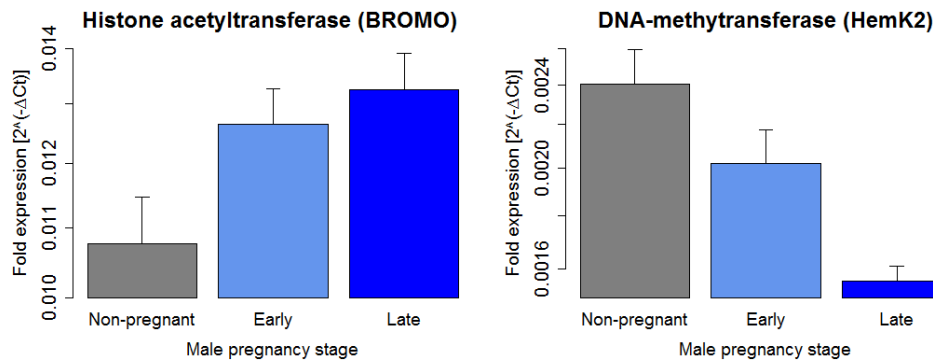


Figure 7: Epigenetic genes. Bar charts demonstrating up/down regulation of specific transcriptional regulation genes (*Histone acetyltransferase (KAT2 A-BROMO)* and *DNA-methyltransferase (HEMK2)*) in the paternal brood pouch tissue of non-pregnant males and pregnant males with increasing brood pouch development (Early and Late stage, N=8 per pregnancy stage).

As immune genes revealed a highly significant effect upon pregnancy stage, it suggests that immune defense is established in the brood pouch tissue during pregnancy. Particularly, transcription level of *C-type lectin*, *Peptidoglycan recognition protein (PGRP)* and *Immunoglobulin light chain* was upregulated during male pregnancy. Hereby, transcript levels of *C-type lectin* and *PGRP* were in both pregnancy stages significantly higher expressed (ANOVA-*lectin*: $X^2 = 10.32$, $p=0.006^*$, NP<Early NP<Late; ANOVA-*PGRP*: $X^2 = 5.85$, $p=0.04^*$, NP<Early NP<Late; Table 2, Figure 8). Both genes code for proteins, which are implicated in innate immunity and responsible for pathogen recognition (Ewart *et al.*, 2001; Dziarski & Gupta, 2006).

Table 2: Non-parametric ANOVA (Kruskal-Wallis) and post hoc test (Wilcoxon sum and rank test) results for each gene revealing a significant effect with respect to increasing pregnancy stages of the brood pouch tissue (Non-pregnant, Early stage and Late stage, N=24).

	Kruskal-Wallis		Wilcoxon rank test	
	$DF = 2$	X^2 chi-square	p -value	Pairwise comparison
C-type lectin		10.32	0.006	NP<Early NP<Late
Immunoglobulin light chain		10.63	0.005	NP<Early Early>Late
Peptidoglycan recognition prot.		05.85	0.040	NP<Early NP<Late
DNA-methyltransferase (HEMK2)		07.34	0.026	NP<Early NP<Late
Histone acetyltransferase (KAT2A)		08.71	0.013	NP>Late

Peptidoglycan recognition proteins (PGRPs) are responsible to recognize bacteria and their unique peptidoglycan cell wall component, mediating host responses to bacterial infections (Dziarski & Gupta, 2006; Li *et al.*, 2007). Li *et al.* (2007) identified high expression of PGRPs in zebrafish eggs, developing embryos, and adult tissues with contact to external environment. In teleosts, PGRPs supposed to have both peptidoglycan-lytic amidase activity

and broad-spectrum bactericidal activity (Li *et al.*, 2007). Likewise, C-type lectins and mannose binding lectins detect and bind sugar chains of the cell-surface of bacteria. Thereby lectins are tagging potential harmful pathogens while activating the complement component system in order to destroy pathogen over phagocytosis (Dodd & Drickamer, 2001; Ewart *et al.*, 2001; Fujita, 2002). Our findings about the presence of C-type lectin in the pouch tissue during pregnancy of *S. typhle* goes in hand with findings of Melamed *et al.* (2005), which could confirm secretion of C-type lectins in the pouch fluid of tiger tail seahorse (*Hippocampus comes*). Therefore, lectins and PGLRPs might be important effector molecules to suppress bacterial growth in the brood pouch tissue throughout all stages of male pregnancy and ensure a high protection of the embryos.

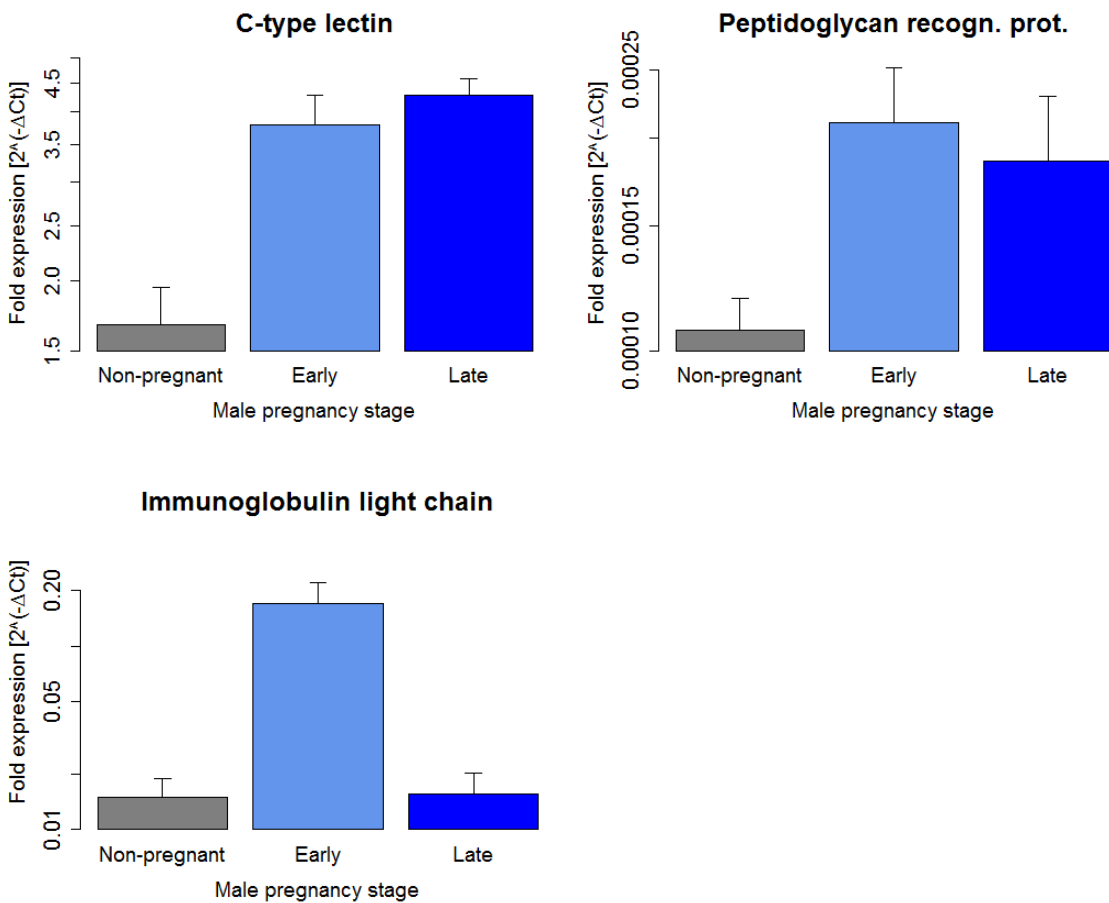


Figure 8: Immune genes. Bar charts demonstrating up or down-regulation of specific immune gene transcripts (*C-type lectin*, *Peptidoglycan recognition protein*, and *Immunoglobulin light chain*) in the paternal brood pouch tissue of non-pregnant males and pregnant males with increasing development (Early and Late stage, N=8 per pregnancy stage).

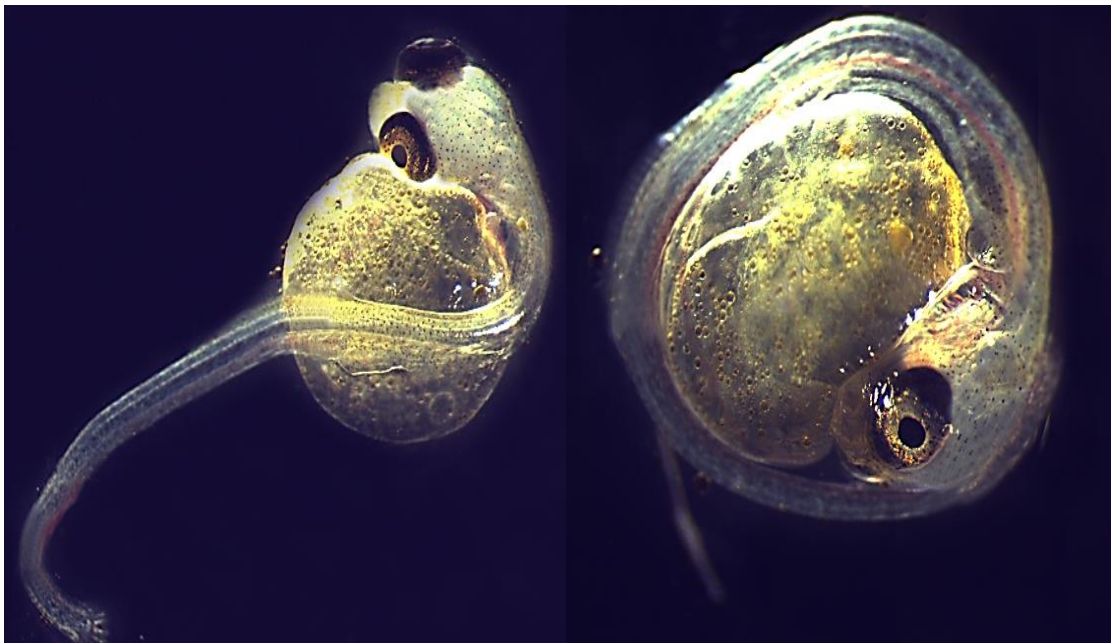
On the contrary, immunoglobulin light chain was solely upregulated in the early stage of male pregnancy (ANOVA-Ig-Lc: $\chi^2 = 10.63$, $p=0.005^*$, NP<Early; Late<Early; Table2, Figure 8). Immunoglobulins are part of the adaptive immune system and soluble immunoglobulins isotypes like IgM identify pathogens (bacteria and viruses) stimulate their removal by opsonisation, agglutination and complement activation (Murphy, 2011). However, the immunoglobulin light chain transcript level was significantly reduced during late pregnancy stage. This indicates *(i.)* a simultaneous transmission of Ig-Lc transcripts to the offspring during nursing over the close connection to the paternal capillaries in the placenta like structure (see general introduction Figures 2- 3), or *(ii)* downregulation due to high risk of detecting their own developing embryos as “non-self”.

Nevertheless, the presence of lectins, PGRPs, Ig-Lc transcripts and resulting immune defense molecules in the paternal brood pouch during male pregnancy could be vital as a large number of fry in an enclosed environment require efficient mechanisms for preventing bacterial colonization. Since the amount of our target immune genes was up-regulated, their mRNA transcripts and/or translated protein molecules could potentially be transmitted over the placenta-like structure to the embryos. Therefore, further studies are required to specifically detect immune gene transcripts or proteins of paternal origin in the embryos (see § Synthesis and § Future perspectives for further discussion).

Acknowledgments

Many thanks to Gisela Schmiedeskamp and Martin Kalbe for their great support with westernblot performance. This work was carried out in collaboration with Martin Kalbe at the Max Planck Institute for Evolutionary Biology (Plön) and Helmholtz Centre for Ocean Research GEOMAR (Kiel).

Synthesis



Synthesis

Title picture of Synthesis: Close-up picture of two broad-nosed-pipefish (*Syngnathus typhle*) yolk-sac larvae, approximately 1 weeks post hatch (Copyright: Anne Beemelmanns).

1. Biparental immune priming over generations

In my thesis I have investigated immune gene expression patterns and immune cell activity of the broad-nosed pipefish (*Syngnathus typhle*) to explore the ability of both parents to transfer immune protection to their progeny. While most species with conventional sex-roles boost their offspring's immune response exclusively via maternal immune priming, I showed that pipefish rely on both, maternal and paternal immune priming. This extraordinary feature of biparental immune priming has to date only been confirmed to occur in the red flour beetle (*Tribolium castaneum*) an invertebrate species (Roth *et al.*, 2010; Zanchi *et al.*, 2011). In my study I could demonstrate that biparental immune priming also arose in vertebrate species (chapter 1) with sex-role reversal and strong paternal investment. So far, it was considered that TGIP in vertebrates is only of major importance during early development, to bridge the time of maturation of the acquired immune system, when selection pressure due to high mortality is greatest (Hasselquist & Nilsson, 2009). Maternally derived antibodies in fish eggs have been shown to decrease after yolk sac consumption in the first weeks post hatch (Swain *et al.*, 2006; Swain & Nayak, 2009). In contrast, the results of my thesis indicate the persistence of immune priming past the maturation of the adaptive immune system (chapter 2). On top of that, I found strong evidence that parental immune priming effects even continued beyond the generation boarder, affecting the immune dynamics of grand-offspring generation (chapter 3). Grand-offspring revealed an altered immune gene expression, if their grandparents had already experienced the bacterial challenge compared to the control group without grandparental bacteria exposure (chapter 3). These findings suggest that experience with pathogens gives not only the direct offspring but subsequently also the grand-offspring an advantage in reacting towards potentially virulent parasites.

Another key result of my thesis were prominent and partially antagonistic bacteria specific immune priming effects towards *Vibrio* spp. and *Tenacibaculum maritimum* bacteria in *S. typhle* (chapter 1, 2). My results clearly indicate that long-term parental immune priming occurred in favor for specific pathogens (chapter 2). One-week-old juveniles revealed a robust but differential immune priming effect against both bacteria species. In contrast, older juveniles (four-month-old) displayed a strongly preserved immune priming effect solely against *Vibrio* bacteria (chapter 2). With the ability to specifically prime on the long-term the next generation only against prevailing and familiar *Vibrio* bacteria occurring in the natural habitat (chapter 2), most likely accomplishes an optimal protection. This bacteria specific immune priming effect was dominating and also covering the parental gender specific effect (chapter 1). The specific bacteria type influenced the strength and degree of interactive

Synthesis

biparental immune priming (chapter 1). Surprisingly, against the initial hypothesis of beneficial additive biparental immune priming when the parental sex received two different bacteria types, I recorded dampened biparental immune priming effects. This is a striking result as our previous underlying study (Roth *et al.* 2012) clearly demonstrated additive biparental immune priming in *S. typhle* in case when both parents received the identical *Vibrio* bacteria treatment. Here, I can only speculate that the dampened biparental immune priming effect might be due to an antagonistic priming pattern according to specific bacteria types. As the pathogen specific immune priming reaction was stronger than the gender specific effect this was probably leading to a compensated instead of additive biparental immune priming effect (chapter 1). As the biparental treatment with two bacteria types did not lead to an additive protection and further caused a severe delay in offspring maturation, it indicates that the underlying trade-off effects are interacting quite complex and it might be a costly tactic.

Strong expression of immunity as well as the maintenance of inducible defenses is costly in terms of energy resources and can lead to resource allocation trade-offs with other important life history traits (Lochmiller & Deerenberg, 2000; Ardia *et al.*, 2012). Channelling the energy resources into additional production and transfer of immune defense molecules to the next generation should therefore be associated with energetic costs. However, in the young F1-generation I firstly recorded only benefits which came with parental immune priming. One-week-old and four-month-old F1-juveniles of parents, which received a parental immune challenge, revealed not only higher immunity, but also a higher body size, mass and hepatosomatic index. This is pointing to an overall higher body condition of immune primed offspring in comparison to the control group (chapter 1 and 2). Yet, costs were firstly found later during sexual maturation of the F1-adults (chapter 3). In accordance, I recorded delayed maturation time of adult F1-males, reduced fecundity and reproduction of adult F1-generation, reduced body size of F2-juveniles in case of parental respectively grandparental bacteria exposure. Thus, it appears that immunological costs were compensated by reduced energy investment into reproduction and size of the second generation. Hence, channelling the energy resources towards the non-genetic transfer of immunity and balancing these benefits with reduced reproduction suggests that biparental immune priming is costly to maintain. These costs might constrain the overall beneficial net output of biparental immune priming. A time shift in sexual maturation and reproduction has severe ecological consequences for *S. typhle* as their mating season during summer is short and early offspring profits from higher water quality and less food competition. Nevertheless, selection for immune priming indicates that there must be an adaptive net influence and total benefits

should outweigh the associated costs (Schmid-Hempel, 2011; Kaufmann *et al.*, 2014). Thus, it seems that producing less offspring later in season with a better shape and immune competence might be the optimal strategy. In fact, Baltic pipefish start to migrate in spring from open water into the seagrass meadows along the coast for reproduction. The habitat and pathogen fauna in which they mate and release their offspring will likely be foreign. Since the abundance of pathogens such as *Vibrio* is increasing with rising water temperature during the summer season (Oberbeckmann *et al.*, 2012), it might be a good strategy to take longer time for acclimatisation to the new parasitic environment before mating. Consequently, individuals will be able to transfer efficient information about prevalent threats, which will become increasingly abundant and virulent within the summer mating season (Schade *et al.*, 2015). In addition, female pipefish are able to sense the immune status of their mates and chose males according to their immunological experience (Landis *et al.*, 2015). As such, pipefish females might favour mates that gained more immunological experiences about their actual habitat, which was leading to sexual selection on delayed maturation and reproduction.

The results of my thesis provide evidence that biparental immune priming gives individuals an advantage whose ancestors experienced pathogens and transfer this knowledge to the following generations. With this strategy parents not only deliver specific protection for their direct offspring and grand-offspring, but it also allows organisms to plastically adapt to the prevailing pathogen environment they will most likely experience. Although it might be beneficial on the individual level, it also involves ecological and evolutionary consequences on population level and may significantly alter the dynamics of host-parasite systems (Mostowy *et al.*, 2012; Tate & Rudolf, 2012). Biparental immune priming across generations, especially when it imposes costs in terms of reduced reproduction, does not necessarily need to diminish the impact of a disease on the population level (Tate & Rudolf, 2012). Theoretical models predict that TGIP across generations, while beneficial on a short-term, can increase parasite prevalence, destabilize coevolutionary dynamics on the long-term and change the spread of epidemics in a population (Tate & Rudolf, 2012; Tidbury *et al.*, 2012). Likewise, it was modelled by Mostowy *et al.* (2012) that non-genetic inheritance can have dramatic effects on the pattern of antagonistic coevolution and might strongly affect cycling behaviour of Red Queen dynamics. They predicted that non-genetic transmission such as TGIP can dampen the phenotype frequency oscillations and simulated that already minimal levels might completely eliminate cycles. The antagonistic oscillation of Red Queen dynamics depend on time-lagged negative frequency dependent selection (Dybdahl & Lively, 1998). Rare parasite genotype alleles have a selective advantage and can spread quickly in

a population, while it takes longer time for the hosts to counter-adapt (Dybdahl & Lively, 1998). Any factor such as non-genetic inheritance that acts to reduce the time lag will in turn reduce the amplitudes of the oscillating cycles (Mostowy *et al.*, 2012). TGIP within one generation increases the phenotypic plasticity of the next generation and will give an advantage for the host to instantly counter-adapt since the exposure to a novel parasite will result in an improved immune reaction. Bacteria specific immune priming mediated by the mothers and fathers should even increase the speed of the spread of immune memory towards the common parasite genotype allele. This phenomenon should slow down the amplitude of the antagonistic oscillation of Red Queen dynamics, while giving the pipefish an advantage in fast clearance of novel pathogens.

On the contrary, costs only payed by the host while performing immune priming may reduce these dampening effect again. Biparental immune priming in combination with two bacteria types might not be additive as the associated costs in terms of reduced reproduction were too high (chapter 2). Further insights into the balance between benefits and costs of biparental bacteria specific immune priming are required to understand the evolutionary origin and maintainance of immune priming as well as its effectiveness under natural conditions. Therefore, the empirical use of population-level measures like survival upon infection with living pathogens in natural populations is needed to understand how exactly these model prediction would be altered. More theoretical and empirical work is necessary to grasp for the complex interaction between costs and benefits of non-genetic biparental and bacteria specific transmission of immune protection.

2. Paternal versus maternal immune priming effects

While fathers and mothers may have similar interests in terms of offspring survival to the prevalent pathogenic risk, they appear to have evolved different strategies to achieve the immune protection of their offspring. As pipefish females invest into the eggs, and males potentially transfer immunity via the placenta-like structure during male pregnancy, I could demonstrate that pipefish mothers and fathers invested to different extend into the immune status of their offspring (chapter 1). As pipefish fathers have an intimate and close connection to their embryos during male pregnancy of approximately 4 – 6 weeks, paternal immune priming effects were dominating over maternal immune priming effects and were more persistent. Hence, one-week-old as well as four-month-old F1-juveniles of fathers that received an immune challenge revealed a similar altered immune gene expression pattern and improved pathogen specific immune cell activity in comparison to offspring with sole

maternal treatment (chapter 1). Nevertheless, maternal effects on immune gene expression of juveniles were detectable but had only an impact on the immune response of one-week-old juveniles (chapter 1). Yet, in accordance with the literature maternal immune priming effects diminished faster over offspring maturation time (Lindholm *et al.*, 2006; Zanchi *et al.*, 2011; Moreau *et al.*, 2012) and came with low specificity than paternal immune priming (chapter 1). Hence, on the long-term grand-offspring with sole grand-paternal immune challenge showed a differential immune gene expression in comparison to grand-offspring whose grandmothers received the bacteria treatment (chapter 3). In general, offspring of paternal respectively grand-paternal treatment groups displayed a similar immune gene expression as offspring of the biparental treatment group (chapter 1 and 3). This indicates that parental immune priming effects in *S. typhle* were predominately driven by the paternal line (chapter 1 and 3). As offspring are born in their father's environment and most probable experience a similar pathogen assembly, the paternal transfer of a solid long-term protection about current pathogens was hence, most likely favoured. This makes the transfer of immunity via the paternal line probable to be adaptive. Hence, both fathers and grandfathers will increase their fitness by altering their phenotype to optimally acclimate offspring to the local parasitic environment (Crean & Bonduriansky, 2014).

Remarkably, I also investigated complementing sex-specific contribution to different pathways of the immune system in juveniles of different age classes (chapter 1, 2, 3). My results showed that F1-juveniles and F2-juveniles both possessed a similar gender-based parental respectively grandparental immune priming pattern (chapter 2 and 3). I found that in both generations immune genes of the innate immune system were favourably affected by the fathers respectively grandfathers whereas genes of the adaptive immune system and complement component system were strongly influenced by the mothers respectively grandmothers. As it is known that in teleost species females prime the adaptive immune system of their offspring by the deposition of immunoglobulins, complement components, and corresponding mRNA transcripts into the eggs (Magnadóttir *et al.*, 2005; Magnadóttir, 2006; Swain *et al.*, 2006; Swain & Nayak, 2009; Zhang *et al.*, 2013), it appears that adaptive immunity in a sex-role reversed fish species is still predominantly driven by the maternal immunological experience.

In contrast, it seems that father's transferred immediate protection via innate immunity during male pregnancy against prevalent pathogens in their environment (chapter 2 and 3). This complementing sex-specific investment in different immune responses should be a very efficient tactic to maintain optimal protection by both parents. How the paternal transmission of preferable innate immunity takes place is still unresolved (see § Future perspectives). However, lectins might play an essential role as C-type lectins were strongly affected upon

the paternal and grand-paternal treatment and were upregulated in the paternal brood pouch tissue (chapters 1, 3, 4, and next paragraph). By using this sex-specific effector strategy maternal and paternal immune priming effects are complementing each other, potentially leading to a beneficial strategy to cope with the local parasitic environment (chapters 1 - 3).

3. How is immune priming mediated over generations?

a.) Epigenetic regulation mechanism as mediator?

In my thesis I assessed the expression of regulation genes, which mediate DNA methylation and histone modifications to investigate the underlying mechanism of trans-generational immune priming. These particular epigenetic processes regulate packing and de-packing of the chromatin structure around histones and with it the accessibility of the transcription machinery to bind the DNA and actively transcribe target genes, for instance immune genes (Foster & Medzhitov, 2009). In accordance, chromatin modifying processes have been shown to be essential regulators of the activity of many inflammatory genes (Foster & Medzhitov, 2009).

By analyzing epigenetic regulation genes that code for chromatin modifying proteins, and thus indirectly regulate immune gene expression, I was aiming to find hints for epigenetic inheritance mechanism of immune priming (chapters 1-3). The results suggest that epigenetic regulation genes were differentially expressed in offspring with parental (chapter 1 and 2) and grandparental (chapter 3) bacteria exposure in contrast to the control groups whose ancestors did not experience an immune challenge. As such, epigenetic modification processes might be involved in transmitting non-genetic information about parental and grandparental bacteria exposure. Interestingly, DNA methylation and histone modification genes were increasingly affected by both parents with the age of their offspring. Starting the observation from one-week old juveniles my targeted epigenetic regulation genes revealed in four-month-old juveniles firstly a dominant expression pattern according to the parental bacteria treatment (chapter 1 and 2). This pattern became even more pronounced in offspring of the second generation (chapter 3). Since DNA methylation and histone modifications are highly dynamic during early development and maturation (Smith & Meissner, 2013; Lee *et al.*, 2015; Wan *et al.*, 2015), epigenetic pattern or stable changes might take time to become fully established. My results indicate that with increasing age of the pipefish epigenetic regulation processes might be important in transferring immune related information across generations mediating long-term protection. Furthermore,

epigenetic regulation genes might be connected in mediating the transfer of acquired immunity about specific bacteria types (chapter 2). In the following I will describe possible epigenetic inheritance mechanism that could permit the transfer of non-genetic information via the maternal and paternal line over two generations in the pipefish *S. typhle*.

Heritable histone modifications?

Histone modifying enzymes place covalent marks on histone tails (acetylation, methylation, ubiquitination, and phosphorylation). Histone acetylation is a positive mark that associates with open, transcriptionally active chromatin, while histone deacetylation marks are leading to condensed and silent chromatin (Berger, 2002). The reaction to repeated pathogen exposure in macrophages involves positive histone marks and chromatin remodeling at specific promoters (Foster & Medzhitov, 2009). As histone modifications are supposed to be heritable across generations (Campos *et al.*, 2014; Gaydos *et al.*, 2014; Jones, 2015), histones might also act as “carriers of epigenetic information” (Ragunathan *et al.*, 2015) for pathogen experiences. In this sense, it was suggested that histone modifications may be associated with immune memory following a viral infection in CD8+T-cells (Youngblood *et al.*, 2010). However, the mechanism responsible for heritable and persistent histone changes remains so far unexplained.

In my thesis I could demonstrate that not only one-week-old F1-juveniles (chapter 1) revealed significant paternal but also that one-week old F2-juveniles (chapter 3) bare sole grand-paternal effects on histone acetylation and deacetylation genes. In particular, F2-juveniles whose grandfathers received an immune challenge with heat-killed bacteria revealed a differential gene expression of Histone methyltransferase (ASH2), Lysine-specific demethylase 6A (*TPR*), Histone-acetyltransferase (*KAT2A-BROMO-domain*), Histone-acetyltransferase HAT1 (*MYST1*), and Histone deacetylase 3 (*HDAC 3*). In addition, Histone-acetyltransferase (*KAT2A-BROMO-domain*), was significantly upregulated in the brood pouch tissue of pregnant males (chapter 4). This consistent pattern of paternal and grand-paternal influences on histone modification genes suggests that the regulation of immune priming over the paternal line might be mediated with heritable marks stored on histones (Ragunathan *et al.*, 2015).

Heritable DNA methylation pattern?

DNA methylation in promotor regions (CpG islands) regulates negatively gene expression and is necessary for all cell differentiation processes (Gama-Sosa *et al.*, 1983; Monk *et al.*, 1987; Razin & Shemer, 1995). DNA methylation patterns are tissue specifically expressed and frequently rearrange during early developmental processes (Gama-Sosa *et al.*, 1983; Monk *et al.*, 1987; Lee *et al.*, 2015). Environmental stress can induce changes in DNA methylation marks, which further are inheritable across generations (Jablonka & Lamb, 2015; Szyf, 2015). To date, it was only shown in plants that pathogen exposure induced stable differential DNA methylation marks of defense-related genes within and across generations (Dorantes-Acosta *et al.*, 2012; Luna *et al.*, 2012), which potentially facilitated the transfer of acquired immunity. Whether the same mechanism could also apply for maternal and paternal trans-generational immune priming observed in vertebrates is likely but remains elusive.

In my thesis I found that DNA methylation genes of offspring with paternal, maternal and biparental bacteria exposure were firstly differentially expressed in four-month-old juveniles (chapter 1) whereby this pattern persisted for offspring of the second generation (chapter 3). Hence, this indicates that DNA methylation might be connected with the regulation of immune gene expression not only of the direct children but also of the grandchildren. For instance, DNA-methyltransferase 3b (*DnMt3b*) and N(6)-adenine-specific DNA-methyltransferase2 (*N6admet*) displayed an altered gene expression upon the grandparental bacteria treatment.

Overall, the results of my thesis thus propose that environmental stressors such as pathogens might leave an epigenetic mark on the genome that can affect immune gene expression of direct offspring and grand-offspring. How and where exactly these DNA methylation marks are established and maintained stays an open question. Further research should focus on parental DNA methylation patterns in promoter regions of immune genes upon pathogen exposure to unravel the mechanisms behind TGIP.

b.) Direct transmission of paternal immune factors

The brood pouch of mature but non-pregnant pipefish is an open structure, accessible to the surrounding water and therefore non-sterile. After eggs are transferred by females, they are fertilized and the brood pouch tissue closes its ventral opening. During pregnancy stages developing embryos get incorporated into the pouch tissue in an enclosed nutrient rich environment that is optimal for bacterial or fungal growth. Therefore, immune defense mechanisms must have been evolved to prevent microbial colonization within the brood pouch that consequently would harm the embryos. A few studies investigated the ongoing molecular mechanism during male pregnancy of individuals from different *Syngnathid* taxa using transcriptome sequencing (Melamed *et al.*, 2005; Small *et al.*, 2013; Whittington *et al.*, 2015). Results of these studies indicated that besides the expression of genes encoding for proteins involved in metabolism, transport, structure and gene regulation, also innate and adaptive immune genes were differentially expressed in the brood pouch tissue, potentially to create an immunological active environment for their embryos. However, as it is already known for mammals, the maternal immune system and especially adaptive immune response must be tightly regulated to prevent the recognition of the embryo as an allograft and hence avoid embryonic rejection (Moffett & Loke, 2006; Walker *et al.*, 2010). Accordingly, on the one side selection pressures should have favoured mechanism to create an optimal balance between production and secretion of immune factors into the brood pouch to avoid bacterial colonization. While on the other side paternal immune system should be suppressed to prevent embryonic rejections. Possible mechanism to keep that balance would be (i) a down-regulation of adaptive immunity due to the lack of MHC II-mediated adaptive immune response as it was suggested by Haase *et al.* (2013), (ii) an up-regulation of alternative non-specific immunity, and (iii) a transfer of immune components directly to the embryos itself during nourishment.

In my thesis I could identify predominantly innate immune genes which displayed a strong paternal or grand-paternal influences and thus might be paternally transferred during male pregnancy. For instance innate immune genes such as allograft inflammation factor (AIF), Interleukin 8 (*IL8*), Lectin protein type I (*lectptl*), Chemokine 7 (*Ck7*), Ik-cytokine (*Ik-cyto*), and Tyroproteinkinase (*Tyroprot*) showed strong grand-paternal effects. Presumably, they play a crucial role in the paternal transmission process (chapter 3). In the following I am going to describe adaptive and innate immune components which showed a significant upregulation in the brood pouch during male pregnancy in *S. typhle* (chapter 4) and paternal (chapter1) or grand-paternal influences (chapter 3). Further I will discuss possible other transferred immune components which were so far not addressed such as the transfer of antimicrobial peptides and specific microbiota (see § Future perspectives).

Paternal transfer of Immunoglobulin?

Immunoglobulin M (IgM) (antibodies) of the acquired immune system (Murphy, 2011) has been shown to be maternally transmitted into fish eggs and paternally through mucus feeding of fry (Mor & Avtalion, 1990; Sin *et al.*, 1994; Swain *et al.*, 2006). Interestingly, none of the recent studies describing the transcriptome of the brood pouch tissue of different seahorse and pipefish species (Melamed *et al.*, 2005; Small *et al.*, 2013; Whittington *et al.*, 2015) reported about the presence of immunoglobulin transcripts in the brood pouch of neither pregnant nor non-pregnant males. In contrast to these findings, we could detect immunoglobulin light chain (Ig-Lc) in the brood pouch tissue, which was however significantly reduced during late pregnancy (chapter 4). Further, we found paternal end grandparental influences on Ig-Lc expression in one-week-old and four-month-old juveniles (chapter 1, 2, 3). Nevertheless, I was struggling to detect soluble immunoglobulin M proteins (light chain) in the plasma of adult pipefish which were repeatedly exposed to heat-killed pathogens (chapter 4). A clear negative evidence for IgM was not easy to perform, as I found mRNA sequences coding for immunoglobulin light chain (chapter 4), but could not detect the functional protein itself. In fact, it remains mysterious whether *S. typhle* owns functional IgM due to the high risk of recognizing their incorporated embryos as allografts or whether fathers are able to transfer IgM to the embryos. Based on the results of my thesis I can only speculate that the antibody-mediated adaptive immunity might be negatively regulated in the brood pouch during pregnancy and that the pipefish could have evolved different potential monomeric subunits of IgM to reduce the high risk of embryo rejection.

Paternal transfer of lectins?

C-type lectins (CTLs) are implicated in innate immunity and responsible for the recognition of carbohydrates of pathogen cell-surfaces leading to the destruction of the pathogen (Dodd & Drickamer, 2001; Fujita, 2002). Since teleost rely profoundly on their innate response, lectins have been found in mucus of the skin, gills and intestine, as well as in the blood and shown to suppress bacterial growth (Ewart *et al.*, 2001). C-type lectins were detected to high quantities in the parental mucus of *Cichlids* with biparental care and are considered to provide antimicrobial defense to parents and offspring at the same time (Chong *et al.*, 2006). Hereafter, C-type lectins might be useful as antimicrobial defense in the brood pouch tissue of *Syngnathids* to ensure protection of their embryos. Indeed, Melamed *et al.* (2005) also found large portion of genes encoding C-type lectins (CTLs), while characterizing the pouch tissue from tiger tail seahorse (*Hippocampus comes*) using transcriptome sequencing. They could identify a gene ("*hcCTL III*") transcribing for signal peptide containing a single C-lectin domain which reveals antimicrobial activity by causing erythrocyte agglutination. Also, Small

et al. (2013) and Whittington *et al.* (2015) discovered more recently the expression of C-type lectin paralogs in the brood pouch during pregnancy of different *Syngnathidae* species.

Likewise, I could verify an upregulated C-type lectin expression in the brood pouch of pregnant males during two key pregnancy stages (chapter 4) and a strong grand-paternal immune priming influence on C-type lectin protein II (chapter 1). Hence, it seems likely that lectins may protect the pipefish embryos from microbial infection and potentially might be paternally mediated to the embryos itself during nursing. The presence of innate immune defense components in the paternal brood pouch, in which the embryos live in an enclosed environment, could therefore be essential to suppress bacterial colonization. As the lectin-mediated pathway relies on the recognition of sugar-chains on the surface of bacteria cell walls (glycogen) it is a rather conserved pathway in comparison to the classical antibody pathway allowing specific recognition (Fujita, 2002). Hence, lectin mediated immune defense could be an adequate mechanism to provide protection without the risk to detect the embedded embryos as non-self.

Future perspectives

The applied targeted approach of measuring 44 genes only allowed me to see a “snapshot” of the ongoing complex gene expression processes in the F1 and F2-juveniles. Therefore, a full transcriptome sequencing approach using RNA-seq sequencing would produce a high “resolution picture” for a better understanding of up- and downregulated immune gene expression and the complex interplay with epigenetic regulation genes. It also would facilitate the identification of important candidate genes which might be involved in the mechanism of immune priming.

Likewise, further insight into complex interactions between costs and benefits under natural conditions are required for an improved understanding of the ultimate fitness advantage of biparental and bacteria-specific immune priming in the pipefish *S. typhle*. Future work should focus on the evaluation of resistance effects and bacterial specificity in a survival experiment by exposing individuals to live bacteria under approximate natural conditions. In this sense, theoretical modelling including all necessary observed parameters would additionally allow decent predictions about evolutionary and ecological consequences of biparental immune priming on population structure and host-parasite dynamics.

Finally, future research should address the physiological mechanisms facilitating the here observed bacteria specific and multigenerational biparental immune priming effects. On the one hand epigenetic mechanisms such as the transmission of differential DNA methylation pattern in promotor regions of specific immune genes could be evaluated by using bisulfide sequencing (Putnam *et al.*, 2015) in combination with methylated DNA immunoprecipitation technique (MeDIP) (Weber *et al.*, 2005; Li *et al.*, 2015). Likewise, the transmission of specific histone acetylation pattern following pathogen exposure could lead to essential new insights into epigenetic maternal and paternal inheritance. This could be realized by flow cytometric technique based on a specific monoclonal antibodies or sensitive probes that recognizes acetylated histone tails (Ronzoni *et al.*, 2005; Sanchez *et al.*, 2015). On the other hand it is necessary to conduct in depth analysis of the potential physiological and cellular mechanism of biparental immune priming and identify maternally and paternally transferred immune components. In the following, I am going to describe two potential future projects concerning the understanding of transmitted paternal immunity.

1. Future project I: Paternal transmission of antimicrobial peptides

Antimicrobial peptides are small hydrophobic molecules that insert into biological membranes and lyse a broad spectrum of pathogens such as bacteria, viruses, fungi and protozoa. In fish, several AMPs like piscidin, defensins, cathelicidins and hepcidin are released in mucus of specific organs (gills, liver, intestine) (Masso-Silva & Diamond, 2014). Some of these antimicrobial peptides have been shown to be present in fertilized eggs and early larval stages (Nam *et al.*, 2010; Sun *et al.*, 2012; Zhang *et al.*, 2013). Therefore, AMPs may represent a key antimicrobial innate immune defense during early developmental stages (Nam *et al.*, 2010; Sun *et al.*, 2012; Zhang *et al.*, 2013). For example hepcidin expression was detected in early fertilized eggs of blunt snout bream (Liang *et al.*, 2013) and β -defensin-like genes (fBDI) were identified in early developmental stages of the olive flounder, *Paralichthys olivaceus* (Nam *et al.*, 2010). Interestingly, studies have investigated that in fish species with strong paternal care (nesting behavior, mouth breeding) and sexually dimorphic traits (anal glands etc.), males coat their eggs with their mucus which contains antimicrobial substances (Giacomello *et al.*, 2006; Pizzolon *et al.*, 2010). These findings indicate that antimicrobial production is a crucial component of male parental care and is facilitating the protection of their offspring against pathogens. Similarly, Sun *et al.* (2012) identified a novel peptide ("HKPLP") from the brooding pouch of *Hippocampus kuda Bleeker*. Likewise, Whittington *et al.* (2015) found expression of hepcidin (HAMP) in the pouch of *Hippocampus abdominalis* both possessing a strong antimicrobial activity against bacteria.

Based on our transcriptomic data (illumina RNA-seq sequencing) obtained from the head kidney, gills and brood pouch tissue of *S. typhle* we could identify sequences of antimicrobial peptides like defensin and hepcidin (Haase & Roth unpublished data). From these transcriptome sequences, we know that also the brood pouch tissue of *S. typhle* contains mRNA transcripts with up to 91% homology to defensin-type proteins and 93% homology to hepcidin of other fish species. Further, preliminary results indicate that the brood pouch tissue of different developmental pregnancy stages contained defensin and hepcidin transcripts. However, an upregulation of these antimicrobial peptides during pregnancy in *S. typhle* could reflect an innate immune mechanism to safeguard and protect transferred eggs or developing embryos against infectious pathogens. Hence, paternal transmission or coating of embryos with antimicrobial substances through enriched mucus might additionally be a likely mechanism (Giacomello *et al.*, 2006; Pizzolon *et al.*, 2010). Recently available transcriptome data of brood pouch tissue and genome data of *S. typhle* could be used in future research to identify and characterize such immune factors more in detail.

2. Future project II: Paternal transmission of specific microbiota

The microbiome is “the ecological community of commensal, symbiotic and pathogenic microorganism that share our body space” (Lederberg & Mccray, 2001). These microbial communities can be specialised to niches within their distinct organs and vary between surface mucosal and intestinal communities (Consortium, 2012). In the sex-role reversed pipefish it is likely that shortly after females transfer their “sterile” eggs into the male’s brood pouch the eggs are colonized by bacteria occurring in the environment of the paternal brood pouch as well as those which were originally present on the chorion (either maternal bacteria or bacteria of the aquatic milieu, or both). The passage of surface bacteria into the gut and early digestive tract colonization has been shown to start when larvae begin to ingest liquid medium (Lauzon *et al.*, 2010; Llewellyn *et al.*, 2014). Several studies reveal evidence that the bacterial taxa of the alimentary tract of first-feeding fries strongly resemble those associated with the food source of their immediate environment (Korsnes *et al.*, 2006; Reid *et al.*, 2009; Lauzon *et al.*, 2010). As the freshly hatched eggs in the brood pouch of the male pipefish are supplied with nutrition and oxygen over a placenta-like structure during male pregnancy and live in the environment shaped by their fathers, the microbiota of the alimentary tract must be strongly paternally influenced. In addition, evidence has been found for a vertical transmission of a pioneering bacteria strain, which was only present in the chorion of the egg, and displayed a dominant genus in the gut microbiota of mature fish (Romero & Navarrete, 2006). Commensal bacteria in the gut intestine interact and significantly influence the immune system (Llewellyn *et al.*, 2014). For instance, they can stimulate toll-like receptors in the intestine and the lymphoid tissue associated with the gut mucosa, triggering thus the production of antibodies against pathogens that may enter the gut (Macpherson & Harris, 2004). Hence, the parental transmission of specific microbiota compositions might be an advantageous mechanism to facilitate a resilient protection. In fish with sex-role reversal a possible vertical maternal transmission of specific microbiome components to eggs during oviposition and an additional paternal transmission during male pregnancy could form the gut microbiota and simultaneously influence the immune tolerance of the offspring. Future studies should address the question whether the microbiota composition of the maternally transferred eggs changes with increasing male pregnancy and if offspring adapt the microbiota composition of their fathers. As gut microbiota is known to promote immunity the paternal transmission of microbiota or vertical transfer of microorganisms could be one possible mechanism to facilitate paternal immune priming in *S. typhle* and influence offspring immunity on the long-term.

Author contributions

Chapter I

Designed the experiment: **AB** and OR. Performed the experiment and lab work: **AB** and OR. Collected and analysed the data: **AB**. Contributed reagents/materials/analysis tools: OR and TBHR. Wrote and contributed to the manuscript **AB** and OR.

Chapter II

Designed the experiment: **AB** and OR. Performed the experiment and lab work: **AB** and OR. Collected and analysed the data: **AB**. Contributed reagents/materials/analysis tools: OR and TBHR. Wrote and contributed to the manuscript: **AB** and OR.

Chapter III

Designed the experiment: **AB** and OR. Performed the experiment and lab work: **AB** and OR. Collected and analysed the data: **AB**. Contributed reagents/materials/analysis tools: OR and TBHR. Wrote and contributed to the manuscript: **AB** and OR and TBHR.

Chapter IV

Designed the experiment: **AB**, MK and OR. Performed the experiment and lab work AB. Contributed reagents/materials/analysis tools: OR and MK. Wrote and contributed to the manuscript: **AB** and OR.

Eidesstattliche Erklärung

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

Biparental immune priming in the broad-nosed pipefish (*Syngnathus typhle*)

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

Die Arbeit wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt.
Dies ist mein einziges und bisher erstes Promotionsverfahren.

Teile dieser Arbeit wurden als Manuskripte in wissenschaftlichen Fachzeitschriften jeweilig mit Olivia Roth als Koautorin eingereicht: Kapitel I in *Zoology*; Kapitel II in *Journal of Evolutionary Biology* und Kapitel III in *Molecular Ecology*.

Anne Beemelmans

Kiel, Januar 2016

List of publications

Publication with coauthor ship:

Roth, O., Klein, V., Beemelmans, A., Scharsack, J.P. & Reusch, T.B. 2012. Male pregnancy and biparental immune priming. *The American Naturalist* **180**: 802-814.

Publication accepted to be published:

Beemelmans, A. and Roth, O. 2016. Biparental immune priming in the pipefish *Syngnathus typhle*. *Zoology*. Accepted to be published.

Publications that are submitted:

Beemelmans, A. and Roth, O. 2016. Grandparental immune priming in the pipefish *Syngnathus typhle*. In review.

Beemelmans, A. and Roth, O. 2016. Bacteria specific immune priming in the pipefish *Syngnathus typhle*. In submission.

Danksagung

Ohne die Unterstützung und Anregung vieler Personen wäre es mir nicht gelungen diese Doktorarbeit fertig zu stellen und daher möchte ich mich an dieser Stelle bei allen bedanken, die mich während dieser Zeit unterstützt haben.

Zuallererst danke ich ganz herzlich meiner Betreuerin **Olivia Roth**, da sie es mir ermöglicht hat an diesem wunderbaren, abwechslungsreichen und spannenden Projekt zu arbeiten. Danke, dass Du mich stets mit vollem Elan motiviert hast, und besonders in turbulenten Zeiten mir immer sofort mit Rat und Tat zur Seite gestanden und mich unterstützt hast. Vor allem danke ich Dir, dass Du es mir ermöglicht hast meine eigenen Ideen und Kreativität in diese Arbeit einzubringen und meine Ziele zu verwirklichen.

Genauso herzlich möchte ich mich bei Prof. Dr. **Thorsten Reusch** bedanken, dass ich in seiner Arbeitsgruppe promovieren durfte und mir die Gelegenheit gab meine Doktorarbeit im Rahmen der IMPRS anzufertigen.

Zudem Danke ich **Martin Kalbe** und **Arne Nolte**, die mich immer bei wissenschaftlichen Fragestellungen unterstützt haben. Dankeschön **Martin**, dass Du mir zum einen so viel zu Fischparasiten und die Anatomie der Fische erklärt hast und zum anderen dein Wissen über Westernblot-Analysen und Proteinaufreinigung mit mir geteilt hast. Zudem danke ich Dir für unentwegte neuen Ideen Antikörper im Plasma der Seenadeln zu finden.

Besonders danke ich den „Pipefish-girls und boy“ der letzten vier Jahre: **Susi, Maude, Sophia, Caterina, Verena K., Verena T. & Joaõ** sowie unserm „semi-Pipefish/Buntbarsch-girl“ **Isa** und ebenfalls „semi-Pipefish/Buntbarsch boy“ **Martin**. Vielen, vielen Dank für die ungezählten Stunden beim Fische fangen, Fische füttern, Aquarien putzen, Fische sezieren, Pipette schwingen im Labor und wieder Aquarien putzen.... Ohne euch wäre diese Arbeit nie zustande gekommen! Ganz besonders möchte ich mich bei unserer lieben Fee **Maude** bedanken! Danke, dass Du mir immer super motiviert, fröhlich und tatkräftig bei jeglichen Arbeiten (sehr zeiteffizient!) geholfen hast. *Merci!*

Ich danke meinen engsten GEOMAR-PhD-Mitstreiterinnen **Isa** und **Andrea** für ihre moralische Unterstützung, tatkräftige Hilfe bei meinen Experimenten, lustige Gespräche während unseren Pausen und Feierabende, gelegentliche gemeinsame Sportstunden und Cuba Libre. Danke für die super schöne Zeit!! Ich danke auch unserer Ex-Geomar-PhD-Mitstreiterin **Franzi**, die es leider nach London verschlagen hat. Vielen Dank für Deinen statistischen Beistand und die aller erste Einführung in die Welt des FLUIDIGMs und in R! Zudem danke ich den „älteren“ PhD-Mitstreitern **Lothar, Miguel, Kai, David, Sören, Corinna, Janina, Burkhard & Matthias** und den „jüngeren“ PhD-Mitstreitern **Melanie, Sophie & Felix** für lustige Gespräche, schöne Mittagspausen und diverse DOKMA- und Weihnachtsfeiern.

Ein ganz großes Dankeschön geht an **Joanna** für unsere vielen schönen wissenschaftlichen und persönlichen Gespräche, das Korrekturlesen, Hilfe bei meiner Probennahme und die gemeinsame Begeisterung fürs Nähen. Ebenfalls danke ich **Caro** und **Till**, für viele gute

Danksagung

Ratschläge und, dass ich mich immer willkommen fühle, wenn ich mit Fragen Euer Büro stürme.

Ganz besonders möchte ich mich bei meinem Büropartner **Hans-Harald Hinrichsen** aka **H³** für eine schöne Zusammenarbeit, viele aufmunternde Gespräche, unterhaltende Anekdoten und die regelmäßige Bereitstellung von Selters bedanken.

Vielen Dank **Rudi** und **Svend** für Eure tatkräftige Hilfe beim vielfachen Auf- und Abbau und der Instandhaltung der Aquarienanlage und bei diversen Computerbelangen. Ein warmer Dank auch an **Diana** und **Katrin** für die Hilfe und Unterstützung während meiner Laborarbeit.

Ein ebenfalls ganz großes Dankeschön geht an unseren einzigartigen Techniker, **Fabian!** Danke, dass Du mir immer hilfsbereit geholfen hast meine Seenadeln zu versorgen und mich ideenreich unterstützt hast die Aquarienanlage für mein großes Seenadel-Experiment aufzubauen und ultimativ kostengünstige und super praktische Features zu entwickeln.

I thank the IMPRS candidates of the year 2012: **Wesseling, Meriem, Luisa, Vienna, Sunna & Ozan!** It was a pleasure to face the challenges of my PhD with you and thank you for nice social events, facing the rather cool Nordic life style of Kiel and happy BBQs in Plön.

Ganz außerordentlich bedanke ich mich bei meinen Mitbewohnerinnen und PhD-Genossinnen **Wesseling** und **Meriem**. Es war eine Freude mit euch zusammenzuwohnen und die Höhen und Tiefen des „Doktorandendaseins“ gemeinsam zu bewältigen. Vielen Dank für die vielen schönen Stunden, die wir gemeinsam verbracht haben und unvergesslichen Kieler Nächte, Ausflüge an den Strand, Fahrradtouren und Nähprojekte, um dem Stress der Doktorarbeit zu entfliegen. Ohne euch hätte ich die vier grauen Winter hier im Norden nicht durchgehalten und die vier nordischen Sommer wären nicht so schön gewesen! Ich danke Euch für diese wunderbare schöne Zeit und Unterstützung in jeglichen Belangen! Meriem, thank you so much for your last minute corrections! Wesseling danke, dass Du immer ein offenes Ohr für mich hattest und ich werde nie vergessen, dass Du mir zu einer für Dich viel zu frühen Tageszeit am Samstagmorgen (während der Windjammerparade der Kieler Woche) geholfen hast Seenadelbabys aus 60 Aquarien zu fischen, bevor sie als Snack von ihren Eltern verspeist werden konnten.

Ich danke meiner **Familie**, dass sie immer für mich da war und mich moralisch und seelisch unterstützt hat. Vor allem danke ich Euch für den stetigen Glauben an mich. Meinen **Eltern** und **Großeltern** danke ich, dass sie mir weitaus mehr als nur ihre Gene weitervererbt haben. Ich danke meiner Schwester und **Phillipp** für aufmunternde, motivierende Gespräche und die wissenschaftliche Unterstützung. Danke **Till** für das Lösen diverser Computerprobleme und danke **Tine** für das Korrekturlesen während der Weihnachtszeit. Ich danke **Johannes** für viele aufheiternde Bilder während meiner Schreibphase.

Mein lieber **Thomas** ich danke Dir, dass du immer an meiner Seite gestanden hast und mich fortwährend sehr verständnisvoll und geduldig während meiner langen Doktorarbeit im fernen Kiel begleitet hast und immer daran geglaubt hast, dass sich es schaffen werde. Ohne Dich wäre ich nie so weit gekommen. Danke!

Bibliography

- Agrawal, A.A., Laforsch, C. & Tollrian, R. 1999. Transgenerational induction of defenses in animals and plants. *Nature* **401**: 60-63.
- Ahnesjö, I. & Craig, J. 2011. The biology of Syngnathidae: pipefishes, seadragons and seahorses. *Journal of Fish Biology* **78**: 1597-1602.
- Alcaide, E., Gil-Sanz, C., Sanjuan, E., Esteve, D., Amaro, C. & Silveira, L. 2001. *Vibrio harveyi* causes disease in seahorse, *Hippocampus* sp. *Journal of Fish Diseases* **24**: 311-313.
- Alder, M.N., Rogozin, I.B., Iyer, L.M., Glazko, G.V., Cooper, M.D. & Pancer, Z. 2005. Diversity and function of adaptive immune receptors in a jawless vertebrate. *Science* **310**: 1970-1973.
- Ardia, D.R., Parmentier, H.K. & Vogel, L.A. 2011. The role of constraints and limitation in driving individual variation in immune response. *Functional Ecology* **25**: 61-73.
- Ardia, D.R., Gantz, J.E., Brent, C. & Strebel, S. 2012. Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. *Functional Ecology* **26**: 732-739.
- Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., Fernandes, G.R., Tap, J., Bruls, T. & Batto, J.-M. 2011. Enterotypes of the human gut microbiome. *Nature* **473**: 174-180.
- Avtalion, R. & Mor, A. 1992. Monomeric IgM is transferred from mother to egg in tilapias. *Israeli Journal of Aquaculture* **44**: 93-98.
- Azzarello, M.Y. 1991. Some questions concerning the Syngnathidae brood pouch. *Bulletin of Marine Science* **49**: 741-747.
- Balcázar, J.L., Gallo-Bueno, A., Planas, M. & Pintado, J. 2010. Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from captive-bred seahorses with disease symptoms. *Antonie van Leeuwenhoek* **97**: 207-210.
- Baron, B. 2012. Breaking the silence: The interplay between transcription factors and DNA methylation, in methylation—From DNA, RNA and histones to diseases and treatment. *InTech, Rijeka, Croatia*.
- Benjamini, Y. & Hochberg, Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society* **57**: 289-300.
- Benn, C.S., Netea, M.G., Selin, L.K. & Aaby, P. 2013. A small jab—a big effect: nonspecific immunomodulation by vaccines. *Trends in immunology* **34**: 431-439.
- Berger, S.L. 2002. Histone modifications in transcriptional regulation. *Current Opinion in Genetics & Development* **12**: 142-148.
- Berger, S.L., Kouzarides, T., Shiekhattar, R. & Shilatifard, A. 2009. An operational definition of epigenetics. *Genes & Development* **23**: 781-783.
- Berglund, A., Rosenqvist, G. & Svensson, I. 1986. Mate choice, fecundity and sexual dimorphism in two pipefish species (Syngnathidae). *Behavioral Ecology and Sociobiology* **19**: 301-307.
- Berglund, A., Rosenqvist, G. & Svensson, I. 1989. Reproductive success of females limited by males in two pipefish species. *American Naturalist*: 506-516.

Bibliography

- Berglund, A. 1993. Risky sex: male pipefishes mate at random in the presence of a predator. *Animal Behaviour* **46**: 169-175.
- Bernardet, J., Campbell, A. & Buswell, J. 1990. Flexibacter maritimus is the agent of 'black patch necrosis' in Dover sole in Scotland. *Diseases of Aquatic Organisms* **8**: 233-237.
- Bestor, T.H. 2000. The DNA methyltransferases of mammals. *Human Molecular Genetics* **9**: 2395-2402.
- Bird, A. 2002. DNA methylation patterns and epigenetic memory. *Genes & Development* **16**: 6-21.
- Birrer, S.C., Reusch, T.B. & Roth, O. 2012. Salinity change impairs pipefish immune defense. *Fish & Shellfish Immunology* **33**: 1238-1248.
- Bitume, E., Bonte, D., Ronce, O., Olivieri, I. & Nieberding, C. 2014. Dispersal distance is influenced by parental and grand-parental density. *Proceedings of the Royal Society of London B: Biological Sciences* **281**: 20141061.
- Blackburn, S. 2014. *Maternal, fetal, & Neonatal Physiology*. Elsevier Health Sciences.
- Bly, J.E., Grimm, A. & Morris, I. 1986. Transfer of passive immunity from mother to young in a teleost fish: haemagglutinating activity in the serum and eggs of plaice, *Pleuronectes platessa* L. *Comparative Biochemistry and Physiology Part A: Physiology* **84**: 309-313.
- Bobe, J. & Labbé, C. 2010. Egg and sperm quality in fish. *General and Comparative Endocrinology* **165**: 535-548.
- Bolte, S., Roth, O., Philipp, E.E., Saphörster, J., Rosenstiel, P. & Reusch, T.B. 2013. Specific immune priming in the invasive ctenophore *Mnemiopsis leidyi*. *Biology Letters* **9**: 20130864.
- Bonduriansky, R. & Day, T. 2009. Nongenetic inheritance and its evolutionary implications. *Annual Review of Ecology, Evolution, and Systematics* **40**: 103.
- Bonduriansky, R. 2012. Rethinking heredity, again. *Trends in Ecology & Evolution* **27**: 330-336.
- Bookout, A.L. & Mangelsdorf, D.J. 2003. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nuclear Records in Signaling* **1**: 102.
- Boots, M. & Bowers, R.G. 2004. The evolution of resistance through costly acquired immunity. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **271**: 715-723.
- Boulinier, T. & Staszewski, V. 2008. Maternal transfer of antibodies: raising immuno-ecology issues. *Trends in Ecology & Evolution* **23**: 282-288.
- Brambell, F. 1969. The transmission of immune globulins from the mother to the foetal and newborn young. *Proceedings of the Nutrition Society* **28**: 35-41.
- Brambell, F.W.R. 1958. The passive immunity of the young mammal. *Biological Reviews* **33**: 488-531.
- Brambell, F.W.R. 1970a. The transmission of passive immunity from mother to young. In: *The transmission of passive immunity from mother to young*.
- Brambell, F.W.R., ed. 1970b. *Transmission of immunity in birds*. Elsevier, New York.
- Brazma, A. & Vilo, J. 2000. Gene expression data analysis. *FEBS Letters* **480**: 17-24.
- Buckley, J., Maunder, R.J., Foey, A., Pearce, J., Val, A.L. & Sloman, K.A. 2010. Biparental mucus feeding: a unique example of parental care in an Amazonian cichlid. *The Journal of Experimental Biology* **213**: 3787-3795.

Bibliography

- Buonocore, F. & Gerdol, M. 2015. Alternative adaptive immunity strategies: coelacanth, cod and shark immunity. *Molecular Immunology*.
- Burgess, S.C. & Marshall, D.J. 2014. Adaptive parental effects: the importance of estimating environmental predictability and offspring fitness appropriately. *Oikos*.
- Burnet, F.M. 1976. A modification of Jerne's theory of antibody production using the concept of clonal selection. *CA: A Cancer Journal for Clinicians* **26**: 119-121.
- Campos, E.I., Stafford, J.M. & Reinberg, D. 2014. Epigenetic inheritance: histone bookmarks across generations. *Trends in Cell Biology* **24**: 664-674.
- Carcupino, M., Baldacci, A., Mazzini, M. & Franzoi, P. 1997. Morphological organization of the male brood pouch epithelium of *Syngnathus abaster* Risso (Teleostea, Syngnathidae) before, during, and after egg incubation. *Tissue and Cell* **29**: 21-30.
- Carcupino, M., Baldacci, A., Mazzini, M. & Franzoi, P. 2002. Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a morphological and ultrastructural comparative study on three anatomically different pouches. *Journal of Fish Biology* **61**: 1465-1480.
- Carone, B.R., Fauquier, L., Habib, N., Shea, J.M., Hart, C.E., Li, R., Bock, C., Li, C., Gu, H. & Zamore, P.D. 2010. Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell* **143**: 1084-1096.
- Chellappa, S., Huntingford, F., Strang, R. & Thomson, R. 1995. Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback. *Journal of Fish Biology* **47**: 775-787.
- Cheng, C.-A., John, J.A.C., Wu, M.-S., Lee, C.-Y., Lin, C.-H., Lin, C.-H. & Chang, C.-Y. 2006. Characterization of serum immunoglobulin M of grouper and cDNA cloning of its heavy chain. *Veterinary Immunology and Immunopathology* **109**: 255-265.
- Chessel, D., Dufour, A.B. & Thioulouse, J. 2004. The ade4 package-I-One-table methods. *R news* **4**: 5-10.
- Chong, K., Joshi, S., Jin, L.T. & Shu-Chien, A.C. 2006. Proteomics profiling of epidermal mucus secretion of a cichlid (*Symphysodon aequifasciata*) demonstrating parental care behavior. *Proteomics* **6**: 2251-2258.
- Chow, P., Jiang, H., Poon, H. & Lee, K. 2003. Embryos sired by males without accessory sex glands induce failure of uterine support: a study of VEGF, MMP and TGF expression in the golden hamster. *Anatomy and Embryology* **206**: 203-213.
- Ci, W. & Liu, J. 2015. Programming and inheritance of parental DNA methylomes in vertebrates. *Physiology* **30**: 63-68.
- Clarke, K. 1993. Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology* **1988**: 117-143.
- Clarke, K.R. & Gorley, R.N., eds. 2006. *Primer v6: User Manual/ Tutorial*. PRIMER-E, Plymouth.
- Clutton-Brock, T.H. 1991. *The evolution of parental care*. University Press, Princeton.
- Consortium, H.M.P. 2012. Structure, function and diversity of the healthy human microbiome. *Nature* **486**: 207-214.
- Contreras-Garduño, J., Rodríguez, M., Rodríguez, M., Alvarado-Delgado, A. & Lanz-Mendoza, H. 2014. Cost of immune priming within generations: trade-off between infection and reproduction. *Microbes and Infection* **16**: 261-267.

Bibliography

- Cooper, M.D. & Alder, M.N. 2006. The evolution of adaptive immune systems. *Cell* **124**: 815-822.
- Craig, J.M. & Bickmore, W.A. 1994. The distribution of CpG islands in mammalian chromosomes. *Nature Genetics* **7**: 376-382.
- Crean, A.J., Dwyer, J.M. & Marshall, D.J. 2012. Fertilization is not a new beginning: the relationship between sperm longevity and offspring performance. *Plos One* **7**: e49167.
- Crean, A.J. & Bonduriansky, R. 2014. What is a paternal effect? *Trends in Ecology & Evolution* **29**: 554-559.
- Curley, J.P., Mashoodh, R. & Champagne, F.A. 2011. Epigenetics and the origins of paternal effects. *Hormones and Behavior* **59**: 306-314.
- Danchin, É., Charmantier, A., Champagne, F.A., Mesoudi, A., Pujol, B. & Blanchet, S. 2011. Beyond DNA: integrating inclusive inheritance into an extended theory of evolution. *Nature Reviews Genetics* **12**: 475-486.
- Díaz, M., Moyano, F., Garcia-Carreno, F., Alarcón, F. & Sarasquete, M. 1997. Substrate-SDS-PAGE determination of protease activity through larval development in sea bream. *Aquaculture International* **5**: 461-471.
- Diepeveen, E.T., Roth, O. & Salzburger, W. 2013. Immune-related functions of the Hivep gene family in East African cichlid fishes. *G3: Genes Genomes Genetics* **3**: 2205-2217.
- Dodd, R.B. & Drickamer, K. 2001. Lectin-like proteins in model organisms: implications for evolution of carbohydrate-binding activity. *Glycobiology* **11**: 71R-79R.
- Dolédec, S. & Chessel, D. 1987. Rythmes saisonniers et composantes stationnelles en milieu aquatique. I: Description d'un plan d'observation complet par projection de variables. *Acta oecologica. Oecologia Generalis* **8**: 403-426.
- Donelson, J., Munday, P., McCormick, M. & Pitcher, C. 2012. Rapid transgenerational acclimation of a tropical reef fish to climate change. *Nature Climate Change* **2**: 30-32.
- Dorantes-Acosta, A.E., Sánchez-Hernández, C.V. & Arteaga-Vazquez, M.A. 2012. Biotic stress in plants: life lessons from your parents and grandparents. *Frontiers in Genetics* **3**: 256.
- Du Pasquier, L. 2006. Germline and somatic diversification of immune recognition elements in Metazoa. *Immunology Letters* **104**: 2-17.
- Dybdahl, M.F. & Lively, C.M. 1998. Host-parasite coevolution: evidence for rare advantage and time-lagged selection in a natural population. *Evolution*: 1057-1066.
- Dziarski, R. & Gupta, D. 2006. The peptidoglycan recognition proteins (PGRPs). *Genome Biology* **7**: 232.
- Eggert, H., Kurtz, J. & Diddens-de Buhr, M.F. 2014. Different effects of paternal trans-generational immune priming on survival and immunity in step and genetic offspring. *Proceedings of the Royal Society of London B: Biological Sciences* **281**: 20142089.
- Egidius, E. 1987. Vibriosis: pathogenicity and pathology. A review. *Aquaculture* **67**: 15-28.
- Ehrlich, P. 1892. Über Immunität durch Vererbung und Säugung. *Zeitschrift für Hygiene und Infektionskrankheiten* **12**: 183-203.
- Ewart, K., Johnson, S. & Ross, N. 2001. Lectins of the innate immune system and their relevance to fish health. *ICES Journal of Marine Science: Journal du Conseil* **58**: 380-385.
- Flajnik, M. & Du Pasquier, L. 2008. Evolution of the immune system. *Fundamental Immunology* **6**: 57-122.

Bibliography

- Flajnik, M.F. & Du Pasquier, L. 2004. Evolution of innate and adaptive immunity: can we draw a line? *Trends in Immunology* **25**: 640-644.
- Flajnik, M.F. 2005. The last flag unfurled? A new immunoglobulin isotype in fish expressed in early development. *Nature Immunology* **6**: 229-230.
- Flajnik, M.F. & Kasahara, M. 2010. Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nature Reviews Genetics* **11**: 47-59.
- Foey, A. & Picchiatti, S. 2014. Immune Defenses of Teleost Fish. *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*: 14-52.
- Foster, S.L. & Medzhitov, R. 2009. Gene-specific control of the TLR-induced inflammatory response. *Clinical Immunology* **130**: 7-15.
- Frank, S.A. 2002. *Immunology and Evolution of Infectious Disease*. Princeton University Press.
- Freitak, D., Heckel, D.G. & Vogel, H. 2009. Dietary-dependent trans-generational immune priming in an insect herbivore. *Proceedings of the Royal Society of London B: Biological Sciences* **276**: 2617-2624.
- Freitak, D., Schmidtberg, H., Dickel, F., Lochnit, G., Vogel, H. & Vilcinskas, A. 2014. The maternal transfer of bacteria can mediate trans-generational immune priming in insects. *Virulence* **5**: 547-554.
- Frette, L., Jørgensen, N.O., Irming, H. & Kroer, N. 2004. *Tenacibaculum skagerrakense* sp. nov., a marine bacterium isolated from the pelagic zone in Skagerrak, Denmark. *International Journal of Systematic and Evolutionary Microbiology* **54**: 519-524.
- Frischknecht, M. 1993. The breeding colouration of male three-spined sticklebacks (*Gasterosteus aculeatus*) as an indicator of energy investment in vigour. *Evolutionary Ecology* **7**: 439-450.
- Frost, P.C., Ebert, D., Larson, J.H., Marcus, M.A., Wagner, N.D. & Zalewski, A. 2010. Transgenerational effects of poor elemental food quality on *Daphnia magna*. *Oecologia* **162**: 865-872.
- Fuchs, R. & Ellinger, I. 2004. Endocytic and transcytotic processes in villous syncytiotrophoblast: role in nutrient transport to the human fetus. *Traffic* **5**: 725-738.
- Fujita, T. 2002. Evolution of the lectin-complement pathway and its role in innate immunity. *Nature Reviews Immunology* **2**: 346-353.
- Gama-Sosa, M.A., Midgett, R.M., Slagel, V.A., Githens, S., Kuo, K.C., Gehrke, C.W. & Ehrlich, M. 1983. Tissue-specific differences in DNA methylation in various mammals. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **740**: 212-219.
- Gasparini, J., McCoy, K.D., Tveraa, T. & Boulinier, T. 2002. Related concentrations of specific immunoglobulins against the Lyme disease agent *Borrelia burgdorferi sensu lato* in eggs, young and adults of the kittiwake (*Rissa tridactyla*). *Ecology Letters* **5**: 519-524.
- Gaydos, L.J., Wang, W. & Strome, S. 2014. H3K27me and PRC2 transmit a memory of repression across generations and during development. *Science* **345**: 1515-1518.
- Gellert, M. 2002. V (D) J Recombination: RAG Proteins, Repair Factors, and Regulation. *Annual Review of Biochemistry* **71**: 101-132.
- Giacomello, E., Marchini, D. & Rasotto, M.B. 2006. A male sexually dimorphic trait provides antimicrobials to eggs in blenny fish. *Biology Letters* **2**: 330-333.

Bibliography

- Goellner, K. & Conrath, U. 2008. Priming: it's all the world to induced resistance. *European Journal of Plant Pathology* **121**: 233-242.
- Gómez-Díaz, E., Jordà, M., Peinado, M.A. & Rivero, A. 2012. Epigenetics of host–pathogen interactions: the road ahead and the road behind.
- Gomulkiewicz, R., Thompson, J.N., Holt, R.D., Nuismer, S.L. & Hochberg, M.E. 2000. Hot spots, cold spots, and the geographic mosaic theory of coevolution. *The American Naturalist* **156**: 156-174.
- Grewal, S.I. & Moazed, D. 2003. Heterochromatin and epigenetic control of gene expression. *Science* **301**: 798-802.
- Grindstaff, J.L., Brodie, E.D. & Ketterson, E.D. 2003. Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **270**: 2309-2319.
- Grindstaff, J.L., Hasselquist, D., Nilsson, J.-Å., Sandell, M., Smith, H.G. & Stjernman, M. 2006. Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity. *Proceedings of the Royal Society B: Biological Sciences* **273**: 2551-2557.
- Guillette Jr, L.J. 1987. The evolution of viviparity in fishes, amphibians and reptiles: An endocrine approach. In: *Hormones and Reproduction in Fishes, Amphibians, and Reptiles*, pp. 523-562. Springer.
- Gutierrez-Galve, L., Stein, A., Hanington, L., Heron, J. & Ramchandani, P. 2015. Paternal depression in the postnatal period and child development: mediators and moderators. *Pediatrics* **135**: e339-e347.
- Haase, D., Roth, O., Kalbe, M., Schmiedeskamp, G., Scharsack, J.P., Rosenstiel, P. & Reusch, T.B. 2013. Absence of major histocompatibility complex class II mediated immunity in pipefish, *Syngnathus typhle*: evidence from deep transcriptome sequencing. *Biology Letters* **9**: 20130044.
- Haase, D., Rieger, J.K., Witten, A., Stoll, M., Bornberg-Bauer, E., Kalbe, M. & Reusch, T.B. 2014. Specific gene expression responses to parasite genotypes reveal redundancy of innate immunity in vertebrates. *Plos One* DOI: 10.1371/journal.pone.0108001
- Hafer, N., Ebil, S., Uller, T. & Pike, N. 2011. Transgenerational effects of food availability on age at maturity and reproductive output in an asexual collembolan species. *Biology Letters: rsbl20110139*.
- Hamilton, R., Siva-Jothy, M. & Boots, M. 2008. Two arms are better than one: parasite variation leads to combined inducible and constitutive innate immune responses. *Proceedings of the Royal Society of London B: Biological Sciences* **275**: 937-945.
- Hanif, A., Bakopoulos, V. & Dimitriadis, G. 2004. Maternal transfer of humoral specific and non-specific immune parameters to sea bream (*Sparus aurata*) larvae. *Fish & Shellfish Immunology* **17**: 411-435.
- Hasselquist, D. & Nilsson, J.-Å. 2009. Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**: 51-60.
- Heijmans, B.T., Tobi, E.W., Stein, A.D., Putter, H., Blauw, G.J., Susser, E.S., Slagboom, P.E. & Lumey, L. 2008. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proceedings of the National Academy of Sciences* **105**: 17046-17049.

Bibliography

- Hellemans, J., Mortier, G., DePaepe, A., Speleman, F. & Vandesompele, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology* **8**: R19.
- Herman, J.J., Sultan, S.E., Horgan-Kobelski, T. & Riggs, C. 2012. Adaptive transgenerational plasticity in an annual plant: grandparental and parental drought stress enhance performance of seedlings in dry soil. *Integrative and Comparative Biology* **52**: 77-88.
- Holbert, M.A. & Marmorstein, R. 2005. Structure and activity of enzymes that remove histone modifications. *Current Opinion in Structural Biology* **15**: 673-680.
- Hordvik, I. 2015. Immunoglobulin Isotypes in Atlantic Salmon, *Salmo Salar*. *Biomolecules* **5**: 166-177.
- Huang, E. & Wells, C.A. 2014. The ground state of innate immune responsiveness is determined at the interface of genetic, epigenetic, and environmental influences. *The Journal of Immunology* **193**: 13-19.
- Huang, S. & Chen, D. 2015. N6-methyladenine: a potential epigenetic mark in eukaryotes. *Oncotarget* **6**: 15744.
- Hunt, J. & Simmons, L. 2000. Maternal and paternal effects on offspring phenotype in the dung beetle *Onthophagus taurus*. *Evolution* **54**: 936-941.
- Jablonka, E. & Raz, G. 2009. Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *The Quarterly Review of Biology* **84**: 131-176.
- Jablonka, E. & Lamb, M.J. 2014. *Evolution in Four Dimensions, revised edition: Genetic, Epigenetic, Behavioral, and Symbolic Variation in the History of Life*. MIT press.
- Jablonka, E. & Lamb, M.J. 2015. The inheritance of acquired epigenetic variations. *International Journal of Epidemiology* dyv020.
- Jaenisch, R. & Bird, A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics* **33**: 245-254.
- Janeway, C., Murphy, K., Travers, P. & Walport, M., eds. 2008. *Immunobiology*. LLC, New York.
- Jiang, L., Zhang, J., Wang, J.-J., Wang, L., Zhang, L., Li, G., Yang, X., Ma, X., Sun, X., Cai, J., Zhang, J., Huang, X., Yu, M., Wang, X., Liu, F., Wu, C.-I., He, C., Zhang, B. & Ci, W. 2013. Sperm, but not oocyte, DNA methylome is inherited by zebrafish early embryos. *Cell* **153**: 773-784.
- Jiménez de Oya, N., Alonso-Padilla, J., Blázquez, A.-B., Escribano-Romero, E., Escribano, J.M. & Saiz, J.-C. 2011. Maternal transfer of antibodies to the offspring after mice immunization with insect larvae-derived recombinant hepatitis E virus ORF-2 proteins. *Virus Research* **158**: 28-32.
- Johnson, K., Flynn, J. & Amend, D. 1982. Duration of immunity in salmonids vaccinated by direct immersion with *Yersinia ruckeri* and *Vibrio anguillarum* bacterins. *Journal of fish diseases* **5**: 207-213.
- Jokela, J. 2010. Transgenerational immune priming as cryptic parental care. *J Animal Ecology* **79**: 305-307.
- Jones, A.G., Rosenqvist, G., Berglund, A. & Avise, J.C. 1999. The genetic mating system of a sex-role-reversed pipefish (*Syngnathus typhle*): a molecular inquiry. *Behavioral Ecology and Sociobiology* **46**: 357-365.
- Jones, B. 2015. Epigenetics: Histones pass the message on. *Nature Reviews Genetics* **16**: 3-3.
- Kaltz, O. & Shykoff, J.A. 1998. Local adaptation in host–parasite systems. *Heredity* **81**: 361-370.

Bibliography

- Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E. & Sasaki, H. 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* **429**: 900-903.
- Kappeler, L. & Meaney, M.J. 2010. Epigenetics and parental effects. *Bioessays* **32**: 818-827.
- Kaufmann, J., Lenz, T.L., Milinski, M. & Eizaguirre, C. 2014. Experimental parasite infection reveals costs and benefits of paternal effects. *Ecology Letters* **17**: 1409-1417.
- Kirkpatrick, M. & Lande, R. 1989. Evolution of Maternal Characters. *Evolution* **43**: 485-503.
- Kokko, H. & Jennions, M.D. 2008. Parental investment, sexual selection and sex ratios. *Journal of evolutionary biology* **21**: 919-948.
- Kolygas, M., Gourzioti, E., Vatsos, I. & Athanassopoulou, F. 2012. Identification of *Tenacibaculum maritimum* strains from marine farmed fish in Greece. *Veterinary Record-English Edition* **170**: 623.
- Kooistra, S.M. & Helin, K. 2012. Molecular mechanisms and potential functions of histone demethylases. *Nature reviews Molecular cell biology* **13**: 297-311.
- Kornienko, E. 2001. Reproduction and development in some genera of pipefish and seahorses of the family Syngnathidae. *Russian Journal of Marine Biology* **27**: S15-S26.
- Korsnes, K., Nicolaisen, O., Skår, C.K., Nerland, A.H. & Bergh, Ø. 2006. Bacteria in the gut of juvenile cod *Gadus morhua* fed live feed enriched with four different commercial diets. *ICES Journal of Marine Science: Journal du Conseil* **63**: 296-301.
- Koskella, B. & Lively, C.M. 2009. Evidence for negative frequency-dependent selection during experimental coevolution of a freshwater snail and a sterilizing trematode. *Evolution* **63**: 2213-2221.
- Kowalczyk, K., Daiss, J. & Halpern, J. 1985. Quantification of maternal-fetal IgG transport in the chicken. *Immunology* **1985**: 755-762.
- Kumar, M., Kumar, K., Jain, S., Hassan, T. & Dada, R. 2013. Novel insights into the genetic and epigenetic paternal contribution to the human embryo. *Clinics* **68**: 05-14.
- Kurtz, J. 2004. Memory in the innate and adaptive immune systems. *Microbes and Infection* **6**: 1410-1417.
- Kurtz, J., Kalbe, M., Aeschlimann, P.B., Häberli, M.A., Wegner, K.M., Reusch, T.B.H. & Milinski, M. 2004. Major histocompatibility complex diversity influences parasite resistance and innate immunity in sticklebacks. *Proceedings of the Royal Society of London B: Biological Sciences* **271**: 197-204.
- Kurtz, J. 2005. Specific memory within innate immune systems. *Trends in Immunology* **26**: 186-192.
- Kurtz, J. & Armitage, S.A.O. 2006. Alternative adaptive immunity in invertebrates. *Trends in Immunology* **27**: 493-496.
- Kurtz, J., Kalbe, M., Langefors, Å., Mayer, I., Milinski, M. & Hasselquist, D. 2007. An Experimental Test of the Immunocompetence Handicap Hypothesis in a Teleost Fish: 11-Ketotestosterone Suppresses Innate Immunity in Three-Spined Sticklebacks. *The American Naturalist* **170**: 509-519.
- Kvarnemo, C., Mobley, K.B., Partridge, C., Jones, A. & Ahnesjö, I. 2011. Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish *Syngnathus typhle*. *Journal of Fish Biology* **78**: 1725-1737.

Bibliography

- Landis, S.H., Sundin, J., Rosenqvist, G., Poirier, M., Jørgensen, G.Ø. & Roth, O. 2015. Female pipefish can detect the immune status of their mates. *Behavioral Ecology and Sociobiology* **69**: 1917-1923.
- Lane, N., Dean, W., Erhardt, S., Hajkova, P., Surani, A., Walter, J. & Reik, W. 2003. Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. *Genesis* **35**: 88-93.
- Lauzon, H., Gudmundsdottir, S., Petursdottir, S., Reynisson, E., Steinarsson, A., Oddgeirsson, M., Bjornsdottir, R. & Gudmundsdottir, B. 2010. Microbiota of Atlantic cod (*Gadus morhua* L.) rearing systems at pre-and posthatch stages and the effect of different treatments. *Journal of Applied Microbiology* **109**: 1775-1789.
- Leavy, O. 2015. Innate immunity: New PAMP discovered. *Nature Reviews Immunology* **15**: 402-403.
- Lederberg, J. & McCray, A. 2001. The Scientist:\Ome Sweet\Omics--A Genealogical Treasury of Words. *The Scientist* **17**.
- Lee, H.J., Lowdon, R.F., Maricque, B., Zhang, B., Stevens, M., Li, D., Johnson, S.L. & Wang, T. 2015. Developmental enhancers revealed by extensive DNA methylome maps of zebrafish early embryos. *Nature communications* **6**.
- Lemke & Lange 1999. Is There a Maternally Induced Immunological Imprinting Phase à la Konrad Lorenz? *Scandinavian Journal of Immunology* **50**: 348-354.
- Lemke, H., Lange, H. & Berek, C. 1994. Maternal immunization modulates the primary immune response to 2-phenyl-oxazolone in BALB/c mice. *European Journal of Immunology* **24**: 3025-3030.
- Lemke, H., Tanasa, R.I., Trad, A. & Lange, H. 2009. Benefits and burden of the maternally-mediated immunological imprinting. *Autoimmunity Review* **8**: 394-399.
- Li, D., Zhang, B., Xing, X. & Wang, T. 2015. Combining MeDIP-seq and MRE-seq to investigate genome-wide CpG methylation. *Methods* **72**: 29-40.
- Li, X., Wang, S., Qi, J., Echtenkamp, S.F., Chatterjee, R., Wang, M., Boons, G.-J., Dziarski, R. & Gupta, D. 2007. Zebrafish peptidoglycan recognition proteins are bactericidal amidases essential for defense against bacterial infections. *Immunity* **27**: 518-529.
- Liang, T., Ji, W., Zhang, G.-R., Wei, K.-J., Feng, K., Wang, W.-M. & Zou, G.-W. 2013. Molecular cloning and expression analysis of liver-expressed antimicrobial peptide 1 (LEAP-1) and LEAP-2 genes in the blunt snout bream (*Megalobrama amblycephala*). *Fish & Shellfish Immunology* **35**: 553-563.
- Lindholm, A.K., Hunt, J. & Brooks, R. 2006. Where do all the maternal effects go? Variation in offspring body size through ontogeny in the live-bearing fish *Poecilia parae*. *Biology Letters* **2**: 586-589.
- Little, T. 2002. The evolutionary significance of parasitism: do parasite-driven genetic dynamics occur *ex silico*? *Journal of Evolutionary Biology* **15**: 1-9.
- Little, T.J., O'Connor, B., Colegrave, N., Watt, K. & Read, A.F. 2003. Maternal Transfer of Strain-Specific Immunity in an Invertebrate. *Current Biology* **13**: 489-492.
- Lively, C.M. & Dybdahl, M.F. 2000. Parasite adaptation to locally common host genotypes. *Nature* **405**: 679-681.
- Llewellyn, M.S., Boutin, S., Hoseinifar, S.H. & Derome, N. 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology* **5**: 54-78.

Bibliography

- Lochmiller, R.L. & Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**: 87-98.
- Lock, J.E. 2012. Transgenerational effects of parent and grandparent gender on offspring development in a biparental beetle species. *Biology Letters* **8**: 408-411.
- Løvoll, M., Kilvik, T., Boshra, H., Bøgwald, J., Sunyer, J.O. & Dalmo, R.A. 2006. Maternal transfer of complement components C3-1, C3-3, C3-4, C4, C5, C7, Bf, and Df to offspring in rainbow trout (*Oncorhynchus mykiss*). *Immunogenetics* **58**: 168-179.
- Lui, K. 2000. Confidence intervals of the simple difference between the proportions of a primary infection and a secondary infection, given the primary infection. *Biomet Journal* **42**: 59-69.
- Luna, E., Bruce, T.J., Roberts, M.R., Flors, V. & Ton, J. 2012. Next-generation systemic acquired resistance. *Plant Physiology* **158**: 844-853.
- Macpherson, A.J. & Harris, N.L. 2004. Interactions between commensal intestinal bacteria and the immune system. *Nature Reviews Immunology* **4**: 478-485.
- Magiafoglou, A. & Hoffmann, A. 2003. Cross-generation effects due to cold exposure in *Drosophila serrata*. *Functional Ecology* **17**: 664-672.
- Magnadóttir, B., Lange, S., Gudmundsdóttir, S., Bagwald, J. & Dalmo, R. 2005. Ontogeny of humoral immune parameters in fish. *Fish Shellfish Immunology* **19**: 429-439.
- Magnadóttir, B. 1998. Comparison of immunoglobulin (IgM) from four fish species. *Icelandic Agricultural Sciences* **12**: 47-59.
- Magnadóttir, B., Lange, S., Gudmundsdóttir, S., Bøgwald, J. & Dalmo, R. 2005. Ontogeny of humoral immune parameters in fish. *Fish & Shellfish Immunology* **19**: 429-439.
- Magnadóttir, B. 2006. Innate immunity of fish (overview). *Fish & Shellfish Immunology* **20**: 137-151.
- Marr, A.K., MacIsaac, J.L., Jiang, R., Airo, A.M., Kobor, M.S. & McMaster, W.R. 2014. *Leishmania donovani* infection causes distinct epigenetic DNA methylation changes in host macrophages. *PloS Pathogen*.
- Marshall, D. & Uller, T. 2007. When is a maternal effect adaptive? *Oikos* **116**: 1957-1963.
- Martin, L.B., Weil, Z.M. & Nelson, R.J. 2008. Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **363**: 321-339.
- Masso-Silva, J.A. & Diamond, G. 2014. Antimicrobial peptides from fish. *Pharmaceuticals* **7**: 265-310.
- Mathern, D.R. & Heeger, P.S. 2015. Molecules great and small: the complement system. *Clinical Journal of the American Society of Nephrology*: CJN. 06230614.
- Matsunaga, T. & Rahman, A. 1998. What brought the adaptive immune system to vertebrates?-The jaw hypothesis and the seahorse. *Immunological reviews* **166**: 177-186.
- May, R. 1974. Larval mortality in marine fishes and the critical period concept. In: *The Early Life History of Fish*, pp. 3-19. Springer.
- McArdle, B. & Anderson, M.J. 2001. Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* **82**: 290-297.
- Medzhitov, R. & Janeway, C.A. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* **91**: 295-298.

Bibliography

- Melamed, P., Xue, Y., Poon, J.F.D., Wu, Q., Xie, H., Yeo, J., Foo, T.W.J. & Chua, H.K. 2005. The male seahorse synthesizes and secretes a novel C-type lectin into the brood pouch during early pregnancy. *Febs Journal* **272**: 1221-1235.
- Mitchell, K.A., Easwaran, H. & Baylin, S.B. 2014. DNMT3B (a de novo DNA methyltransferase) epigenetically regulates gene expression, independent of its DNA methyltransferase activity. *Cancer Research* **74**: 4779-4779.
- Mitchell, S.E. & Read, A.F. 2005. Poor maternal environment enhances offspring disease resistance in an invertebrate. *Proceedings of the Royal Society B: Biological Sciences* **272**: 2601-2607.
- Moffett, A. & Loke, C. 2006. Immunology of placentation in eutherian mammals. *Nature Reviews Immunology* **6**: 584-594.
- Molinier, J., Ries, G., Zipfel, C. & Hohn, B. 2006. Transgeneration memory of stress in plants. *Nature* **442**: 1046-1049.
- Monk, M., Boubelik, M. & Lehnert, S. 1987. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* **99**: 371-382.
- Mor, A. & Avtalion, R. 1990. Transfer of antibody activity from immunized mother to embryo in tilapias. *Journal of Fish Biology* **37**: 249-255.
- Moreau, J., Martinaud, G., Troussard, J.P., Zanchi, C. & Moret, Y. 2012. Trans-generational immune priming is constrained by the maternal immune response in an insect. *Oikos* **121**: 1828-1832.
- Moret, Y. 2006. 'Trans-generational immune priming': specific enhancement of the antimicrobial immune response in the mealworm beetle, *Tenebrio molitor*. *Proceedings of the Royal Society B: Biological Sciences* **273**: 1399-1405.
- Mostowy, P. & Engelstädter, J. 2012. Host-parasite coevolution induces selection for condition-dependent sex. *Journal of Evolutionary Biology* **25**: 2033-2046.
- Mostowy, R., Engelstädter, J. & Salathé, M. 2012. Non-genetic inheritance and the patterns of antagonistic coevolution. *BMC Evolutionary Biology* **12**: 93.
- Mousseau, T.A. & Fox, C.W. 1998a. Maternal Effects as Adaptations for Transgenerational Phenotypic. *Maternal Effects as Adaptations*: 159.
- Mousseau, T.A. & Fox, C.W. 1998c. The adaptive significance of maternal effects. *Trends in Ecology & Evolution* **13**: 403-407.
- Mukherjee, K., Fischer, R. & Vilcinskas, A. 2012. Histone acetylation mediates epigenetic regulation of transcriptional reprogramming in insects during metamorphosis, wounding and infection. *Frontiers in Zoology* **9**: 25.
- Mukherjee, K., Twyman, R.M. & Vilcinskas, A. 2015. Insects as models to study the epigenetic basis of disease. *Progress in Biophysics and Molecular Biology* **118**: 69-78.
- Mulero, I., García-Ayala, A., Meseguer, J. & Mulero, V. 2007. Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: A minireview. *Aquaculture* **268**: 244-250.
- Munday, P.L., Warner, R.R., Monro, K., Pandolfi, J.M. & Marshall, D.J. 2013. Predicting evolutionary responses to climate change in the sea. *Ecology Letters* **16**: 1488-1500.
- Murphy, K. 2011. *Janeway's immunobiology*. Garland Science.
- Musthaq, S.K.S. & Kwang, J. 2014. Evolution of specific immunity in shrimp—A vaccination perspective against white spot syndrome virus. *Developmental & Comparative Immunology* **46**: 279-290.

Bibliography

- Nakahigashi, K., Kubo, N., Narita, S.-i., Shimaoka, T., Goto, S., Oshima, T., Mori, H., Maeda, M., Wada, C. & Inokuchi, H. 2002. HemK, a class of protein methyl transferase with similarity to DNA methyl transferases, methylates polypeptide chain release factors, and hemK knockout induces defects in translational termination. *Proceedings of the National Academy of Sciences* **99**: 1473-1478.
- Nam, B.-H., Moon, J.-Y., Kim, Y.-O., Kong, H.J., Kim, W.-J., Lee, S.-J. & Kim, K.-K. 2010. Multiple β -defensin isoforms identified in early developmental stages of the teleost *Paralichthys olivaceus*. *Fish & Shellfish Immunology* **28**: 267-274.
- Norouzitallab, P., Biswas, P., Baruah, K. & Bossier, P. 2014. Multigenerational immune priming in an invertebrate pathogenetic *Artemia* to a pathogenic *Vibrio campbelli*. *Fish & Shellfish Immunology* **42**: 426-429.
- Oberbeckmann, S., Fuchs, B.M., Meiners, M., Wichels, A., Wiltshire, K.H. & Gerdtz, G. 2012. Seasonal dynamics and modeling of a *Vibrio* community in coastal waters of the North Sea. *Microbial Ecology* **63**: 543-551.
- Okamoto, Y., Shinjo, K., Shimizu, Y., Sano, T., Yamao, K., Gao, W., Fujii, M., Osada, H., Sekido, Y. & Murakami, S. 2014. Hepatitis virus infection affects DNA methylation in mice with humanized livers. *Gastroenterology* **146**: 562-572.
- Okano, M., Bell, D.W., Haber, D.A. & Li, E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**: 247-257.
- Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J. & Suggests, M. 2007. The vegan package. *Community ecology package*: 631-637.
- Palti, Y. 2011. Toll-like receptors in bony fish: from genomics to function. *Developmental & Comparative Immunology* **35**: 1263-1272.
- Patterson, R., Youngner, J.S., Weigle, W.O. & Dixon, F.J. 1962. Antibody production and transfer to egg yolk in chickens. *The Journal of Immunology* **89**: 272-278.
- Pembrey, M.E., Bygren, L.O., Kaati, G., Edvinsson, S., Northstone, K., Sjöström, M. & Golding, J. 2005. Sex-specific, male-line transgenerational responses in humans. *European Journal of Human Genetics* **14**: 159-166.
- Pembrey, M.E. 2010. Male-line transgenerational responses in humans. *Human Fertility* **13**: 268-271.
- Perry, M. & Chalkley, R. 1982. Histone acetylation increases the solubility of chromatin and occurs sequentially over most of the chromatin. A novel model for the biological role of histone acetylation. *Journal of Biological Chemistry* **257**: 7336-7347.
- Peters, A.H. & Schübeler, D. 2005. Methylation of histones: playing memory with DNA. *Current Opinion in Cell Biology* **17**: 230-238.
- Pham, L.N., Dionne, M.S., Shirasu-Hiza, M. & Schneider, D.S. 2007. A Specific Primed Immune Response in *Drosophila* Is Dependent on Phagocytes. *PLoS Pathog* **3**: e26.
- Picchietti, S., Abelli, L., Buonocore, F., Randelli, E., Fausto, A.M., Scapigliati, G. & Mazzini, M. 2006. Immunoglobulin protein and gene transcripts in sea bream (*Sparus aurata* L.) oocytes. *Fish and Shellfish Immunology* **20**: 398-404.
- Pigliucci, M. & Müller, G.B., eds. 2010. *Evolution - the extended synthesis*. MIT Press, Cambridge.
- Pigliucci, M. & Finkelman, L. 2014. The Extended (Evolutionary) Synthesis Debate: Where Science Meets Philosophy. *BioScience*: biu062.
- Pizzolon, M., Giacomello, E., Marri, L., Marchini, D., Pascoli, F., Mazzoldi, C. & Rasotto, M.B. 2010. When fathers make the difference: efficacy of male sexually selected antimicrobial glands in enhancing fish hatching success. *Functional Ecology* **24**: 141-148.

Bibliography

- Potok, M.E., Nix, D.A., Parnell, T.J. & Cairns, B.R. 2013. Reprogramming the maternal zebrafish genome after fertilization to match the paternal methylation pattern. *Cell* **153**: 759-772.
- Poulin, R. & Thomas, F. 2008. Epigenetic effects of infection on the phenotype of host offspring: parasites reaching across host generations. *Oikos* **117**: 331-335.
- Press, C.M. & Evensen, Ø. 1999. The morphology of the immune system in teleost fishes. *Fish & Shellfish Immunology* **9**: 309-318.
- Putnam, E., Nguyen, L., Chung, H., Shi, P., Sun, X., Van Eden, M.E. & Jia, X.-Y. 2015. A targeted bisulfite sequencing method combining microfluidics-based PCR with Next-Gen sequencing. *Cancer Research* **75**: 2956-2956.
- Ragunathan, K., Jih, G. & Moazed, D. 2015. Epigenetic inheritance uncoupled from sequence-specific recruitment. *Science* **348**: 1258699.
- Ramos, R., Garnier, R., González-Solís, J. & Boulinier, T. 2014. Long Antibody Persistence and Transgenerational Transfer of Immunity in a Long-Lived Vertebrate. *The American Naturalist* **184**: 764-776.
- Rando, O.J. 2012. Daddy issues: paternal effects on phenotype. *Cell* **151**: 702-708.
- Rassoulzadegan, M., Grandjean, V., Gounon, P., Vincent, S., Gillot, I. & Cuzin, F. 2006. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature* **441**: 469-474.
- Rassoulzadegan, M. & Cuzin, F. 2015. Epigenetic heredity: RNA-mediated modes of phenotypic variation. *Annals of the New York Academy of Sciences* **1341**: 172-175.
- Rauta, P.R., Nayak, B. & Das, S. 2012. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunology Letters* **148**: 23-33.
- Rauta, P.R., Samanta, M., Dash, H.R., Nayak, B. & Das, S. 2014. Toll-like receptors (TLRs) in aquatic animals: signaling pathways, expressions and immune responses. *Immunology Letters* **158**: 14-24.
- Ravelli, G.-P., Stein, Z.A. & Susser, M.W. 1976. Obesity in young men after famine exposure in utero and early infancy. *New England Journal of Medicine* **295**: 349-353.
- Razin, A. & Shemer, R. 1995. DNA methylation in early development. *Human Molecular Genetics* **4**: 1751-1755.
- Reid, H.I., Treasurer, J.W., Adam, B. & Birkbeck, T.H. 2009. Analysis of bacterial populations in the gut of developing cod larvae and identification of *Vibrio logei*, *Vibrio anguillarum* and *Vibrio splendidus* as pathogens of cod larvae. *Aquaculture* **288**: 36-43.
- Reid, J.M., Arcese, P., Keller, L.F. & Hasselquist, D. 2006. Long-term maternal effect on offspring immune response in song sparrows *Melospiza melodia*. *Biology Letters* **2**: 573-576.
- Reik, W., Dean, W. & Walter, J. 2001. Epigenetic reprogramming in mammalian development. *Science* **293**: 1089-1093.
- Reuman, P., Paganini, C., Ayoub, E. & Small, P. 1983. Maternal-infant transfer of influenza-specific immunity in the mouse. *The Journal of Immunology* **130**: 932-936.
- Reusch, T.B. 2014. Climate change in the oceans: evolutionary versus phenotypically plastic responses of marine animals and plants. *Evolutionary Applications* **7**: 104-122.
- Ripley, J.L. & Foran, C.M. 2006. Differential parental nutrient allocation in two congeneric pipefish species (Syngnathidae: *Syngnathus* spp.). *Journal of Experimental Biology* **209**: 1112-1121.

Bibliography

- Ripley, J.L. 2009. Osmoregulatory role of the paternal brood pouch for two *Syngnathus* species. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **154**: 98-104.
- Ripley, J.L. & Foran, C.M. 2009. Direct evidence for embryonic uptake of paternally-derived nutrients in two pipefishes (Syngnathidae: *Syngnathus* spp.). *Journal of Comparative Physiology B* **179**: 325-333.
- Romero, J. & Navarrete, P. 2006. 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). *Microbial Ecology* **51**: 422-430.
- Ronzoni, S., Faretta, M., Ballarini, M., Pelicci, P. & Minucci, S. 2005. New method to detect histone acetylation levels by flow cytometry. *Cytometry Part A* **66**: 52-61.
- Rosengaus, R.B., Maxmen, A.B., Coates, L.E. & Traniello, J.F. 1998. Disease resistance: a benefit of sociality in the dampwood termite *Zootermopsis angusticollis* (Isoptera: Termopsidae). *Behavioral Ecology and Sociobiology* **44**: 125-134.
- Rossiter, M.C. 1996. Incidence and consequences of inherited environmental effects. *Annual Review of Ecology, Evolution, and Systematics* **27**: 451-476.
- Roth, O., Sadd, B.M., Schmid-Hempel, P. & Kurtz, J. 2009. Strain-specific priming of resistance in the red flour beetle, *Tribolium castaneum*. *Proceedings of the Royal Society of London B: Biological Sciences* **276**: 145-151.
- Roth, O., Joop, G., Eggert, H., Hilbert, J., Daniel, J., Schmid-Hempel, P. & Kurtz, J. 2010. Paternally derived immune priming for offspring in the red flour beetle, *Tribolium castaneum*. *Journal of Animal Ecology* **79**: 403-413.
- Roth, O., Scharsack, J., Keller, I. & Reusch, T.B. 2011. Bateman's principle and immunity in a sex-role reversed pipefish. *Journal of Evolutionary Biology* **24**: 1410-1420.
- Roth, O., Keller, I., Landis, S.H., Salzburger, W. & Reusch, T.B. 2012a. Hosts are ahead in a marine host-parasite coevolutionary arms race: innate immune system adaptation in pipefish *Syngnathus typhle* against *Vibrio* phylotypes. *Evolution* **66**: 2528-2539.
- Roth, O., Klein, V., Beemelmans, A., Scharsack, J.P. & Reusch, T.B. 2012. Male pregnancy and biparental immune priming. *The American Naturalist* **180**: 802-814.
- Sadd, B.M., Kleinlogel, Y., Schmid-Hempel, R. & Schmid-Hempel, P. 2005. Trans-generational immune priming in a social insect. *Biology Letters* **1**: 386-388.
- Sadd, B.M. & Schmid-Hempel, P. 2009. A distinct infection cost associated with trans-generational priming of antibacterial immunity in bumble-bees. *Biology Letters* **5**: 798-801.
- Salinas, I. 2015. The Mucosal Immune System of Teleost Fish. *Biology* **4**: 525-539.
- Salmela, H., Amdam, G.V. & Freitak, D. 2015. Transfer of Immunity from Mother to Offspring Is Mediated via Egg-Yolk Protein Vitellogenin. *PLoS Pathog* **11**: e1005015.
- Sanchez, O.F., Williamson, D., Cai, L. & Yuan, C. 2015. A sensitive protein-based sensor for quantifying histone acetylation levels. *Talanta* **140**: 212-218.
- Schade, F.M., Raupach, M.J. & Wegner, K.M. 2015. Seasonal variation in parasite infection patterns of marine fish species from the Northern Wadden Sea in relation to interannual temperature fluctuations. *Journal of Sea Research*.
- Scheiners, S.M. 2014. Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics* **24**: 35-68.

Bibliography

- Schluter, S., Bernstein, R., Bernstein, H. & Marchalonis, J. 1998. 'Big Bang' emergence of the combinatorial immune system. *Developmental and Comparative Immunology* **23**: 107-111.
- Schmid-Hempel, P. & Ebert, D. 2003. On the evolutionary ecology of specific immune defense. *Trends in Ecology & Evolution* **18**: 27-32.
- Schmid-Hempel, P. 2005a. Natural insect host-parasite systems show immune priming and specificity: puzzles to be solved. *BioEssays* **27**: 1026-1034.
- Schmid-Hempel, P. 2005b. Evolutionary ecology of insect immune defenses. *Annual Review of Entomology* **50**: 529-551.
- Schmid-Hempel, P. 2011. *Evolutionary parasitology: the integrated study of infections, immunology, ecology, and genetics*. Oxford University Press, New York.
- Seppola, M., Johnsen, H., Mennen, S., Myrnes, B. & Tveiten, H. 2009. Maternal transfer and transcriptional onset of immune genes during ontogenesis in Atlantic cod. *Developmental & Comparative Immunology* **33**: 1205-1211.
- Shama, L.N.S. & Wegner, K.M. 2014. Grandparental effects in marine sticklebacks: transgenerational plasticity across multiple generations. *Journal of Evolutionary Biology* **27**: 2297-2307.
- Sheldon, B.C. & Verhulst, S. 1996. Ecological immunology: costly parasite defenses and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution* **11**: 317-321.
- Simister, N.E. 2003. Placental transport of immunoglobulin G. *Vaccine* **21**: 3365-3369.
- Sin, Y., Ling, K. & Lam, T. 1994. Passive transfer of protective immunity against ichthyophthiriasis from vaccinated mother to fry in tilapias, *Oreochromis aureus*. *Aquaculture* **120**: 229-237.
- Smale, S.T., Tarakhovsky, A. & Natoli, G. 2014. Chromatin contributions to the regulation of innate immunity. *Annual Review of Immunology* **32**: 489-511.
- Small, C.M., Harlin-Cognato, A.D. & Jones, A.G. 2013. Functional similarity and molecular divergence of a novel reproductive transcriptome in two male-pregnant Syngnathus pipefish species. *Ecology and Evolution* **3**: 4092-4108.
- Smith, Z.D. & Meissner, A. 2013. DNA methylation: roles in mammalian development. *Nature Reviews Genetics* **14**: 204-220.
- Star, B., Nederbragt, A.J., Jentoft, S., Grimholt, U., Malmstrøm, M., Gregers, T.F., Rounge, T.B., Paulsen, J., Solbakken, M.H. & Sharma, A. 2011. The genome sequence of Atlantic cod reveals a unique immune system. *Nature* **477**: 207-210.
- Stein, L.R. & Bell, A.M. 2014. Paternal programming in sticklebacks. *Animal Behaviour* **95**: 165-171.
- Stenvik, J. & Jørgensen, T.Ø. 2000. Immunoglobulin D (IgD) of Atlantic cod has a unique structure. *Immunogenetics* **51**: 452-461.
- Stölting, K.N. & Wilson, A.B. 2007. Male pregnancy in seahorses and pipefish: beyond the mammalian model. *BioEssays* **29**: 884-896.
- Sun, D., Wu, S., Jing, C., Zhang, N., Liang, D. & Xu, A. 2012. Identification, synthesis and characterization of a novel antimicrobial peptide HKPLP derived from Hippocampus kuda Bleeker. *The Journal of Antibiotics* **65**: 117-121.
- Sun, J.C., Ugolini, S. & Vivier, E. 2014. Immunological memory within the innate immune system. *The EMBO journal*.

Bibliography

- Sunday, J.M., Bates, A.E. & Dulvy, N.K. 2012. Thermal tolerance and the global redistribution of animals. *Nature Climate Change* **2**: 686-690.
- Sunyer, J.O. 2013. Fishing for mammalian paradigms in the teleost immune system. *Nature Immunology* **14**: 320-326.
- Suzuki, M., Nakagawa, Y., Harayama, S. & Yamamoto, S. 2001. Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: proposal for Tenacibaculum gen. nov. with Tenacibaculum maritimum comb. nov. and Tenacibaculum ovolyticum comb. nov., and description of Tenacibaculum mesophilum sp. nov. and Tenacibaculum amyolyticum sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **51**: 1639-1652.
- Suzuki, Y., Orito, M., Furukawa, K. & Aida, K. 1994. Existence of Low Molecular Weight Immunoglobulin M in Carp Eggs. *Fisheries science* **60**: 159-162.
- Swain, P., Nayak, S., Sahu, A., Mohapatra, B. & Meher, P. 2002. Bath immunisation of spawn, fry and fingerlings of Indian major carps using a particulate bacterial antigen. *Fish & Shellfish Immunology* **13**: 133-140.
- Swain, P., Dash, S., Bal, J., Routray, P., Sahoo, P., Sahoo, S., Saurabh, S., Gupta, S. & Meher, P. 2006. Passive transfer of maternal antibodies and their existence in eggs, larvae and fry of Indian major carp, Labeo rohita (Ham.). *Fish & Shellfish Immunology* **20**: 519-527.
- Swain, P. & Nayak, S. 2009. Role of maternally derived immunity in fish. *Fish & Shellfish Immunology* **27**: 89-99.
- Szyf, M. 2015. Nongenetic inheritance and transgenerational epigenetics. *Trends in molecular medicine* **21**: 134-144.
- Takemura, A. 1996. Immunohistochemical localization of lysozyme in the prelarvae of tilapia, Oreochromis mossambicus. *Fish & Shellfish Immunology* **6**: 75-77.
- Tate, A.T. & Rudolf, V.H. 2012. Impact of life stage specific immune priming on invertebrate disease dynamics. *Oikos* **121**: 1083-1092.
- Thioulouse, J., Dolédec, S., Chessel, D. & Olivier, J. 1995. ADE software: multivariate analysis and graphical display of environmental data. *Software per l'ambiente*: 57-62.
- Tidbury, H.J., Pedersen, A.B. & Boots, M. 2011. Within and transgenerational immune priming in an insect to a DNA virus. *Proceedings of the Royal Society of London B: Biological Sciences* **278**: 871-876.
- Tidbury, H.J., Best, A. & Boots, M. 2012. The epidemiological consequences of immune priming. *Proceedings of the Royal Society B: Biological Sciences* **279**: 4505-4512.
- Tort, L., Balasch, J. & Mackenzie, S. 2003. Fish immune system. A crossroads between innate and adaptive responses. *Inmunología* **22**: 277-286.
- Uller, T. 2008. Developmental plasticity and the evolution of parental effects. *Trends in Ecology & Evolution* **23**: 432-438.
- Uribe, C., Folch, H., Enriquez, R. & Moran, G. 2011. Innate and adaptive immunity in teleost fish: a review. *Veterinarni Medicina* **56**: 486-503.
- Van Valen, L. 1974. Molecular evolution as predicted by natural selection. *Journal of molecular evolution* **3**: 89-101.
- Veenendaal, M.V., Painter, R.C., Rooij, S., Bossuyt, P.M., Post, J., Gluckman, P.D., Hanson, M.A. & Roseboom, T.J. 2013. Transgenerational effects of prenatal exposure to the 1944-45 Dutch famine. *BJOG: An International Journal of Obstetrics & Gynaecology* **120**: 548-554.

Bibliography

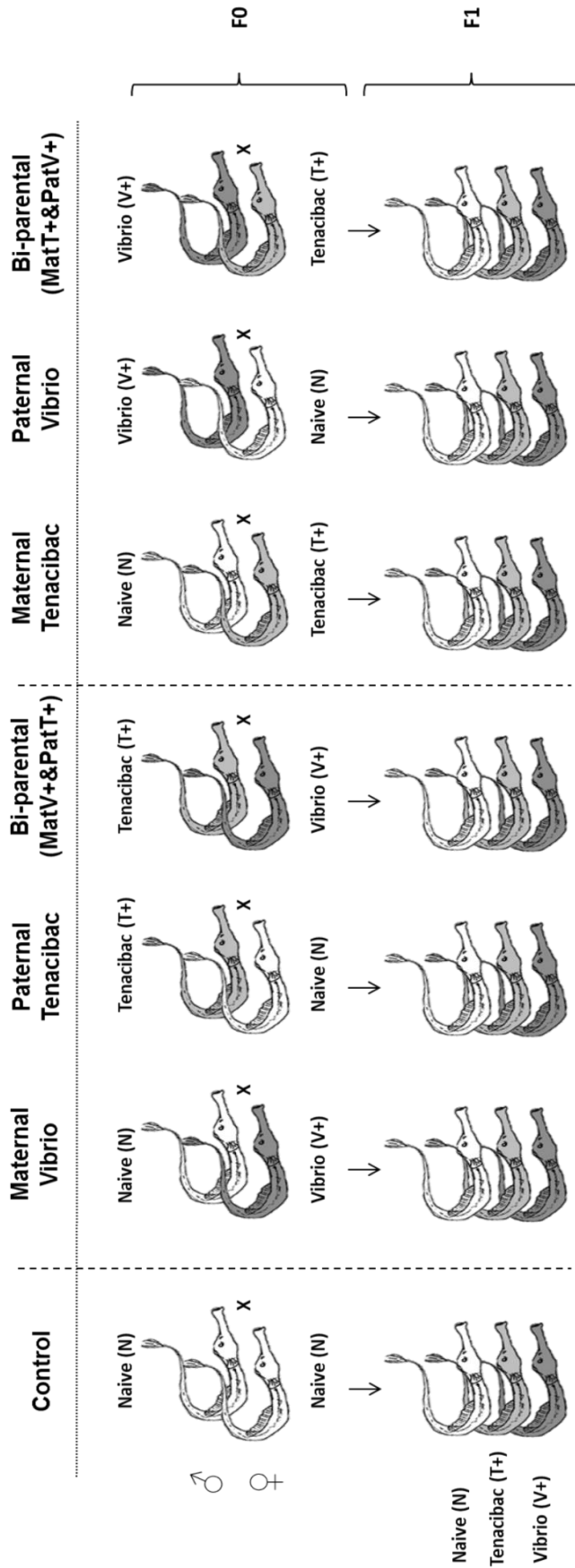
- Vincent, A.C., Berglund, A. & Ahnesjö, I. 1995. Reproductive ecology of five pipefish species in one eelgrass meadow. *Environmental Biology of Fishes* **44**: 347-361.
- von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. & Wittzell, H. 1999. Good genes, oxidative stress and condition-dependent sexual signals. *Proc R Soc B* **266**: 1-12.
- Waddington, C.H. 1953. *Epigenetics and evolution*. Symp. Soc. Exp. Biol.
- Wade, M.J. 1998. The evolutionary genetics of maternal effects. *Maternal effects as adaptations*: 5-21.
- Wade, P.A., Pruss, D. & Wolffe, A.P. 1997. Histone acetylation: chromatin in action. *Trends in Biochemical Sciences* **22**: 128-132.
- Walker, C.G., Meier, S., Littlejohn, M.D., Lehnert, K., Roche, J.R. & Mitchell, M.D. 2010. Modulation of the maternal immune system by the pre-implantation embryo. *BMC genomics* **11**: 474.
- Wan, J., Oliver, V.F., Wang, G., Zhu, H., Zack, D.J., Merbs, S.L. & Qian, J. 2015. Characterization of tissue-specific differential DNA methylation suggests distinct modes of positive and negative gene expression regulation. *BMC Genomics* **16**: 49
- Wang, G., Zhang, S. & Wang, Z. 2009. Responses of alternative complement expression to challenge with different combinations of *Vibrio anguillarum*, *Escherichia coli* and *Staphylococcus aureus*: evidence for specific immune priming in amphioxus *Branchiostoma belcheri*. *Fish & Shellfish Immunology* **26**: 33-39.
- Wang, Z. & Zhang, S. 2010. The role of lysozyme and complement in the antibacterial activity of zebrafish (*Danio rerio*) egg cytosol. *Fish & Shellfish Immunology* **29**: 773-777.
- Wassarman, P.M., Jovine, L. & Litscher, E.S. 2001. A profile of fertilization in mammals. *Nature Cell Biology* **3**: E59-E64.
- Watson, F.L., Pu, R., Thomas, F., Lamar, D.L., Hughes, M., Kondo, M. & Schmucker, D. 2005. Extensive diversity of Ig-superfamily proteins in the immune system of insects. *Science* **309**: 1874-1878.
- Weber, M., Davies, J.J., Wittig, D., Oakeley, E.J., Haase, M., Lam, W.L. & Schuebeler, D. 2005. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nature Genetics* **37**: 853-862.
- Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Pääbo, S., Rebhan, M. & Schübeler, D. 2007. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature Genetics* **39**: 457-466.
- Whittington, C.M., Griffith, O.W., Qi, W., Thompson, M.B. & Wilson, A.B. 2015. Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Molecular Biology and Evolution* **32**: 3114-3131.
- Whyte, S.K. 2007. The innate immune response of finfish—a review of current knowledge. *Fish & Shellfish Immunology* **23**: 1127-1151.
- Wilson, A. & Orr, J. 2011. The evolutionary origins of Syngnathidae: pipefishes and seahorses. *Journal of Fish Biology* **78**: 1603-1623.
- Wilson, A.B., Ahnesjö, I., Vincent, A.C. & Meyer, A. 2003. The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family Syngnathidae). *Evolution* **57**: 1374-1386.
- Wilson, A.J. & Réale, D. 2006. Ontogeny of additive and maternal genetic effects: lessons from domestic mammals. *The American Naturalist* **167**: E23-E38.

Bibliography

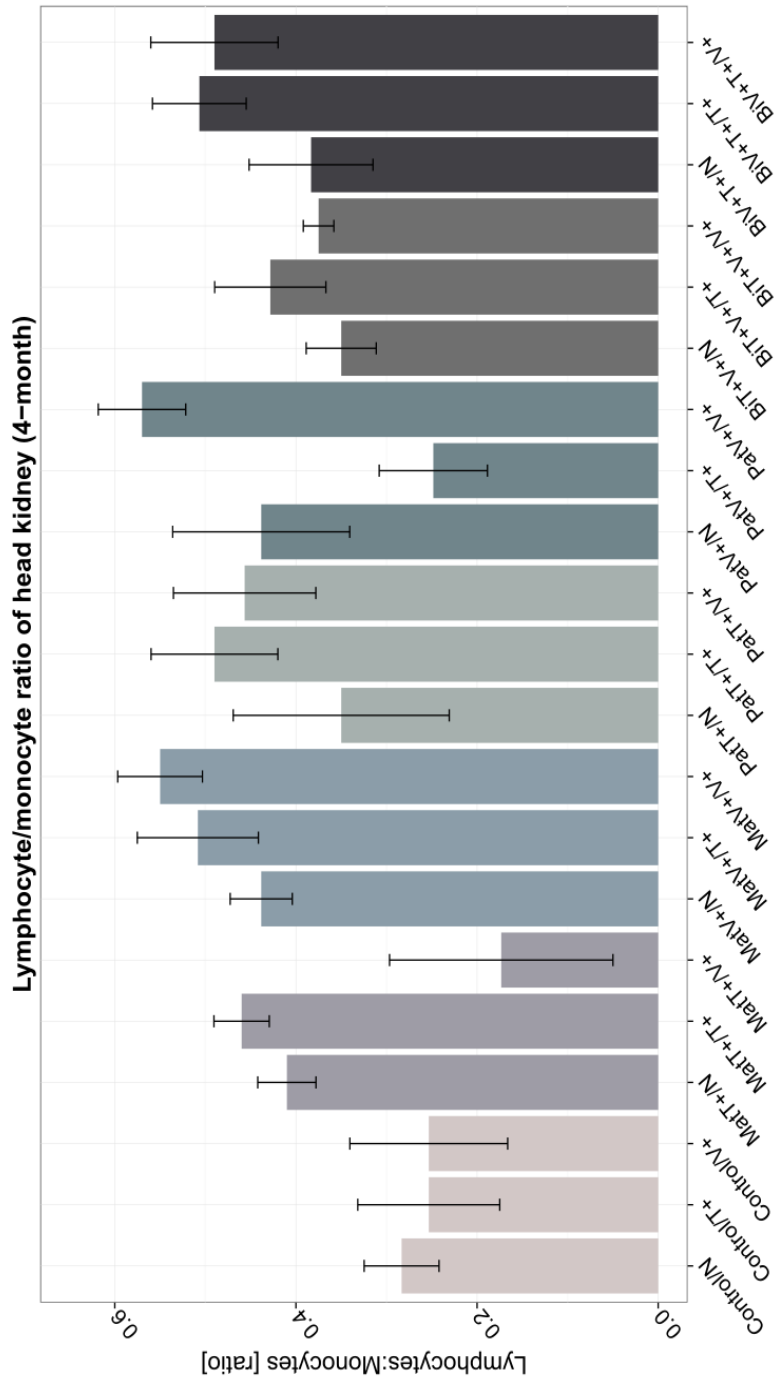
- Wilson, K. & Cotter, S.C. 2013. Host-parasite interactions and the evolution of immune defense. *Adverse Study of Behaviour* **45**: 81-174.
- Windsor, D.A. 1998. Controversies in parasitology, Most of the species on Earth are parasites. *International journal for parasitology* **28**: 1939-1941.
- Workenhe, S.T., Rise, M.L., Kibenge, M.J. & Kibenge, F.S. 2010. The fight between the teleost fish immune response and aquatic viruses. *Molecular Immunology* **47**: 2525-2536.
- Wourms, J.P. & Lombardi, J. 1992. Reflections on the evolution of piscine viviparity. *American Zoologist* **32**: 276-293.
- Wu, G., Li, M., Liu, Y., Ding, Y. & Yi, Y. 2015. The specificity of immune priming in silkworm, *Bombyx mori*, is mediated by the phagocytic ability of granular cells. *Journal of Insect Physiology* **81**: 60-68.
- Youngblood, B., Davis, C.W. & Ahmed, R. 2010. Making memories that last a lifetime: heritable functions of self-renewing memory CD8 T-cells. *International Immunology*: dxq437.
- Youngson, N.A. & Whitelaw, E. 2008. Transgenerational epigenetic effects. *Annual Review of Genomics and Human Genetics* **9**: 233-257.
- Zanchi, C., Troussard, J.P., Martinaud, G., Moreau, J. & Moret, Y. 2011. Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. *Journal of Animal Ecology* **80**: 1174-1183.
- Zapata, A., Diez, B., Cejalvo, T., Gutierrez-de Frias, C. & Cortes, A. 2006. Ontogeny of the immune system of fish. *Fish & Shellfish Immunology* **20**: 126-136.
- Zhang, S., Wang, Z. & Wang, H. 2013. Maternal immunity in fish. *Developmental & Comparative Immunology* **39**: 72-78.
- Zhang, Y. & Reinberg, D. 2001. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes & development* **15**: 2343-2360.

Appendices

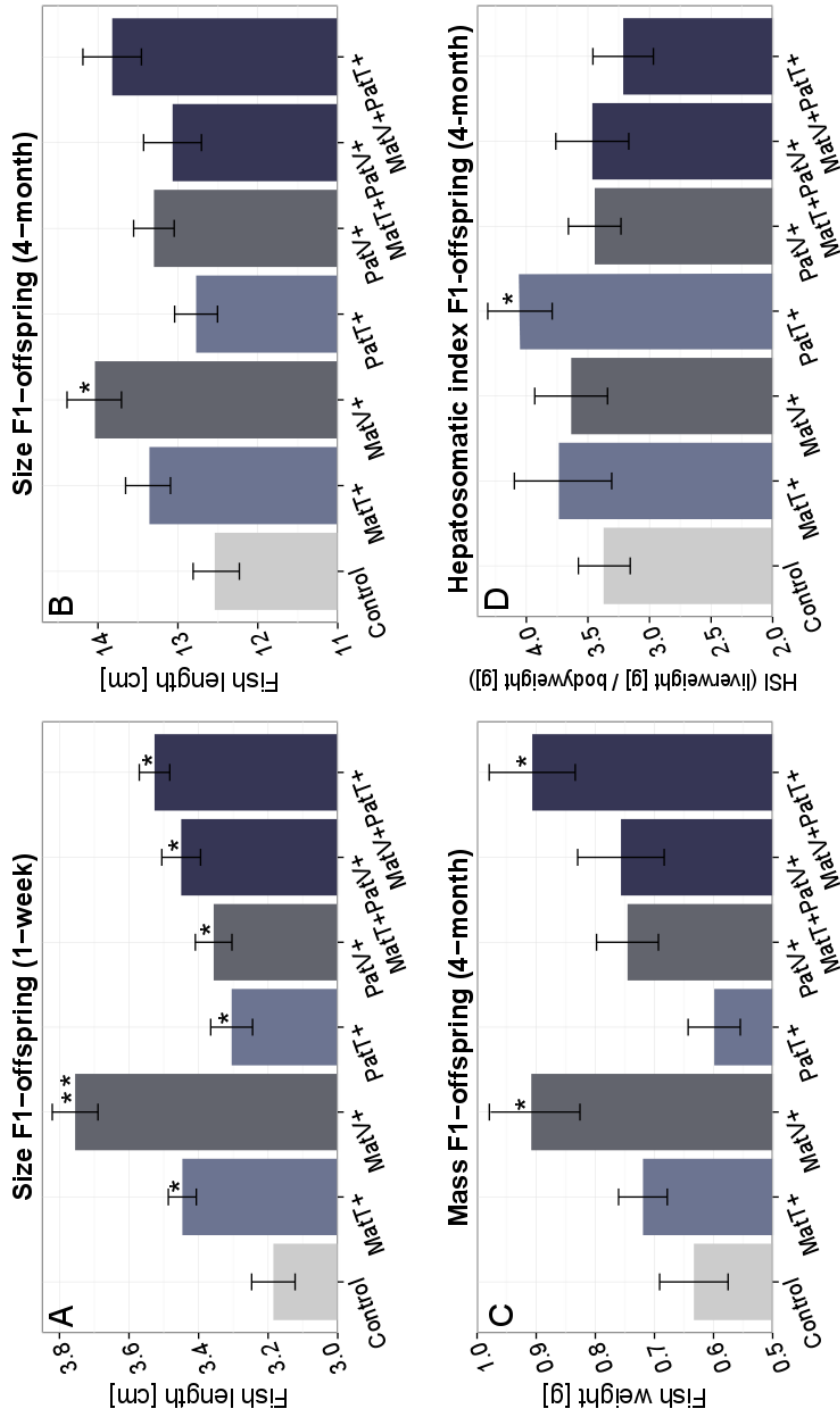
Appendix Chapter I



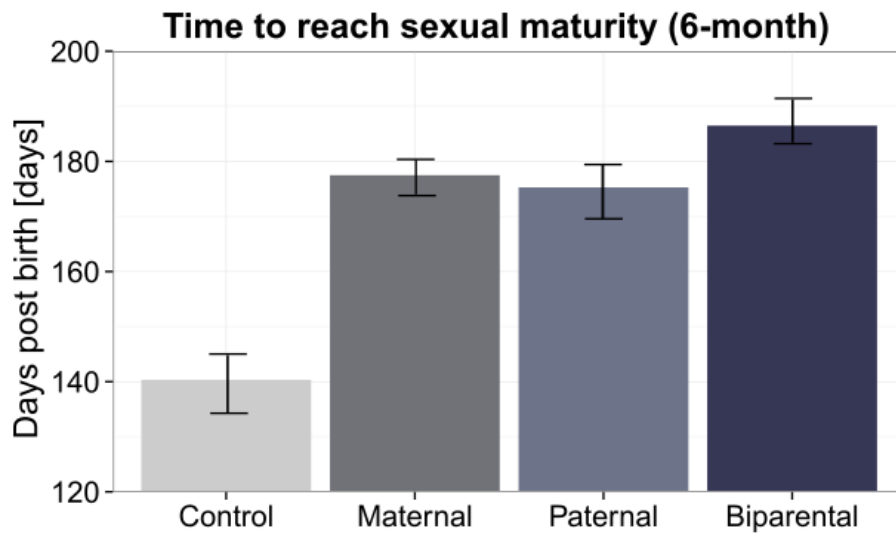
Supplement Figure S1: Experimental design to explore maternal, paternal and biparental immune priming effects and parental bacteria specificity in the pipefish *Syngnathus typhle* over one generation. In total seven parental treatment groups were applied whereby each sex of the parent was exposed either to a different bacteria strain or stayed without any treatment: **1. Control** (both parents naive (N)), **2. only maternal *Vibrio* (MatV+) or 3. *Tenacibaculum* (MatT+)**, **4. only paternal *Vibrio* (PatV+) or 5. *Tenacibaculum* (PatT+)**, both parents received different bacteria exposures **6. Biparental (MatV+PatT+)** and **7. Biparental (MatT+PatV+)**. F1 offspring were one-week and four-month post birth applied to the homologous or heterologous bacteria challenges as their parents or received no challenge as control. To test for uni-parental immune priming effects the parental treatment groups were pooled into following four: **1. Control:** Naive (control); **2. Maternal:** Maternal *Vibrio* (Mat V+) & Maternal *Tenacibaculum* (MatT+); **3. Paternal *Vibrio* (PatV+) & *Tenacibaculum* (PatT+)** **4. Biparental Bi-(MatV+PatT+) & Bi-(MatT+PatV+)**.



Supplement Figure 2: Lymphocyte/monocyte ratio in head kidney of four-month-old juveniles (N=126) grouped by F0-parents x F1-offspring interaction terms. Barplot to visualize maternal and paternal bacteria specificity effects based on lymphocyte/monocyte ratio in the head kidney of four month old F1-juveniles. The first term of the annotation indicates the F0-parental treatment (parental control (Control), maternal *Vibrio* (MatV+) and *Tenacibaculum* (MatT+), paternal *Vibrio* (PatV+) and *Tenacibaculum* (PatT+), and bi-parental (Bi-(MatV+PatT+) / Bi-(MatT+PatV+)) and the second term indicates the respective F1-offspring treatment (*Vibrio* (V+), *Tenacibaculum* (T+) and control (N)). Error bars are representing standard error of the mean (\pm sem).



Supplement Figure 3: Parental treatment effects based on life-history parameters of one-week-old juveniles (N=420) and four-month-old juveniles (N=126). Barplots are grouped according to F0-parental treatments (parental control (Control), maternal *Vibrio* (MatV+) and *Tenacibaculum* (MatT+), paternal *Vibrio* (PatV+) and *Tenacibaculum* (PatT+), and biparental (Bi-MatV+PatT+)/Bi-(MatT+PatV+)). Respective error bars are representing standard error of the mean (+/-sem). Different panels display **A**: size of one-week-old juveniles, **B**: size of four-month-old juveniles, **C**: mass (body weight) of four-month-old juveniles, **D**: hepatosomatic index (HSI) of four-month-old juveniles. Asterix * demonstrates significant difference compared to control group. **S3A**: Double asterix ** demonstrates significant difference between control group and other treatment groups.



Supplement Figure 4: Parental treatment effects on time for F1-males to reach sexual-maturity (N=170). Barplot is grouped according to F0-parental treatments (parental control, maternal, paternal and biparental and representing time (days post birth) of F1-males to create a functional brood pouch tissue and reach sexual maturity. Respective error bars are representing standard error of the mean (+/-sem).

Supplement Table 1: Results from PERMANOVA analysis of gene expression, immune cell counts and life-history traits of one-week and four-month old F1-juveniles. Multivariate PERMANOVA analysis to assess the effect and interaction of the two fixed factors F0-parents and F1-offspring, including the nested factor nested in F0-parents ('family' (for one-week-old) and 'sampling day' (for four-month-old)) and size as covariate on relative gene expression data (-ΔCt-values), proportion of immune cells (%) and life-history parameters. Each based on a Bray-Curtis distance matrix with p-values obtained by 999 permutations. Significant p-values are marked in bold letters (significance code: 0.1 > p-value ≥ 0.05 trend •, < 0.001 **, 0.001 ***, 0.01 *), whereas "ns" indicates no statistical difference. PERMANOVA analysis was conducted for all target genes (44) and divided into following functional gene categories: genes of the innate immune system (13), of innate and adaptive immune system (5), the adaptive immune system (8), complement component genes (3), methylation/demethylation genes (9), and acetylation/deacetylation genes (5). Further for immune cell count measurements (proportion of lymphocytes, of monocytes, and ratio of lymphocytes/monocytes) of the head kidney and the blood, as well as life-history parameters (size, mass condition factor (CF) and hepatosomatic index (HSI)).

Age	Response Variables	Model		F0-parents			F1-offspring			F0-parents x F1-offspring			F0-parents : Nested factor			Size							
		Total	Residuals	Df	F.Model	Pr(F)	Sig	Df	F.Model	Pr(F)	Sig	Df	F.Model	Pr(F)	Sig	Df	F.Model	Pr(F)	Sig				
1	All genes [44]	419	377	6	11.17	<0.001	***	2	16.09	<0.001	***	12	1.86	<0.001	***	21	16.02	<0.001	***	1	9.53	<0.001	***
	Immune genes [29]	419	377	6	12.44	<0.001	***	2	18.36	<0.001	***	12	1.75	<0.001	***	21	14.81	<0.001	***	1	7.89	<0.001	***
	Innate [13]	419	377	6	12.50	<0.001	***	2	14.08	<0.001	***	12	1.65	0.002	**	21	15.84	<0.001	***	1	9.44	<0.001	***
	Innate&Adaptive [5]	419	377	6	16.29	<0.001	***	2	20.18	<0.001	***	12	1.39	0.058	.	21	8.80	<0.001	***	1	4.98	0.002	**
	Adaptive [8]	419	377	6	10.08	<0.001	***	2	2.94	0.006	**	12	1.44	0.030	*	21	16.99	<0.001	***	1	4.104	0.003	**
	Complement [3]	419	377	6	10.63	<0.001	***	2	45.65	<0.001	***	12	2.31	<0.001	***	21	13.84	<0.001	***	1	5.459	0.004	**
	Epigenetic [15]	419	377	6	6.61	<0.001	***	2	6.10	<0.001	***	12	2.12	<0.001	***	21	19.44	<0.001	***	1	15.457	<0.001	***
	Methylation [9]	419	377	6	5.64	<0.001	***	2	4.42	0.003	**	12	2.22	0.002	**	21	17.62	<0.001	***	1	21.180	<0.001	***
	Acetylation [5]	419	377	6	9.58	<0.001	***	2	8.74	<0.001	***	12	1.90	0.011	*	21	23.75	<0.001	***	1		ns	
	4	All genes [44]	125	98	6	3.21	<0.001	***	2	2.91	0.003	**	12		ns		7	2.81	<0.001	***	1		ns
Immune genes [29]		125	98	6	3.07	<0.001	***	2	3.25	<0.001	***	12		ns		7	2.63	<0.001	***	1		ns	
Innate [13]		125	98	6	2.41	<0.001	***	2	4.12	<0.001	***	12		ns		7	3.29	<0.001	***	1		ns	
Innate&Adaptive [5]		125	98	6	4.12	<0.001	***	2	2.90	0.012	*	12		ns		7	2.11	0.010	**	1		ns	
Adaptive [8]		125	98	6	3.36	<0.001	***	2	2.33	0.030	*	12		ns		7	2.23	0.002	**	1		ns	
Complement [3]		125	98	6	3.93	<0.001	***	2		ns		12		ns		7	2.37	0.012	*	1		ns	
Epigenetic [15]		125	98	6	3.50	<0.001	***	2		ns		12		ns		7	3.54	<0.001	***	1		ns	
Methylation [9]		125	98	6	3.82	<0.001	***	2		ns		12		ns		7	3.47	<0.001	***	1		ns	
Acetylation [5]		125	98	6	3.42	0.002	**	2		ns		12		ns		7	4.26	<0.001	***	1		ns	
Immune cells [6]		125	97	6	5.94	<0.001	***	2	4.02	<0.001	***	12		ns		7	8.46	<0.001	***	1		ns	
Immune cells HK [3]	125	97	6	7.29	<0.001	***	2	6.53	<0.001	***	12	2.57	0.005	**	7	6.69	<0.001	***	1	6.86	0.006	**	
Immune cells blood [3]	125	97	6	4.66	<0.001	***	2	3.84	0.002	**	12		ns		7	8.45	<0.001	***	1		ns		
Size/Mass/CF/HSI		125	98	6	2.56	0.004	**	2		ns		12		ns		7	0.77	ns		1		ns	

Supplement Table 2: Results from ANOSIM analysis of 29 immune genes to test for F0-parental and F1-offspring treatment effects in one-week-old F1-juveniles. Multivariate ANOSIM analysis to assess differences in the gene expression profiles per parental treatment groups applying pairwise comparison on relative gene expression data ($-\Delta\text{Ct}$ -values) based on a Bray-Curtis distance matrix and 999 permutations. Significant p-values are marked in bold letters, whereas “ns” indicates no statistical difference. Shown are global R and global significance values over two main treatments (parents and offspring) and their interaction followed by pairwise comparisons between parental control (Control), paternal *Vibrio* (PatV+) and *Tenacibaculum* (PatT+), maternal *Vibrio* (MatV+) and *Tenacibaculum* (MatT+), respective biparental (Bi-(MatV+PatT+) and Bi-(MatT+PatV+)) treatment groups. ANOSIM analysis was conducted for all target genes (44) and divided in following functional gene categories: genes of the innate immune system (13), of innate and adaptive immune system (5), the adaptive immune system (8) and complement component genes (3).

ANOSIM	Immune genes [29]	Innate [13]	Innate & Adaptive [5]	Adaptive [8]	Complement [3]
F0-parents (7 treatments)					
<i>Global R</i>	0.093	0.078	0.099	0.079	0.046
<i>Significance level</i>	0.001	0.001	0.001	0.001	0.001
Bi-(MatV+PatT+) x Bi-(MatT+PatV+)	0.004	0.042	0.004	0.002	ns
Bi-(MatV+PatT+) x MatV+	0.010	0.034	0.001	0.048	ns
Bi-(MatV+PatT+) x MatT+	0.001	0.006	0.001	0.001	ns
Bi-(MatV+PatT+) x PatV+	0.001	0.001	0.001	0.001	0.002
Bi-(MatV+PatT+) x PatT+	ns	ns	ns	ns	ns
Bi-(MatV+PatT+) x Control	0.005	0.005	0.003	0.015	ns
Bi-(MatT+PatV+) x MatV+	0.001	0.003	0.001	0.001	0.001
Bi-(MatT+PatV+) x MatT+	0.001	0.017	0.001	0.002	0.009
Bi-(MatT+PatV+) x PatV+	0.001	0.001	0.001	0.001	0.001
Bi-(MatT+PatV+) x PatT+	0.001	0.001	0.002	0.005	0.042
Bi-(MatT+PatV+) x Control	0.001	0.003	0.001	0.008	ns
MatV+ x MatT+	0.001	0.002	0.001	0.001	ns
MatV+ x PatV+	0.001	0.001	0.004	0.006	ns
MatV+ x PatT+	0.001	0.001	0.010	0.006	0.001
MatV+ x Control	0.002	0.001	0.003	0.007	ns
MatT+ x PatV+	0.001	0.002	0.001	0.001	0.028
MatT+ x PatT+	0.005	0.005	0.001	0.006	0.035
MatT+ x Control	0.001	0.001	0.001	0.001	ns
PatV+ x PatT+	0.001	0.001	0.001	0.001	0.003
PatV+ x Control	0.001	0.001	0.001	0.001	0.008
PatT+ x Control	0.001	0.001	0.009	0.007	ns
F1-offspring (3 treatments)					
<i>ANOSIM-Global R</i>	0.061	0.070	0.074	0.001	0.087
<i>Significance level</i>	0.001	0.001	0.001	0.409	0.001
F1-V+ x F1-T+	ns	ns	ns	ns	ns
F1-V+ x F1-N	0.001	0.001	0.001	ns	0.001
F1-T+ x F1-N	0.001	0.001	0.001	ns	0.001
F0-parents x F1-offspring					
<i>ANOSIM-Global R</i>	0.149	0.118	0.130	0.076	0.098
<i>Significance level</i>	0.001	0.001	0.001	0.001	0.001
Bi-(MatV+PatT+)/F1-V+ X Bi-(MatV+PatT+)/F1-T+	ns	ns	ns	ns	ns
Bi-(MatV+PatT+)/F1-V+ X Bi-(MatT+PatV+)/F1-V+	ns	ns	ns	ns	ns
Bi-(MatV+PatT+)/F1-V+ X Bi-(MatT+PatV+)/F1-T+	ns	ns	0.008	ns	ns
Bi-(MatV+PatT+)/F1-T+ X Bi-(MatT+PatV+)/F1-V+	ns	ns	ns	ns	ns
Bi-(MatV+PatT+)/F1-T+ X Bi-(MatT+PatV+)/F1-T+	0.006	0.032	ns	0.043	0.035
Bi-(MatT+PatV+)/F1-V+ X Bi-(MatT+PatV+)/F1-T+	ns	ns	ns	ns	ns
Bi-(MatV+PatT+)/F1-N X Bi-(MatT+PatV+)/F1-V+	0.001	0.001	0.001	ns	0.004
Bi-(MatV+PatT+)/F1-N X Bi-(MatT+PatV+)/F1-T+	0.003	0.015	0.001	ns	0.018
Bi-(MatV+PatT+)/F1-V+ X Bi-(MatT+PatV+)/F1-N	0.001	0.001	0.001	ns	0.001
Bi-(MatV+PatT+)/F1-T+ X Bi-(MatV+PatT+)/F1-N	0.002	0.023	0.001	ns	0.001
Bi-(MatV+PatT+)/F1-V+ X Bi-(MatV+PatT+)/F1-N	0.002	0.049	0.001	ns	0.005
Bi-(MatV+PatT+)/F1-T+ X Bi-(MatT+PatV+)/F1-N	0.001	0.003	0.001	0.104	0.001
Bi-(MatV+PatT+)/F1-N X Bi-(MatT+PatV+)/F1-N	0.028	0.044	0.047	0.363	ns
Bi-(MatT+PatV+)/F1-V+ X Bi-(MatT+PatV+)/F1-N	0.001	0.001	0.001	ns	0.007
Bi-(MatT+PatV+)/F1-T+ X Bi-(MatT+PatV+)/F1-N	0.027	ns	0.003	ns	ns
Bi-(MatV+PatT+)/F1-V+ X MatV+/F1-V+	0.023	ns	0.004	0.038	ns
Bi-(MatV+PatT+)/F1-V+ X MatV+/F1-T+	ns	ns	0.005	0.014	ns
Bi-(MatV+PatT+)/F1-V+ X MatT+/F1-V+	0.006	0.052	0.004	0.002	ns
Bi-(MatV+PatT+)/F1-V+ X MatT+/F1-T+	0.002	0.013	0.004	0.004	ns
Bi-(MatV+PatT+)/F1-V+ X PatV+/F1-V+	0.002	0.006	0.001	0.002	ns
Bi-(MatV+PatT+)/F1-V+ X PatV+/F1-T+	0.009	0.048	0.001	0.025	0.031

Appendix Chapter I

Bi-(MatV+PatT+)/F1-V+ X PatT+/F1-V+	ns	ns	ns	ns	ns
Bi-(MatV+PatT+)/F1-V+ X PatT+/F1-T+	ns	ns	ns	ns	ns
Bi-(MatV+PatT+)/F1-T+ X MatV+/F1-V+	0.005	0.007	0.010	0.050	ns
Bi-(MatV+PatT+)/F1-T+ X MatV+/F1-T+	0.038	0.024	0.029	ns	ns
Bi-(MatV+PatT+)/F1-T+ X MatT+/F1-V+	0.024	ns	0.001	0.002	ns
Bi-(MatV+PatT+)/F1-T+ X MatT+/F1-T+	0.009	0.033	0.002	ns	ns
Bi-(MatV+PatT+)/F1-T+ X PatV+/F1-V+	0.003	0.004	0.002	0.072	ns
Bi-(MatV+PatT+)/F1-T+ X PatV+/F1-T+	0.005	0.006	0.002	ns	ns
Bi-(MatV+PatT+)/F1-T+ X PatT+/F1-V+	0.024	0.018	ns	ns	ns
Bi-(MatV+PatT+)/F1-T+ X PatT+/F1-T+	ns	ns	ns	ns	ns
Bi-(MatT+PatV+)/F1-T+ X PatV+/F1-V+	0.001	0.023	0.001	0.001	ns
Bi-(MatT+PatV+)/F1-T+ X PatV+/F1-T+	0.001	trend	0.001	0.001	0.001
Bi-(MatT+PatV+)/F1-V+ X MatV+/F1-V+	0.001	0.002	0.003	0.005	0.007
Bi-(MatT+PatV+)/F1-V+ X MatV+/F1-T+	0.008	0.019	0.046	0.028	0.006
Bi-(MatT+PatV+)/F1-T+ X PatT+/F1-V+	0.002	0.003	0.003	0.029	ns
Bi-(MatT+PatV+)/F1-T+ X PatT+/F1-T+	0.002	0.005	0.014	ns	ns
Bi-(MatT+PatV+)/F1-V+ X MatT+/F1-V+	0.005	ns	0.001	0.037	ns
Bi-(MatT+PatV+)/F1-V+ X MatT+/F1-T+	0.006	trend	0.001	ns	0.021
Bi-(MatT+PatV+)/F1-V+ X PatV+/F1-V+	0.004	ns	0.007	0.003	ns
Bi-(MatT+PatV+)/F1-V+ X PatV+/F1-T+	0.005	ns	0.011	0.015	0.005
Bi-(MatT+PatV+)/F1-V+ X PatT+/F1-V+	0.041	0.027	ns	ns	ns
Bi-(MatT+PatV+)/F1-V+ X PatT+/F1-T+	ns	ns	ns	ns	ns
Bi-(MatT+PatV+)/F1-T+ X MatV+/F1-V+	0.001	0.002	0.001	0.001	0.001
Bi-(MatT+PatV+)/F1-T+ X MatV+/F1-T+	0.001	ns	0.001	0.002	0.002
Bi-(MatT+PatV+)/F1-T+ X MatT+/F1-V+	0.002	0.007	0.001	0.002	0.018
Bi-(MatT+PatV+)/F1-T+ X MatT+/F1-T+	0.001	0.018	0.001	0.072	0.002
Bi-(MatT+PatV+)/F1-T+ X MatT+/F1-N	0.001	0.005	0.001	0.032	0.099
Bi-(MatT+PatV+)/F1-T+ X PatV+/F1-N	0.002	0.074	0.016	0.001	0.014
Bi-(MatT+PatV+)/F1-T+ X PatT+/F1-N	0.004	0.011	0.005	0.007	ns
Bi-(MatT+PatV+)/F1-T+ X MatV+/F1-N	0.014	ns	0.005	0.006	ns
Bi-(MatV+PatT+)/F1-V+ X MatV+/F1-N	0.018	ns	0.002	ns	0.061
Bi-(MatV+PatT+)/F1-V+ X MatT+/F1-N	0.001	0.002	0.001	0.001	0.001
Bi-(MatV+PatT+)/F1-V+ X PatV+/F1-N	0.002	0.003	0.003	0.001	0.015
Bi-(MatV+PatT+)/F1-V+ X PatT+/F1-N	0.005	0.021	0.039	0.029	0.015
Bi-(MatV+PatT+)/F1-T+ X MatV+/F1-N	0.009	0.022	0.003	ns	0.029
Bi-(MatV+PatT+)/F1-T+ X MatT+/F1-N	0.001	0.003	0.001	0.007	0.001
Bi-(MatV+PatT+)/F1-T+ X PatV+/F1-N	0.004	0.003	0.010	0.040	0.039
Bi-(MatV+PatT+)/F1-T+ X PatT+/F1-N	0.002	0.003	0.014	ns	0.003
Bi-(MatT+PatV+)/F1-V+ X MatV+/F1-N	0.002	0.001	0.001	ns	ns
Bi-(MatT+PatV+)/F1-V+ X MatT+/F1-N	0.001	0.001	0.001	ns	0.008
Bi-(MatT+PatV+)/F1-V+ X PatV+/F1-N	0.001	0.001	0.001	0.008	0.013
Bi-(MatT+PatV+)/F1-V+ X PatT+/F1-N	0.001	0.003	0.002	ns	0.028
Bi-(MatV+PatT+)/F1-N X MatV+/F1-N	ns	ns	0.011	ns	ns
Bi-(MatV+PatT+)/F1-N X MatT+/F1-N	0.010	0.034	0.001	ns	ns
Bi-(MatV+PatT+)/F1-N X PatV+/F1-N	0.001	0.001	0.001	0.003	0.001
Bi-(MatV+PatT+)/F1-N X PatT+/F1-N	ns	ns	trend	0.020	ns
Bi-(MatV+PatT+)/F1-N X MatV+/F1-V+	0.001	0.001	0.001	0.006	0.001
Bi-(MatV+PatT+)/F1-N X MatV+/F1-T+	0.001	0.003	0.001	0.007	0.001
Bi-(MatV+PatT+)/F1-N X MatT+/F1-V+	0.001	0.001	0.001	0.004	0.001
Bi-(MatV+PatT+)/F1-N X MatT+/F1-T+	0.001	0.002	0.001	ns	0.001
Bi-(MatV+PatT+)/F1-N X PatV+/F1-V+	0.001	0.001	0.001	0.033	0.001
Bi-(MatV+PatT+)/F1-N X PatV+/F1-T+	0.001	0.001	0.001	ns	0.001
Bi-(MatV+PatT+)/F1-N X PatT+/F1-V+	0.001	0.009	0.001	0.024	0.004
Bi-(MatV+PatT+)/F1-N X PatT+/F1-T+	0.001	0.005	0.001	ns	0.001
Bi-(MatT+PatV+)/F1-N X MatV+/F1-V+	0.001	0.001	0.001	0.001	0.001
Bi-(MatT+PatV+)/F1-N X MatV+/F1-T+	0.001	0.002	0.001	0.007	0.001
Bi-(MatT+PatV+)/F1-N X MatV+/F1-N	0.003	0.020	0.009	0.044	ns
Bi-(MatT+PatV+)/F1-N X MatT+/F1-V+	0.001	0.003	0.001	0.001	0.001
Bi-(MatT+PatV+)/F1-N X MatT+/F1-T+	0.001	0.006	0.001	ns	0.001
Bi-(MatT+PatV+)/F1-N X MatT+/F1-N	0.001	0.029	0.001	0.039	ns
Bi-(MatT+PatV+)/F1-N X PatV+/F1-V+	0.001	0.001	0.001	0.001	0.001
Bi-(MatT+PatV+)/F1-N X PatV+/F1-T+	0.001	0.001	0.001	0.001	0.001
Bi-(MatT+PatV+)/F1-N X PatV+/F1-N	0.001	0.005	0.018	0.005	0.003
Bi-(MatT+PatV+)/F1-N X PatT+/F1-V+	0.001	0.001	0.001	0.021	0.009
Bi-(MatT+PatV+)/F1-N X PatT+/F1-T+	0.001	0.001	0.001	trend	0.002
Bi-(MatT+PatV+)/F1-N X PatT+/F1-N	0.004	0.008	0.004	0.013	0.049
Bi-(MatT+PatV+)/F1-N X Control/F1-V+	0.001	0.001	0.001	0.004	0.001
Bi-(MatT+PatV+)/F1-N X Control/F1-T+	0.001	0.001	0.001	ns	0.001
Bi-(MatT+PatV+)/F1-N X Control/F1-N	0.003	0.013	0.004	ns	ns
Bi-(MatV+PatT+)/F1-N X Control/F1-V+	0.001	0.001	0.001	0.046	0.001
Bi-(MatV+PatT+)/F1-N X Control/F1-T+	0.001	0.006	0.001	ns	0.001

Appendix Chapter I

Bi-(MatV+PatT+)/F1-N X Control/F1-N	ns	ns	0.009	ns	ns
Bi-(MatT+PatV+)/F1-V+ X Control/F1-V+	0.047	0.020	ns	ns	ns
Bi-(MatT+PatV+)/F1-V+ X Control/F1-T+	0.034	0.036	0.014	ns	ns
Bi-(MatT+PatV+)/F1-V+ X Control/F1-N	0.003	0.014	0.001	0.012	ns
Bi-(MatT+PatV+)/F1-T+ X Control/F1-V+	0.001	0.001	0.001	0.008	ns
Bi-(MatT+PatV+)/F1-T+ X Control/F1-T+	0.001	0.003	0.001	0.042	ns
Bi-(MatT+PatV+)/F1-T+ X Control/F1-N	0.005	0.031	0.003	0.001	ns
Bi-(MatV+PatT+)/F1-V+ X Control/F1-V+	0.023	0.008	0.019	0.005	ns
Bi-(MatV+PatT+)/F1-V+ X Control/F1-T+	ns	ns	0.027	ns	ns
Bi-(MatV+PatT+)/F1-V+ X Control/F1-N	0.006	0.046	0.003	0.005	ns
Bi-(MatV+PatT+)/F1-T+ X Control/F1-V+	0.015	0.001	ns	trend	ns
Bi-(MatV+PatT+)/F1-T+ X Control/F1-T+	0.042	0.010	ns	ns	ns
Bi-(MatV+PatT+)/F1-T+ X Control/F1-N	0.013	0.056	0.004	0.040	0.024

Supplement Table 3: Results from ANOSIM analysis of 29 immune genes to test for F0-parental and F1-offspring treatment effects in four-month-old F1-juveniles. Multivariate ANOSIM analysis to assess differences in the gene expression profiles per parental treatment groups applying pairwise comparison on relative gene expression data ($-\Delta\text{Ct}$ -values) based on a Bray-Curtis distance matrix and 999 permutations. Significant p-values are marked in bold letters (significance code: 0.1>p-value \geq 0.05 trend •, <0.001***, 0.001**, 0.01*), whereas “ns” indicates no statistical difference. Shown are global R and global significance values over two main treatments (F0-parents and F1-offspring) and their interactions. These are followed by pairwise comparisons between parental control (Control), paternal *Vibrio* (PatV+) and *Tenacibaculum* (PatT+), maternal *Vibrio* (MatV+) and *Tenacibaculum* (MatT+), respective biparental (Bi-(MatV+PatT+); Bi-(MatT+PatV+)) treatment groups as well as pairwise comparison between direct offspring treatment *Vibrio* (F1-V+), *Tenacibaculum* (F1-T+), and Naïve (F1-N). ANOSIM analysis was conducted for all target genes (44) and divided in following functional gene categories: genes of the innate immune system (13), of innate and adaptive immune system (5), the adaptive immune system (8), complement component genes (3).

ANOSIM	Immune genes [29]	Innate [13]	Innate & Adaptive [5]	Adaptive [8]	Complement [3]
F0-parents (7 treatments)					
Global R	0.156	0.076	0.074	0.179	0.132
Significance level	0.001	0.010	0.008	0.001	0.001
Bi-(MatV+PatT+) x Bi-(MatT+PatV+)	0.003	0.002	ns	0.001	ns
Bi-(MatV+PatT+) x MatV+	0.006	0.028	ns	0.004	ns
Bi-(MatV+PatT+) x MatT+	0.008	ns	0.011	ns	ns
Bi-(MatV+PatT+) x PatV+	0.027	ns	ns	0.003	ns
Bi-(MatV+PatT+) x PatT+	ns	ns	ns	ns	ns
Bi-(MatV+PatT+) x Control	0.029	0.015	ns	0.013	0.004
Bi-(MatT+PatV+) x MatV+	0.011	0.002	0.076	0.015	ns
Bi-(MatT+PatV+) x MatT+	0.002	0.001	0.005	0.002	ns
Bi-(MatT+PatV+) x PatV+	ns	ns	ns	ns	ns
Bi-(MatT+PatV+) x PatT+	0.003	0.014	ns	0.007	ns
Bi-(MatT+PatV+) x Control	0.001	0.008	ns	0.001	0.006
MatV+ x MatT+	0.001	0.006	0.009	0.001	0.047
MatV+ x PatV+	ns	ns	ns	0.015	ns
MatV+ x PatT+	0.044	0.037	ns	ns	ns
MatV+ x Control	0.001	0.004	0.009	0.001	0.001
MatT+ x PatV+	0.001	0.031	0.002	0.036	0.003
MatT+ x PatT+	0.006	ns	0.004	0.014	ns
MatT+ x Control	0.010	ns	0.008	0.044	0.067
PatV+ x PatT+	0.028	ns	ns	0.032	0.048
PatV+ x Control	0.001	0.064	ns	0.001	0.001
PatT+ x Control	ns	ns	ns	0.012	0.024
F1-offspring (3 treatments)					
ANOSIM-Global R	0.056	0.076	0.013	0.027	ns
Significance level	0.001	0.001	0.048	0.006	ns
F1-V+ x F1-T+	ns	ns	ns	0.030	ns
F1-V+ x F1-N	0.001	0.001	0.042	0.027	ns
F1-T+ x F1-N	0.001	0.001	0.039	0.008	ns
F0-parents x F1-offspring (interaction)					
	ns	ns	ns	ns	ns

Appendix Chapter I

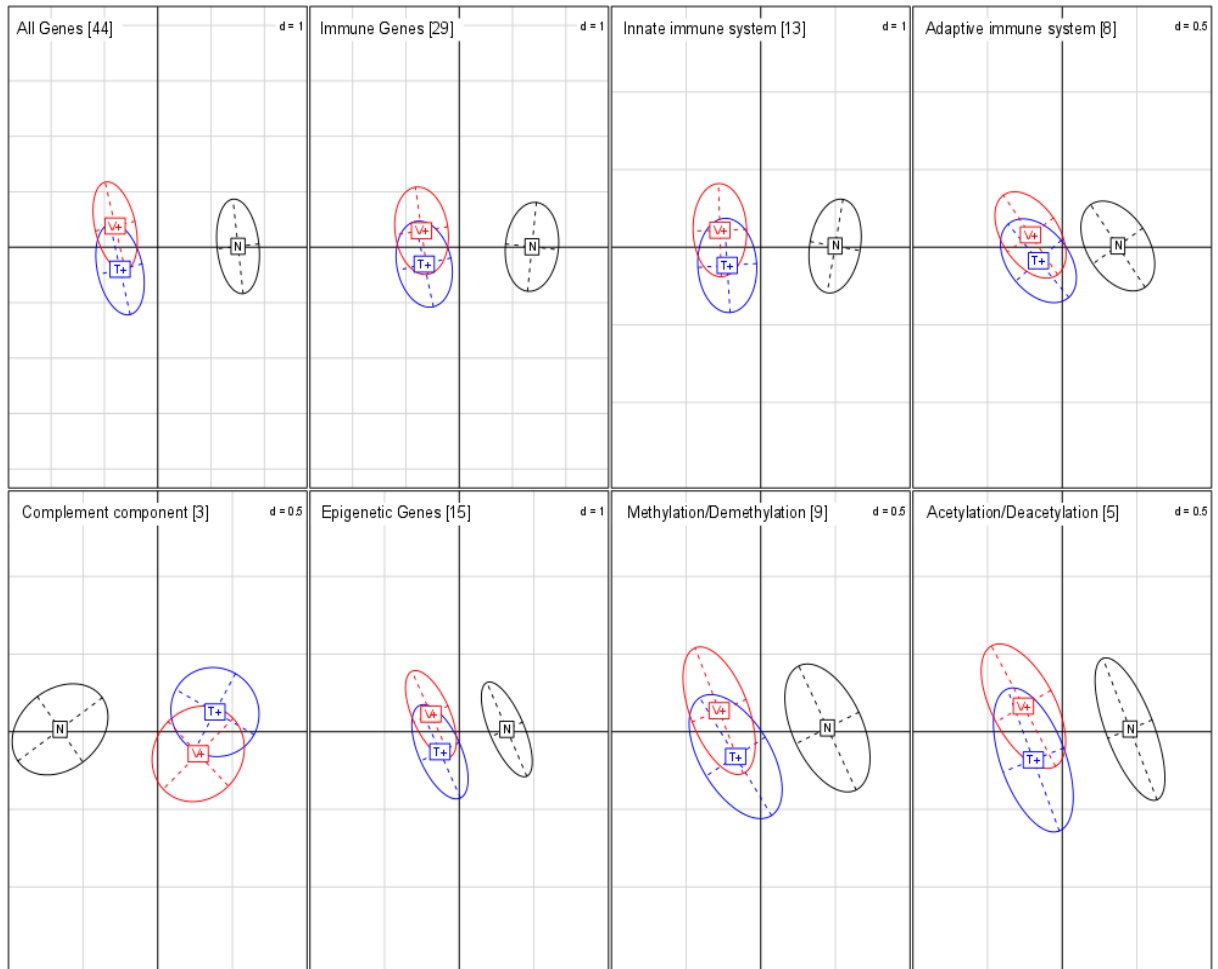
Supplement Table 4: Results from ANOSIM analysis of 15 epigenetic genes to test for F0-parental treatment effects in one-week-old F1-juveniles and four-month-old F1-juveniles. Multivariate ANOSIM analysis to assess differences in the gene expression profiles per parental treatment groups applying pairwise comparison on relative gene expression data ($-\Delta Ct$ -values) based on a Bray-Curtis distance matrix and 999 permutations. Significant p-values are marked in bold letters, whereas “ns” indicates no statistical difference. Shown are global R and global significance values for the parental treatment effect (F0-parents). These are followed by pairwise comparisons between parental control, paternal, maternal, and biparental treatment groups.

ANOSIM	1 Week			4 Month		
	Epigenetic [15]	Methylation [9]	Acetylation [5]	Epigenetic [15]	Methylation [9]	Acetylation [5]
F0-parents (4 Groups)						
ANOSIM-Global R	0.020	0.022	0.021	0.048	0.038	0.065
Significance level	0.014	0.009	0.011	0.001	0.003	0.001
Biparental x Maternal	0.002	0.001	0.010	ns	ns	ns
Biparental x Paternal	0.004	0.004	0.013	ns	ns	ns
Biparental x Control	0.049	0.043	ns	0.001	0.001	0.001
Maternal x Paternal	0.019	0.003	0.034	ns	ns	ns
Maternal x Control	ns	ns	ns	0.006	0.017	0.002
Paternal x Control	ns	ns	0.046	0.001	0.003	0.001

Supplement Table 5: Results from univariate statistical analysis of life-history parameters and immune cell count measurements in one-week-old, four-month-old and six-month-old F1-juveniles. Univariate testing using ANOVAs for each response variable and TukeyHSD post-hoc test was applied to test the effect of the two fixed factors F0-parents and F1-offspring, including their interaction effects. Corresponding levels of the fixed factor ‘F0-parents’ is parental control (Control), paternal *Vibrio* (PatV+) and *Tenacibaculum* (PatT+), maternal *Vibrio* (MatV+) and *Tenacibaculum* (MatT+), biparental (Bi-(MatV+PatT+)/Bi-(MatT+PatV+)) treatment groups. Levels of fixed factor ‘F1-offspring’ is *Vibrio* (F1-V+), *Tenacibaculum* (F1-T+) and control (F1-N). Significant p-values are marked in bold letters (significance code: <0.001***, 0.001**, 0.01*). Response variables measured were body size of one-week and four-month-old juveniles, for the latter also mass, hepatosomatic index (HIS), lymphocyte/monocyte ratio (L/M-ratio) of blood and head kidney (HK). Time post birth to reach sexual maturity was measured for approx. six-month-old F1-offspring.

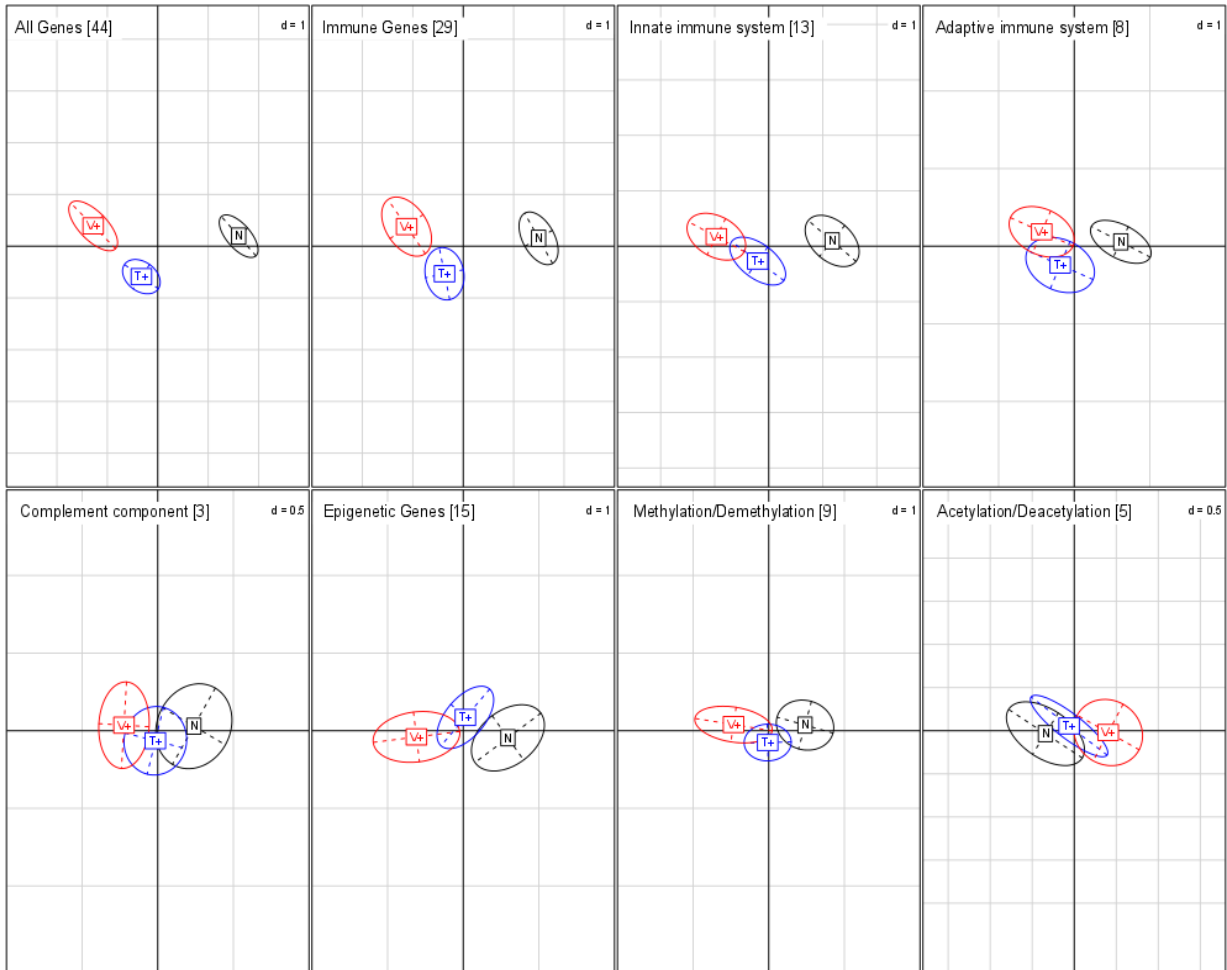
ANOVA	Factor	Df	Pr(>F)	F-value	Sig	TukeyHSD (Posthoc)
Residuals 378	One-week-old					
Size	F0-Parents	6	30.04	< 0.001	***	Control < MatT+; Control < MatV+; Control < PatV+; Control < Bi-(MatT+PatV+); Control < Bi-(MatV+PatT+); MatT+ < MatV+; MatT+ > PatT+; MatV+ > PatT+; MatV+ > PatV+; PatT+ < Bi-(MatT+PatV+); PatT+ < Bi-(MatV+PatT+); PatV+ < Bi-(MatV+PatT+); MatV+ > Bi-(MatT+PatV+); MatV+ > Bi-(MatV+PatT+)
Residuals 98	Four-month-old					
Body Size	F0-Parents	6	2.85	0.013	*	Control < MatV+
Body Mass	F0-Parents	6	4.43	0.001	***	Control < Bi-(MatV+PatT+); Control < MatV+; PatT+ < MatV+; PatT+ < Bi-(MatV+PatT+)
HIS	F0-Parents	6	2.39	0.034	*	Control < PatT+
Residuals 73	Four-month-old					
L/M-ratio blood	F0-Parents	6	5.15	<0.001	***	Control < PatV+
L/M-ratio HK	F0-Parents	6	6.42	<0.001	***	Control < MatV+; Control < Bi(MatV+PatT+); Control < PatT+; Control < PatV+; Control < Bi(MatT+PatV+); MatT+ < MatV+
L/M-ratio blood	F1-offspring	2	7.89	0.001	***	F1-N < F1V+
L/M-ratio HK	F0-Parents x F1-offspring	12	2.98	0.002	**	ControlxF1N < PatV+xF1V+; ControlxF1N < MatV+xF1V+; ControlxF1T+ < MatV+xF1V+; ControlxF1T+ < PatV+xF1V+; ControlxF1V+ < PatV+xF1V+; ControlxF1V+ < MatV+xF1V+; MatT+x F1V+ < MatV+x F1T+; PatV+xF1T+ < MatV+xF1V+; MatT+xF1V+ < PatV+xF1V+; PatV+xF1T+ < PatV+xF1V+
Residuals 168	six-month old					
Time to reach sexual maturity	F0-Parents	3		0.001	***	Control < Mat; Control < Pat; Control < Bi; Pat < Bi; Mat < Bi

Appendix Chapter II



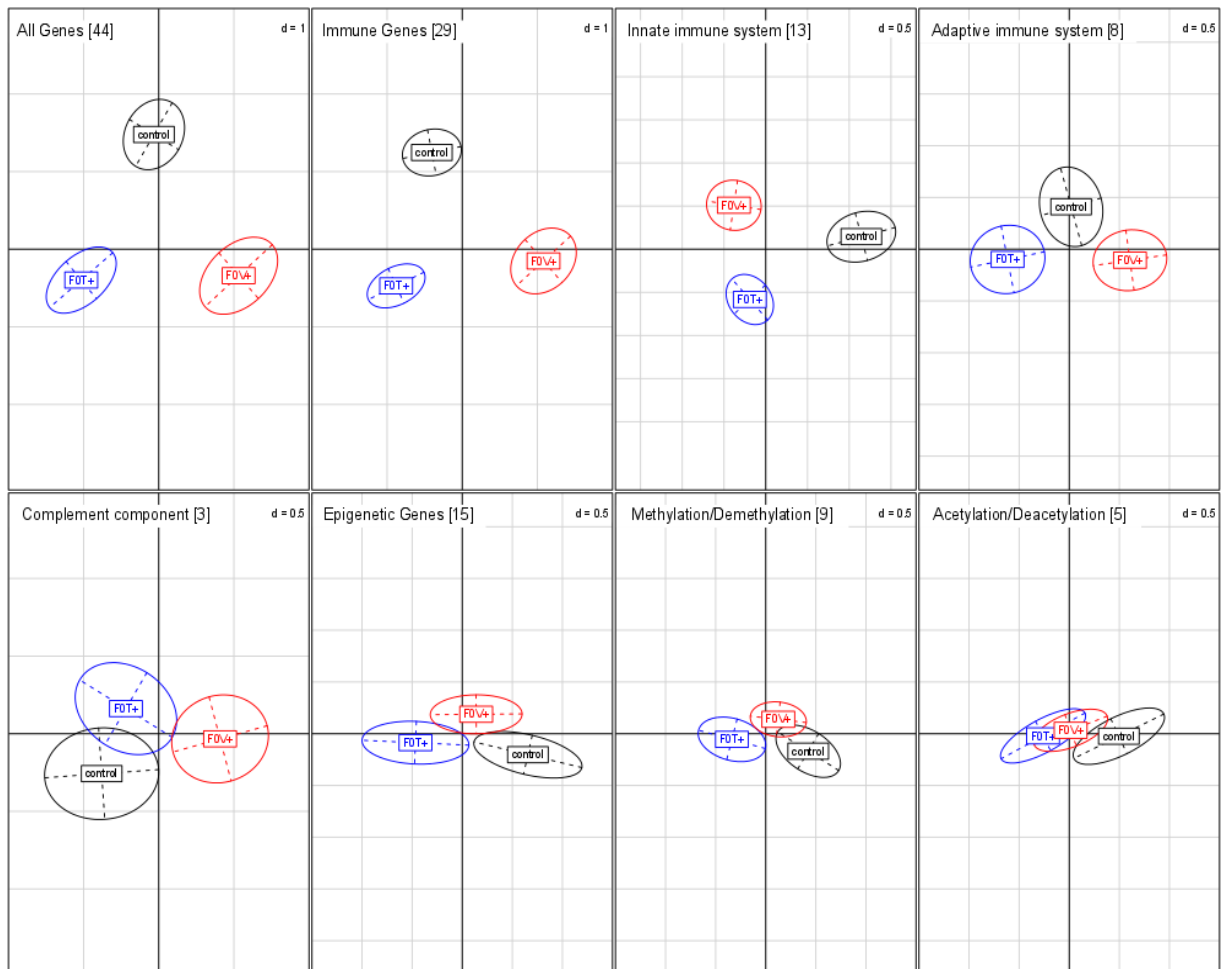
Supplement Figure 1: Between Component Analysis (BCA) to analyse acute F1-offspring treatment effect in one-week-old juveniles (N=300). BCA plot demonstrate gene expression pattern per F1-offspring treatment (F1-Vibrio (V+) in red, F1-Tenacibaculum (T+) in blue, F1-Naïve (N) in black). Analysis was conducted for following gene categories: all target genes (44), all immune genes (29), genes of the innate immune system (13), genes of the adaptive immune system (8), complement component genes (3), epigenetic genes (15), DNA and histone methylation/demethylation genes (9), histone acetylation/deacetylation genes (5). The scale of the graph is given by a grid, which size is given in the upper right corner.

Appendix Chapter II



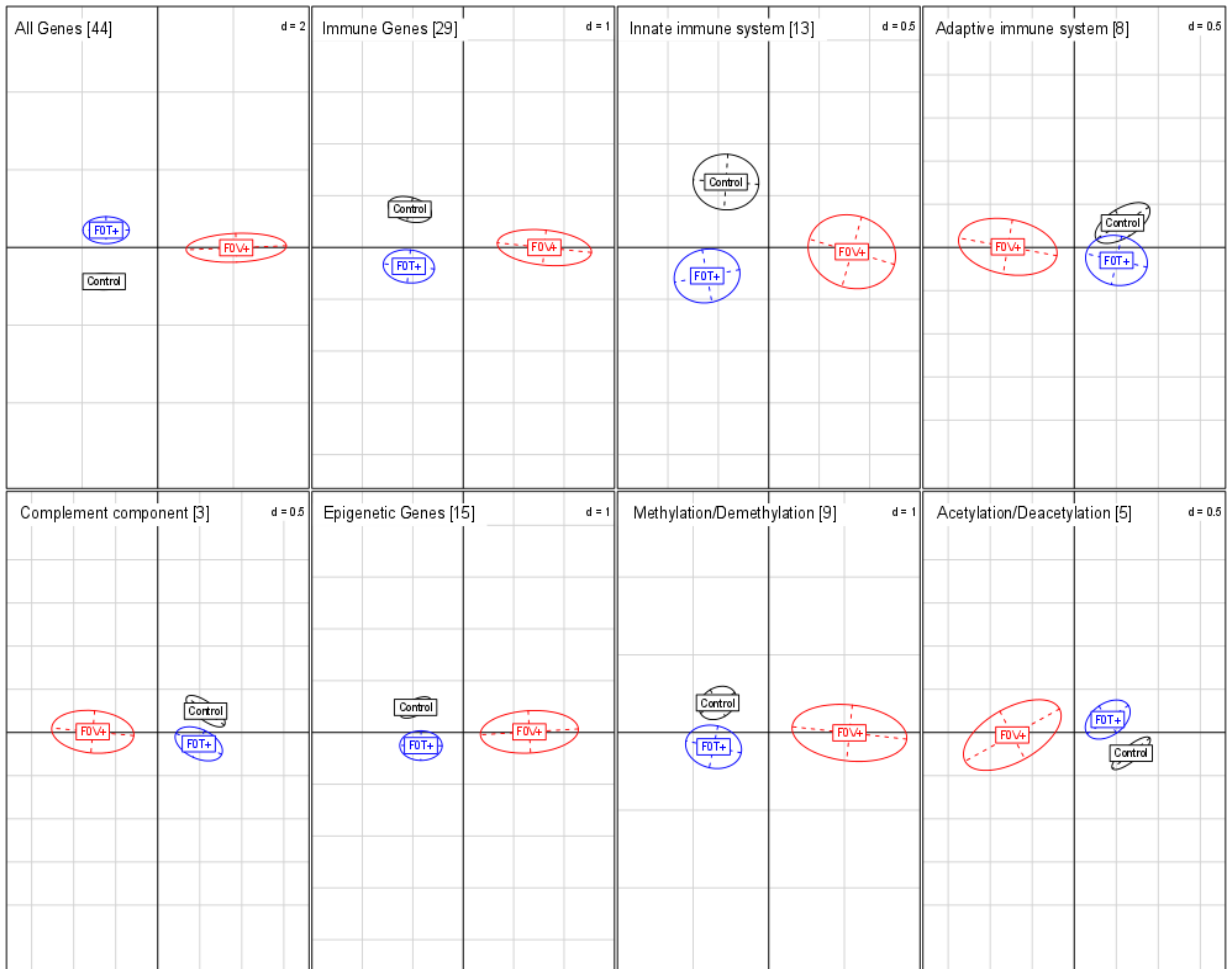
Supplement Figure 2: Between Component Analysis (BCA) to analyse acute F1-offspring treatment effect in four-month-old juveniles (N=90). BCA plot demonstrate gene expression pattern per F1-offspring treatment (F1-Vibrio (V+) in red, F1-Tenacibaculum (T+) in blue, F1-Naïve (N) in black). Analysis was conducted for following gene categories: all target genes (44), all immune genes (29), genes of the innate immune system (13), genes of the adaptive immune system (8), complement component genes (3), epigenetic genes (15), DNA and histone methylation/demethylation genes (9), histone acetylation/deacetylation genes (5). The scale of the graph is given by a grid, which size is given in the upper right corner.

Appendix Chapter II

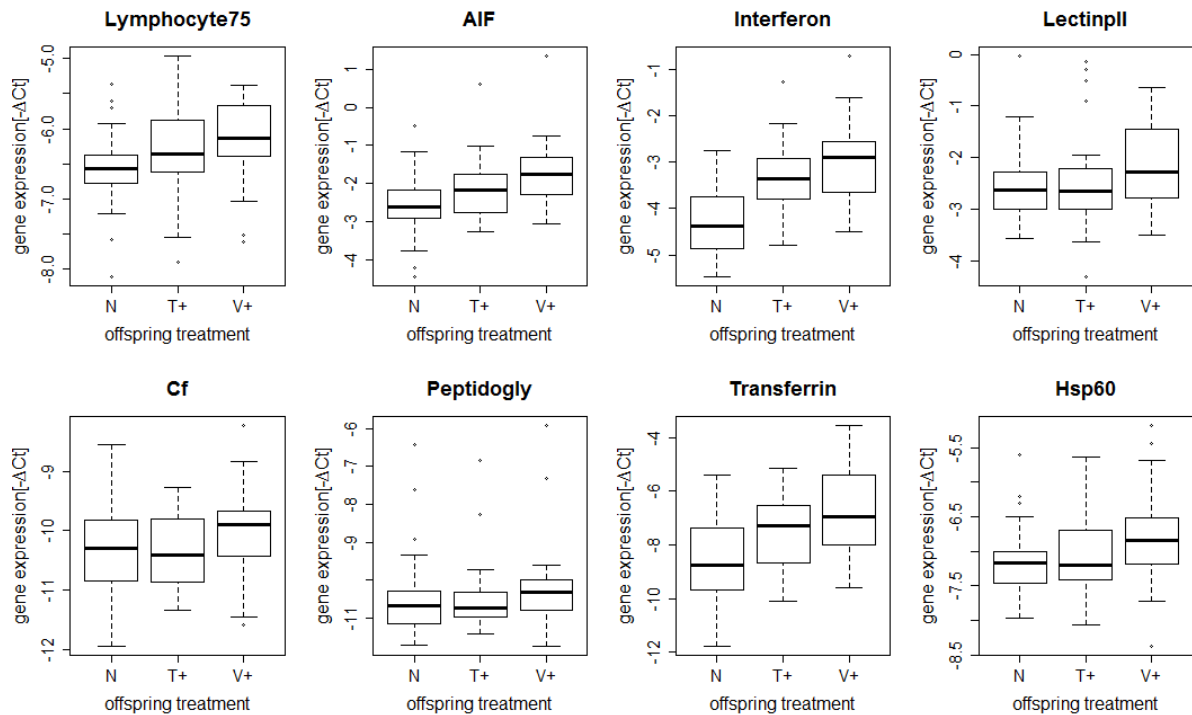


Supplement Figure 3: Between Component Analysis (BCA) to analyse F0-parental treatment effect in one-week-old juveniles (N=300). BCA plot demonstrate gene expression pattern per F0-parental bacteria treatment (F0-Vibrio (F0-V+) in red; F0-Tenacibaculum (F0-T+) in blue; F0-Naïve (F0-N) in black). Analysis was conducted for following gene categories: all target genes (44), all immune genes (29), genes of the innate immune system (13), genes of the adaptive immune system (8), complement component genes (3), epigenetic genes (15), DNA and histone methylation/demethylation genes (9), histone acetylation/deacetylation genes (5). The scale of the graph is given by a grid, which size is given in the upper right corner

Appendix Chapter II

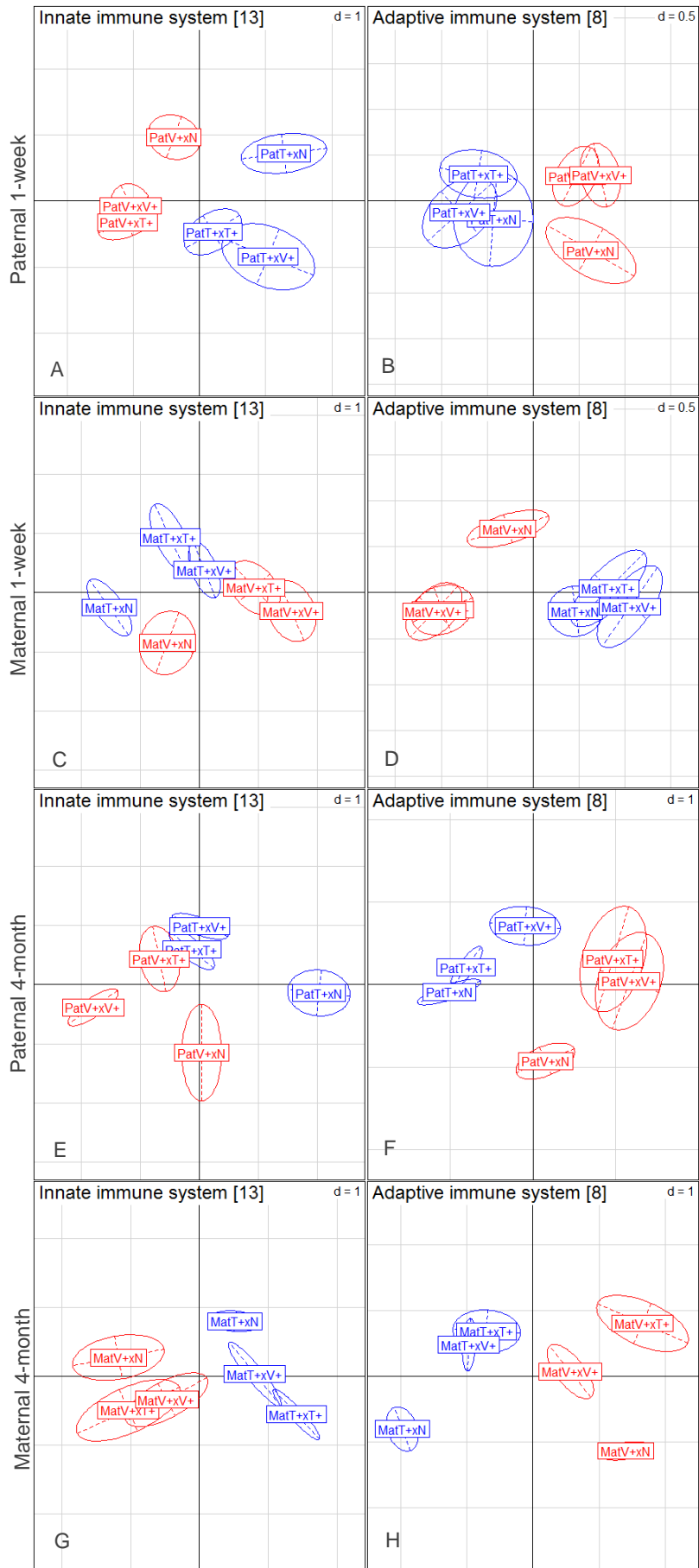


Supplement Figure 4: Between Component Analysis (BCA) to analyse F0-parental treatment effect in four-month-old juveniles (N=90). BCA plot demonstrate gene expression pattern per F0-parental bacteria treatment (F0-Vibrio (F0-V+) in red; F0-Tenacibaculum (F0-T+) in blue; F0-Naïve (F0-N) in black). Analysis was conducted for following gene categories: all target genes (44), all immune genes (29), genes of the innate immune system (13), genes of the adaptive immune system (8), complement component genes (3), epigenetic genes (15), DNA and histone methylation/demethylation genes (9), histone acetylation/deacetylation genes (5). The scale of the graph is given by a grid, which size is given in the upper right corner.



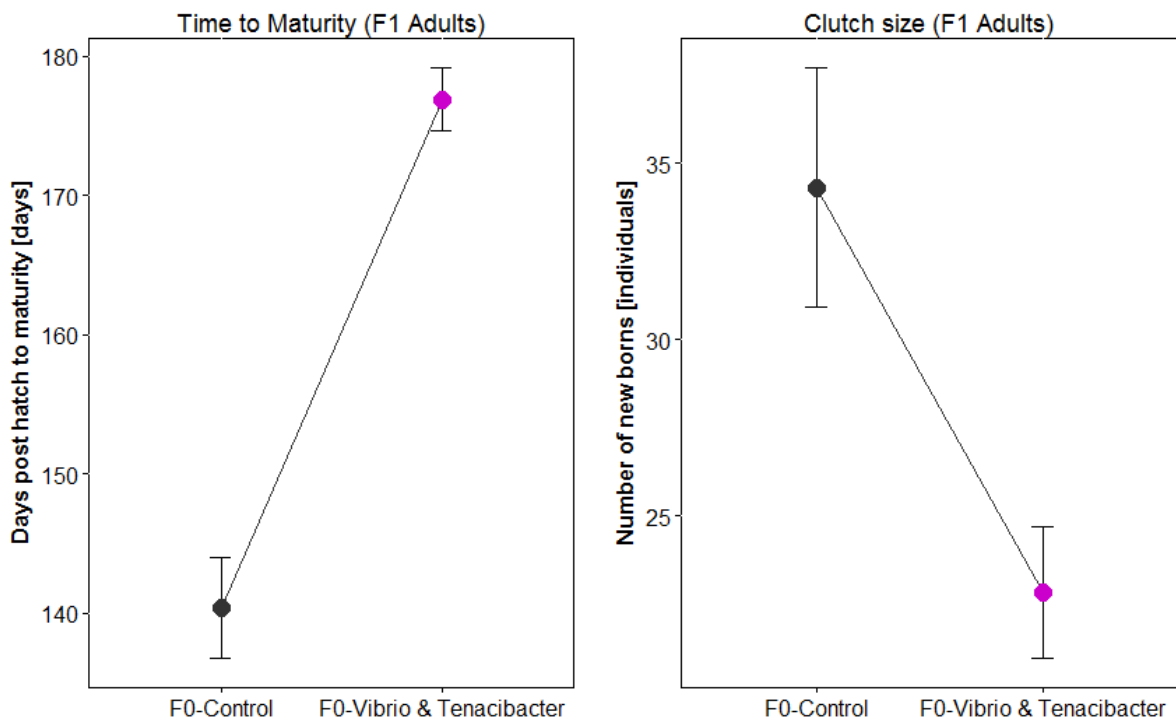
Supplement Figure 5: F1-offspring treatment effect based on immune gene expression of four-month-old juveniles (N=90). Relative gene expression ($-\Delta\text{Ct}$ -values) depicted per F1-offspring treatment for eight immune genes revealing *Vibrio* specificity (*Lymphocyte antigen 78*, *Allograft inflammation factor*, *Interferon*, *Lectin protein II*, *Coagulation factor*, *Peptidoglycan recognition protein*, *Transferrin*, and *Heatshock Protein 60*). Gene expression is pictured per F0-offspring treatment: control group without any treatment (Naïve), immune challenge with heat-killed *Vibrio* (V+) and *Tenacibaculum* (T+) bacteria.

Appendix Chapter II



Supplement Figure 6: Paternal and maternal specificity effects based on innate (13) and adaptive immune (8) gene expression in one-week (N=300) and four-month-old juveniles (N=90). Depicted groups are either paternal or maternal treatment x F1-offspring treatment (control group without any treatment (Naïve), immune challenge with heat-killed *Vibrio* (V+) and *Tenacibaculum* (T+) bacteria.

Panel A: paternal specificity effect based on innate immune genes of one-week-old F1-juveniles
Panel B: paternal specificity effect based on adaptive immune genes of one-week-old F1-juveniles
Panel C: maternal specificity effect based on innate immune genes of one-week-old F1-juveniles
Panel D: maternal specificity effect based on adaptive immune genes of one-week-old F1-juveniles
Panel E: paternal specificity effect based on innate immune genes of four-month-old F1-juveniles
Panel F: paternal specificity effect based on adaptive immune genes of four-month-old F1-juveniles
Panel G: maternal specificity effect based on innate immune genes of four-month-old F1-juveniles
Panel H: maternal specificity effect based on adaptive immune genes of four-month-old F1-juveniles.



Supplement Figure 7: Parental treatment effects on time for F1-males to reach sexual-maturity and clutch size of adult F1-generation. Plots are depicted according to F0-parental treatments (parental control (Control), and pooled parental bacteria challenged groups (Vibrio & Tenacibaculum) displaying time (days post birth) of F1-males to create a functional brood pouch tissue and reach sexual maturity. Respective error bars are representing standard error of the mean (+/-sem).

Supplement Table 1: Results from PERMANOVA and ANOSIM analysis to test for F0-parental and F1-offspring treatment effects in one-week-old F1-juveniles. Multivariate ANOSIM analysis to assess differences in the gene expression profiles per parental treatment groups applying pairwise comparison on relative gene expression data ($-\Delta\Delta Ct$ -values) based on a Bray-Curtis distance matrix and 999 permutations. Significant p-values are marked in bold letters (significance code: $<0.001^{***}$, 0.001^{**} , 0.01^* , $0.1 >p\text{-value} \geq 0.05$ trend, "ns" means not significant). Shown are significance values over two main treatments (F0-parents and F1-offspring) and their interaction followed by pairwise comparisons (ANOSIM) noted as Level in (%) values between parental control (Control), parental *Vibrio* (V+) and *Tenacibaculum* (T+), treatment groups. Investigated gene categories are genes all target genes (44), all immune genes (29), genes of the innate immune system (13), genes of the adaptive immune system (8), complement component genes (3), epigenetic genes (15), DNA and histone methylation/demethylation genes (9), histone acetylation/deacetylation genes (5).

	All genes genes $<0.001^{***}$	Immune genes genes $<0.001^{***}$	Innate genes genes $<0.001^{***}$	Innate & Adaptive genes genes $<0.001^{***}$	Adaptive genes genes $<0.001^{***}$	Compl. genes genes $<0.001^{***}$	Epigen. genes genes 0.001^{**}	De/Methyl. genes genes 0.024^*	De/Acetyl. genes genes $<0.001^{***}$
F0-Parents - PERMANOVA	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	0.001^{**}	0.027^*	0.002^{**}
Pairwise comparison (%)									
F0 V+ x F0 T+	0.1	0.1	0.1	0.1	0.1	0.1	1	1.4	1.5
F0 V+ x F0 control	0.3	0.1	0.1	0.8	0.4	0.6	49	63.7	12.2
F0 T+ x F0 control	3.3	0.3	0.1	6.2	6.0	23.8	74.3	72.1	30.5
F1-offspring - PERMANOVA	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	0.03996^*	$<0.001^{***}$	0.001^{**}	0.027^*	0.002^{**}
Pairwise comparison (%)									
F1 V+ x F1 T+	95	93.9	79.3	96.7	75	22.2	73.1	80.5	48.7
F1 V+ x F1 N	0.1	0.1	0.1	0.1	3.8	0.1	0.9	1.5	0.5
F1 T+ x F1 N	0.1	0.1	0.1	0.1	12.9	0.1	0.5	1	0.1
F0-Parents x F1-offspring	0.0519	0.0839	ns	0.083	ns	0.0492^*	0.054	ns	0.006^{**}
Pairwise comparison (%)									
F0V+/F1V+ x F0V+/F1T+	ns	ns	ns	ns	ns	86.3	ns	ns	99.9
F0V+/F1V+ x F0V+/F1N	ns	ns	ns	ns	ns	0.6	ns	ns	2.5
F0V+/F1V+ x F0T+/F1V+	ns	ns	ns	ns	ns	14.5	ns	ns	31.9
F0V+/F1V+ x F0T+/F1T+	ns	ns	ns	ns	ns	34.3	ns	ns	33.3
F0V+/F1V+ x F0T+/F1N	ns	ns	ns	ns	ns	0.1	ns	ns	13.6
F0V+/F1T+ x F0V+/F1N	ns	ns	ns	ns	ns	0.1	ns	ns	0.7
F0V+/F1T+ x F0T+/F1V+	ns	ns	ns	ns	ns	1.3	ns	ns	8.9
F0V+/F1T+ x F0T+/F1T+	ns	ns	ns	ns	ns	14	ns	ns	19.9
F0V+/F1T+ x F0T+/F1N	ns	ns	ns	ns	ns	0.1	ns	ns	4.8
F0V+/F1N x F0T+/F1V+	ns	ns	ns	ns	ns	18.4	ns	ns	7.9
F0V+/F1N x F0T+/F1T+	ns	ns	ns	ns	ns	11.8	ns	ns	3.4
F0V+/F1N x F0T+/F1N	ns	ns	ns	ns	ns	0.1	ns	ns	42.1
F0T+/F1V+ x F0T+/F1T+	ns	ns	ns	ns	ns	12.3	ns	ns	24.6
F0T+/F1V+ x F0T+/F1N	ns	ns	ns	ns	ns	0.1	ns	ns	24.3
F0T+/F1T+ x F0T+/F1N	ns	ns	ns	ns	ns	0.1	ns	ns	13.2

Supplement Table 2: Results from PERMANOVA and ANOSIM analysis to test for F0-parental and F1-offspring treatment effects in four-month-old F1-juveniles. Multivariate ANOSIM analysis to assess differences in gene expression, immune cell and life-history measurements per parental treatment groups applying pairwise comparison based on a Bray-Curtis distance matrix and 999 permutations. Significant p-values are marked in bold letters (significance code: $<0.001^{***}$, 0.001^{**} , 0.01^* , $0.1 > p\text{-value} \geq 0.05$ trend, "ns" not significant). Shown are significance values over two main treatments (F0-parents and F1-offspring) and their interaction followed by pairwise comparisons (ANOSIM) depicted as Level in (%) between parental control (Control), parental *Vibrio* (V+), and *Tenacibaculum* (T+) treatment groups. Investigated gene categories are all target genes (44), all immune genes (29), genes of the innate immune system (13), genes of the adaptive immune system (8), complement component genes (3), epigenetic genes (15), DNA and histone methylation/demethylation genes (9), histone acetylation/deacetylation genes (5), lymphocyte and monocyte counts of blood and head kidney (each 3). Life history traits: body size, mass, condition factor, and hepatosomatic index (4).

	All genes	Immune genes	Innate genes	Innate & Adaptive genes	Adaptive genes	Comp. genes	Epig. genes	DeMethyl. genes	De/Acety. genes	Immune cells	Cells-hk	Cells-blood	Life-history
F0-parents - PERMANOVA	$<0.001^{***}$	0.001^{**}	$<0.001^{***}$	0.004^{**}	0.011^*	0.001^{**}	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$	$<0.001^{***}$
<i>Pairwise comparison (%)</i>													
F0 V+ x F0 T+	0.01	0.04	0.30	2.30	0.90	0.05	0.01	0.02	0.03	0.01	0.01	0.30	23.90
F0 V+ x F0 control	0.01	0.01	0.05	0.04	0.01	0.01	0.01	0.01	0.01	0.01	0.05	0.01	0.5
F0 T+ x F0 control	22.5	25.6	26.7	25.3	18.5	3.3	8.7	19.2	6.7	0.01	0.01	0.01	0.2
F1-offspring - PERMANOVA	0.005^{**}	$<0.001^{***}$	$<0.001^{***}$	0.002^{**}	ns	ns	ns	ns	ns	0.001^{**}	$<0.001^{***}$	0.001^{**}	ns
<i>Pairwise comparison (%)</i>													
F1 V+ x F1 T+	24.3	23.9	14.7	84.2	ns	ns	ns	ns	ns	12.20	82.0	57.9	ns
F1 V+ x F1 N	0.01	0.01	0.01	0.30	ns	ns	ns	ns	ns	0.30	0.01	1.30	ns
F1 T+ x F1 N	0.10	0.02	0.04	0.30	ns	ns	ns	ns	ns	0.04	0.01	1.80	ns
F0-parents x F1-offspring	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Supplement Table 3: Results from univariate statistical analysis of life-history parameters and immune cell count measurements in one-week-old, four-month-old and six-month-old F1-juveniles. Univariate testing using ANOVAs for each response variable and TukeyHSD post-hoc test was applied to test the effect of the two fixed factors F0-parents and F1-offspring, including their interaction effects. Corresponding levels of the fixed factor `F0-parents` is parental control (Control), parental *Vibrio* (V+) and *Tenacibaculum* (T+) treatment groups. Levels of fixed factor `F1-offspring` is *Vibrio* (F1-V+), *Tenacibaculum* (F1-T+) and control (F1-N). Significant p-values are marked in bold letters (significance code: <0.001***, 0.001**, 0.01*, 0.1>p-value≥0.05 trend ●). Response variables measured were body size of one-week and four-month-old juveniles, for the latter also body mass, hepatosomatic index (HIS), lymphocyte/monocyte ratio (L/M-ratio) of blood and head kidney (HK).

	F0-parents DF = 2		F1-offspring DF = 2		F0-parents x F1-offspring DF = 4	
	ANOVA Pr(F)	Tukey post hoc	ANOVA Pr(F)	Tukey HSD	ANOVA Pr(F)	Tukey HSD
<i>Residuals 55</i>						
Lymphocyte counts-blood	<0.001 ***	C<V+ C<T+ ns	0.1143 ns	ns	0.814 .	ns
Monocyte counts-blood	<0.001 ***	C>V+ C>T+ V+<T+ ns	0.002 **	N<V+ N<T+	0.035 ns	ns
Lymphocyte/Monocyte-ratio blood	<0.001 ***	C<V+ C<T+ V+>T+ ns	<0.001 ***	N>V+ N>T+	0.302 ns	ns
Lymphocyte counts-head kidney	0.1252	ns ns ns	0.002 **	ns N>T+	0.701 ns	ns
Monocyte counts head kidney	<0.001 ***	C>V+ C>T+ ns	<0.001 ***	N>V+ N>T+	0.569 ns	ns
Lymphocyte/Monocyte-ratio head kidney	<0.001 ***	C<V+ C<T+ ns	0.219 ns	ns	0.074 .	F0V+:V+ > F0V+:T+
<i>Residuals 273</i>						
Size one-week old	<0.001 ***	C<V+ C<T+ T+<V+				
<i>Residuals 74</i>						
Size 4month old	0.012 *	C<V+ ns ns				
Weight four-month-old	0.003 **	C<V+ ns T+<V+				
CF1 four-month-old	0.083 ns	ns ns ns				
CF2 four-month-old	0.067 ns	ns ns ns				
HIS four-month-old	0.003 **	(C<V+). C<T+ ns				

Supplement Table 4: Results from PERMANOVA analysis to test for F0-paternal and F0-maternal specificity effects in one-week-old and four month old F1-juveniles. Multivariate analysis to assess differences in immune gene expression per parental (paternal and maternal) treatment groups on relative immune gene expression data ($-\Delta\text{Ct}$ -values) based on a Bray-Curtis distance matrix and 999 permutations. Significant p-values are marked in bold letters (significance code: $<0.001^{***}$, 0.001^{**} , 0.01^* , $0.1 > p\text{-value} \geq 0.05$ trend \bullet), whereas “ns” indicates no statistical difference. Shown are significance values over two main treatments (F0-parents and F1-offspring) and their interaction. Investigated gene categories are genes of the innate immune system (13), genes of the adaptive immune system (8), complement component genes (3).

Age	Response Variables	Total Df	Residuals Df	R ²	F1-parents		F0-offspring		F1-parents x F0-offspring		F1-parents : Nested Factor		
					Df	F.Model	Pr(>F)	Df	F.Model	Pr(>F)	Df	F.Model	Pr(>F)
1 W E E K	Innate	119	108	0.456	1	25.714	<0.001	2	5.036	<0.001	6	15.172	<0.001
	Adaptive	119	108	0.458	1	7.409	<0.001	2	1.683	0.116	6	19.214	<0.001
	Complement	119	108	0.489	1	17.704	<0.001	2	8.935	<0.001	6	11.893	<0.001
	Innate	119	108	0.479	1	11.85	<0.001	2	6.431	<0.001	6	14.994	<0.001
	Adaptive	119	108	0.489	1	23.45	<0.001	2	1.797	0.057	6	13.81	<0.001
	Complement	119	108	0.467	1	7.56	<0.001	2	21.327	<0.001	6	11.47	<0.001
4 M O N T H	Innate	30	23	0.647	1	2.608	0.022	2	1.967	0.039	2	2.028	0.042
	Adaptive	30	23	0.682	1	2.626	0.041	2	0.916	0.512	2	2.736	0.013
	Complement	30	23	0.677	1	5.064	0.019	2	0.259	0.853	2	2.014	0.121
	Innate	30	23	0.639	1	2.282	0.092	2	0.597	0.729	2	4.002	0.002
	Adaptive	30	23	0.583	1	8.94	<0.001	2	0.687	0.643	2	0.721	0.621
	Complement	30	23	0.633	1	7.764	0.002	2	0.6	0.626	2	2.022	0.12

Appendix Chapter II

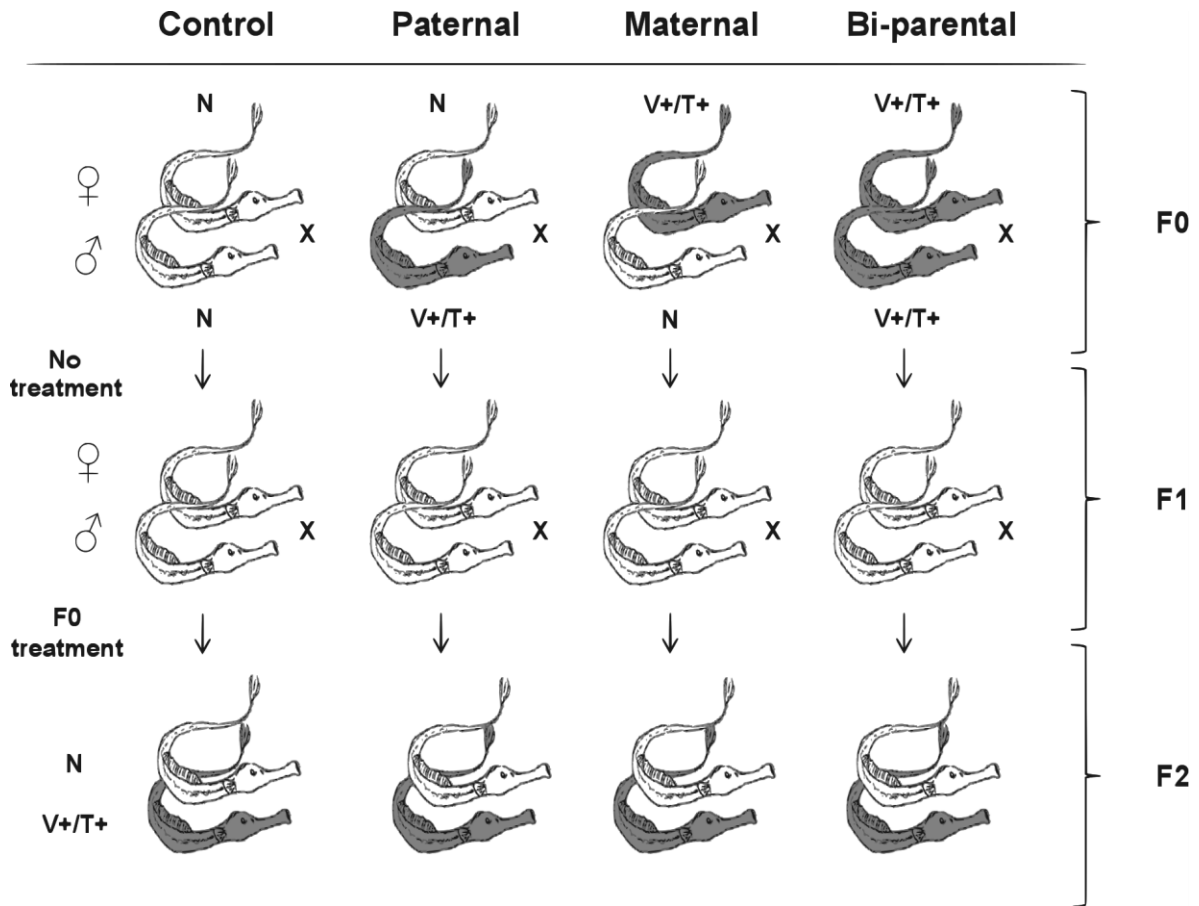
Supplement Table 5: Linear Mixed effect model to test for F0-parental effects in time of maturation of adult pipefish males and clutch size of six-month old F1-offspring.

<i>Six-month-old F1-offspring</i>		numDF	denDF	F-value	p-value
	Time to reach Sexual maturity	(Intercept)	1	126	29648.78
F0-parents		1	7	325.0	<.0001
<i>Six-month-old F1-offspring</i>		numDF	denDF	F-value	p-value
	Clutch size	(Intercept)	1	15	204.80
F0-parents		1	7	7.95	0.0257

Supplement Table 6: Correlation analysis between immune genes and monocyte as well as lymphocyte counts in head kidney and blood from four-month-old juveniles. By using a Pearson correlation matrix each single gene ($-\Delta\text{Ct}$ -values) was correlated with each immune cell count.

Monocytes head kidney	Monocytes blood	Lymphocytes head kidney and blood
Lectin protein II $R^2=0.26^{**}, 0.0138$	Lectin protein I $R^2=0.28^{**}, 0.038$	Complement component 1 $R^2=-0.25^*, 0.016; -0.28^{**}, 0.007$
Complement component 3 $R^2=0.35^{***}, <0.001$	Complement component 3 $R^2=0.23^{***}, 0.0109$	HIVEP3 $R^2=-0.23^*, 0.031$
Interferon $R^2=0.25^{**}, 0.019$	Complement component 1 $R^2=0.34^{***}, <0.001$	
Peptidoglycan $R^2=0.30^{**}, 0.004$	Ik-cytokine $R^2=0.23^*, 0.029$	
Tyrosine kinase $R^2=0.23^*, 0.032$	Lymphantigen 75 $R^2=-0.22^*, 0.038$	
Lectin protein I $R^2=0.20(.trend), 0.057$	Lectin protein II $R^2=0.18(.trend), 0.0928$	
Transferrin $R^2=0.18(.trend), 0.096$	Interleukin 10 $R^2=0.21(.trend), 0.051$	
	HIVEP3 $R^2=0.19(.trend), 0.074$	
	Peptidoglycan $R^2=0.19(.trend), 0.070$	
	Integrin $R^2=0.18(.trend), 0.096$	

Appendix Chapter III

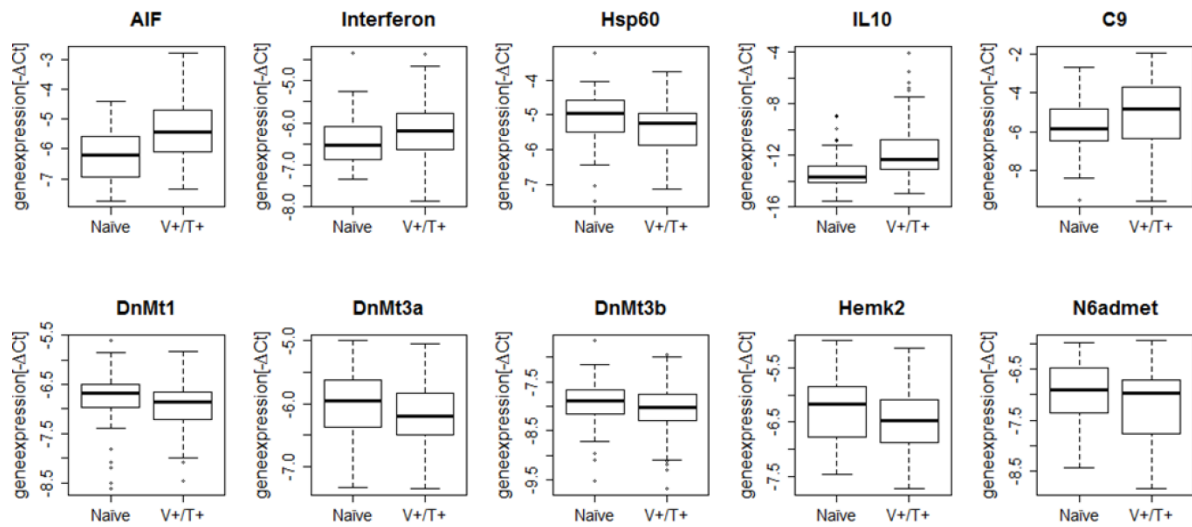


Supplement 1: Experimental design. The parental generation (F0) was vaccinated using a combination of heat-killed immunological novel *Vibrio spp* (V+) and *Tenacibaculum maritimum* (T+) bacteria, or were left naïve (N) as control. Immune-challenged mature pipefish were used in following mating design: 1. Control: [♀N x ♂N]; 2. Paternal: [♀N x ♂V+/T+]; 3. Maternal: [♀V+/T+ x ♂N] and 4. Biparental: [♀V+/T+ x ♂V+/T+] and kept according to their mating pairs (families) in separate 36x80L semi-flow through aquaria (16 family replicates per parental bacteria treatment and 8 per control group; 56 families). F1 individuals were crossed within former parental treatment groups but left immunologically naïve (out of each of the 4 parental treatment groups 5 families were chosen to do F1-crosses resulting in 20 F1 families). In spring 2014, F2-juveniles were exposed one-week post birth to the same heat-killed *Vibrio* (V+) and *Tenacibaculum* (T+) bacteria used for the F0-generation or left naïve (per F1-crossing 4 families produced F2 offspring resulting in 16 F1-families. Out of each family 12 individuals were chosen for the direct immune challenge. Per F2-offspring treatment (V+/ T+/ naïve) 4 individual replicates were used; resulting in a total of 192 samples).

Supplement 2: Names and function of 44 target genes and 4 housekeeping genes assessed for gene expression measurements.

Gene	Gene name	Function	Category	Reference
CD45	CD45 (leukocyte common antigen)	Regulation of T-cell and B-cell antigen receptor signaling	Adaptive	NCBI, UniProt
HIVEP2	Human immunodeficiency virus type1 enhancer2	Transcription factor, V(D)J recombination, MHC enhancer binding	Adaptive	(Diepeveen et al., 2013)
HIVEP3	Human immunodeficiency virus type1 enhancer3	Transcription factor, V(D)J recombination, MHC enhancer binding	Adaptive	(Diepeveen et al., 2013)
IgM-1c	Immunoglobulin light chain	Recognition of antigen or pathogen	Adaptive	NCBI, UniProt
Integ-Bt	Integrin-beta 1	Cell signaling and adhesion of immunoglobulin	Adaptive	NCBI, UniProt
Lymph75	Lymphocyte antigen 75	Directs captured antigens to lymphocytes	Adaptive	(Birrer et al., 2012)
Lymphcyt	Lymphocyte cytosolic protein 2	Positive role in promoting T-cell development and activation	Adaptive	NCBI, UniProt
TAP	Tap-binding protein (Tapasin)	Transport of antigenic peptides, peptide loading on MHC I	Adaptive	NCBI, UniProt
AIF	Allograft inflammation factor	Inflammatory responses, allograft rejection, activation of macrophages	Innate	(Roth et al., 2012)
Calreculin	Calreculin	Chaperone, promotes phagocytosis and clearance of apoptotic cells	Innate	NCBI, UniProt
Cf	Coagulation factor II	Blood clotting and inflammation response	Innate	(Birrer et al., 2012)
IL8	Interleukin 8	Neutrophil chemotactic factor, phagocytosis, inflammatory activity	Innate	NCBI, UniProt
Intf	Interferon induced transmembrane protein 3	Negative regulation of viral entry into host cell, antiviral response	Innate	NCBI, UniProt
Kin	Kinesin	Intracellular transport	Innate	(Roth et al., 2012)
LectplI	Lectin protein type I	Pathogen recognition receptors (C-type lectin type I)	Innate	NCBI, UniProt
LectplII	Lectin protein type II	Pathogen recognition receptors (C-type lectin type II)	Innate	NCBI, UniProt
Nlrnp	Natural resistance-assoc macrophage protein	Macrophage activation	Innate	(Roth et al., 2012)
Pepdgly	Peptidoglycan recognition protein II	Recognition of peptidoglycan on bacteria (gram+) cell wall	Innate	NCBI, UniProt
Tranferrin	Transferrin	Iron withholding (prevents bacterial outgrowth)	Innate	NCBI, UniProt
Tspo	Translocator protein	Inflammatory response	Innate	NCBI, UniProt
Hsp60	Heat shock protein 60	Chaperone, general stress response	Innate/stress	(Roth et al., 2012)
Ck7	Chemokine 7	Chemotaxis for leukocytes, monocytes, neutrophils, blood cells	Innate & Adaptive	NCBI, UniProt
Ik-cytokine	Ik cytokine(RED-protein)	Inhibits interferon gamma mediated downregulation of MHCII	Innate & Adaptive	NCBI, UniProt
IL10	Interleukin 10	Regulation of macrophage activity, suppresses lymphocyte response	Innate & Adaptive	(Birrer et al., 2012)
LPS-TNF	LPS-induced TNF- α factor (LITAF)	Mediating cytokine (TNF- α) expression in LPS-induced response	Innate & Adaptive	NCBI, UniProt
Tyroprot	Tyrosinekinase	Cytokine receptor signaling pathway, T and B cell activation	Innate & Adaptive	NCBI, UniProt
C1q-sco	Recognition sub-component (C1q)	C1q complex, helps in forming antigen-antibody complex	Complement	NCBI, Wikipedia
C3	Complement component 3	Opsonization of bacteria, activation of complement system	Complement	(Birrer et al., 2012)
C9	Complement component 9	Membrane attack complex (MAC), lysis of bacteria	Complement	(Roth et al., 2012)
TAF8	Transcription-initiation-factor 8	Transcription factor positive regulation of transcription	Gene activation	NCBI, UniProt
ASH2	Histone methyltransferase (ASH2)	Histone methyltransferase (SET1-ASH2 complex)	Gene activation	NCBI, UniProt
TPR	Lysine-specific demethylase 6A	Histone demethylation (H3K27me3)	Gene activation	NCBI, UniProt
BROMO	Histone-acetyltransferase (KAT2A)	Histone acetylation (KAT2A-Bromodomain)	Gene activation	NCBI, UniProt
MYST1	Histone-acetyltransferase (HAT1)	Histone acetylation (MYST1-domain)	Gene activation	NCBI, UniProt
HDAC1	Histone deacetylase 1-like	Histone deacetylation (hydrolysis of N(6)-acetyl-lysine residues)	Gene silencing	NCBI, UniProt
HDAC3	Histone deacetylase 3-like	Histone deacetylation (hydrolysis of N(6)-acetyl-lysine residues)	Gene silencing	NCBI, UniProt
HDAC6	Histone deacetylase 6-like	Histone deacetylation (deacetylation lysine residues of core histones)	Gene silencing	NCBI, UniProt
JmJC-PHD	Lysine-specific-demethylase 5B	Histone demethylation (demethylates Lys-4(H3K4me))	Gene silencing	NCBI, UniProt
NO66	Lysine-specific-histone demethylase NO66	Histone demethylation (demethylates Lys-4(H3K4me),(H3K36me))	Gene silencing	NCBI, UniProt
DnM1	DNA-Methyltransferase 1	DNA methyltransferase (C5-methylcytosine)-maintenance methylation	Gene silencing	NCBI, UniProt
DnM13a	DNA-Methyltransferase 3a	DNA methyltransferase (C5-methylcytosine)-de novo methylation	Gene silencing	NCBI, UniProt
DnM13b	DNA-Methyltransferase 3b	DNA methyltransferase (C5-methylcytosine)-de novo methylation	Gene silencing	NCBI, UniProt
HemK2	HemK-methyltransferase family member 2	DNA methyltransferase (N6-methyladenine)	Gene silencing	NCBI, UniProt
N6admet	N(6)-adenine-specific DNA methyltransferase 2	DNA methyltransferase (N6-methyladenine)	unknown	NCBI, UniProt
DDP-gly	DDP-glycosyltransferase	Metabolisation process (natural glycosidic linkages)	Housekeeping	NCBI, UniProt
G6PD	Glucose 6-phosphate dehydrogenase (G6PD)	Metabolisation process (pentose phosphate pathway)	Housekeeping	NCBI, UniProt
Ribop	Ribosomal protein	Translation process	Housekeeping	NCBI, UniProt
Ubi	Ubiquitin	Labels regulatory proteins for degradation	Housekeeping	(Birrer et al., 2012)

Appendix Chapter III



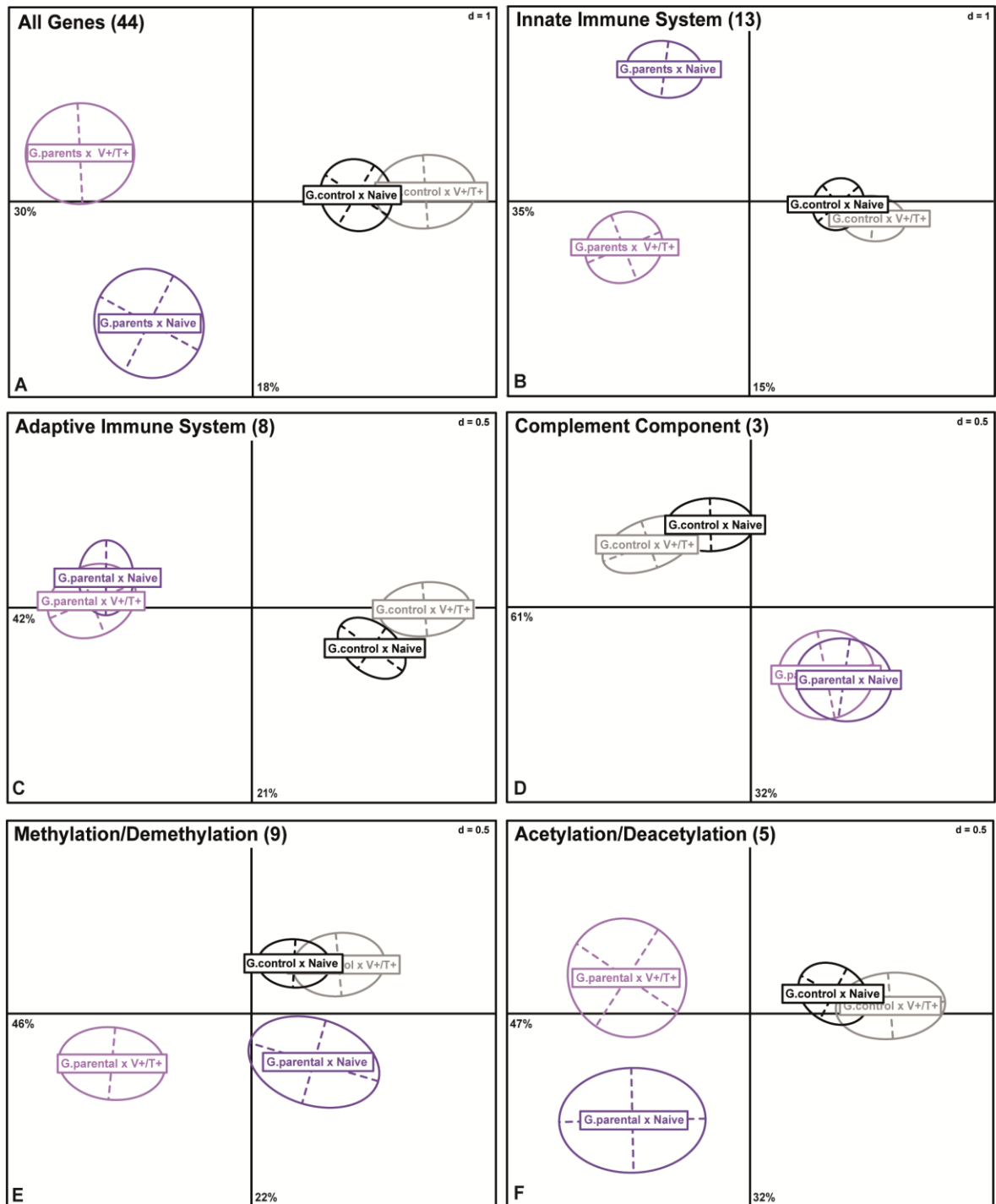
Supplement 3: Grand-offspring effect. Relative gene expression data ($-\Delta\text{Ct}$ -values) for 5 immune genes and 5 methylation genes with F2-grand-offspring effect. Juvenile grand-offspring treatment 20 hrs after immune challenge (N= 192). Gene expression is pictured per grand-offspring treatment: control group without any treatment (Naive), immune challenge with heat-killed *Vibrio* (V+) and *Tenacibaculum* (T+) strains.

Appendix Chapter III

Supplement 4: SIMPER results of all target genes. Contribution (%) results per gene gives information about how much variation each gene is contributing to the differences within grandparental control, grand-paternal, grand-maternal and grand-biparental treatment groups.

Grand-control		Grand-paternal		Grand-maternal		Grand-bi-parental	
Gene	(%)	Gene	(%)	Gene	(%)	Gene	(%)
MYST1	0.82	CD45	1.18	Lectptl	0.48	Ck7	0.87
Lectptll	0.87	Cf	1.52	IgM-Ic	1.21	Pepdgly	1.4
Calrcul	1.14	HIVEP2	1.52	Nramp	1.32	Lectptl	1.43
N6admet	1.16	AIF	1.54	Lectptll	1.36	MYST1	1.48
No66	1.23	MYST1	1.57	Kin	1.38	IgM-Ic	1.49
TSPO	1.5	Tyroprot	1.59	TAP	1.48	IL10	1.5
Hsp60	1.57	C3	1.61	IL10	1.49	Tranfe	1.51
Ik-cytokine	1.59	DnMt3b	1.66	HDAC3	1.56	No66	1.52
C1Q-SCO	1.64	IL8	1.69	Intf	1.63	Lymphcyt	1.7
HDAC1	1.67	Nramp	1.73	HDAC6	1.68	Intf	1.74
LPS-TNF	1.69	HIVEP3	1.74	IL8	1.76	C9	1.75
DnMt1	1.72	TSPO	1.79	ASH	1.77	TAP	1.76
Kin	1.8	TAP	1.8	Cf	1.79	IL8	1.81
BROMO	1.83	C1Q-SCO	1.9	Ik-cytokine	1.8	LPS-TNF	1.93
Lymphcyt	1.83	TAF8	2.02	HemK2	1.85	N6admet	1.95
Cf	1.86	Lymphcyt	2.04	Tranfe	1.9	Lymph75	1.97
HDAC3	1.89	Ck7	2.07	DnMt3a	1.98	Calrcul	2.07
DnMt3a	1.94	LPS-TNF	2.09	Ck7	1.99	HDAC1	2.2
Integ-Bt	1.95	Calrcul	2.09	Tyroprot	2	Integ-Bt	2.22
DnMt3b	2.02	C9	2.1	TPR	2.01	CD45	2.26
Nramp	2.07	Kin	2.15	BROMO	2.04	HIVEP3	2.28
HIVEP2	2.07	BROMO	2.2	LPS-TNF	2.27	TAF8	2.35
CD45	2.08	HDAC3	2.27	HIVEP2	2.3	TPR	2.38
HemK2	2.12	Integ-Bt	2.28	TAF8	2.41	ASH	2.41
IgM-Ic	2.2	Lectptll	2.28	JmjC-PHD	2.42	Cf	2.41
JmjC-PHD	2.32	HDAC1	2.34	AIF	2.43	JmjC-PHD	2.44
HIVEP3	2.32	Tranfe	2.47	DnMt3b	2.47	Nramp	2.49
C3	2.33	Lymph75	2.48	TSPO	2.48	TSPO	2.5
Lymph75	2.36	Pepdgly	2.5	DnMt1	2.51	HDAC3	2.51
HDAC6	2.36	HDAC6	2.51	Lymphcyt	2.52	HIVEP2	2.54
ASH	2.4	DnMt3a	2.56	Hsp60	2.55	AIF	2.59
TAF8	2.42	DnMt1	2.58	Lymph75	2.57	BROMO	2.63
Tyroprot	2.59	Hsp60	2.59	HIVEP3	2.71	HemK2	2.66
C9	2.76	Intf	2.67	N6admet	2.73	DnMt3b	2.81
IL8	2.8	ASH	2.73	C1Q-SCO	2.74	Tyroprot	2.82
TPR	2.82	No66	2.76	C3	2.81	Hsp60	2.86
AIF	2.89	N6admet	2.84	Integ-Bt	2.86	C3	2.87
Intf	2.91	TPR	2.84	CD45	2.91	C1Q-SCO	2.87
IL10	3.14	Ik-cytokine	2.85	C9	2.94	DnMt1	2.88
Pepdgly	3.57	HemK2	2.96	Pepdgly	2.98	Lectptll	3
Tranfe	3.62	JmjC-PHD	2.99	No66	3.48	Ik-cytokine	3.15
TAP	3.81	Lectptl	3.1	HDAC1	3.54	HDAC6	3.24
Ck7	4.88	IgM-Ic	3.76	MYST1	4.39	DnMt3a	3.29
Lectptl	5.42	IL10	4.01	Calrcul	4.51	Kin	3.46

Appendix Chapter III

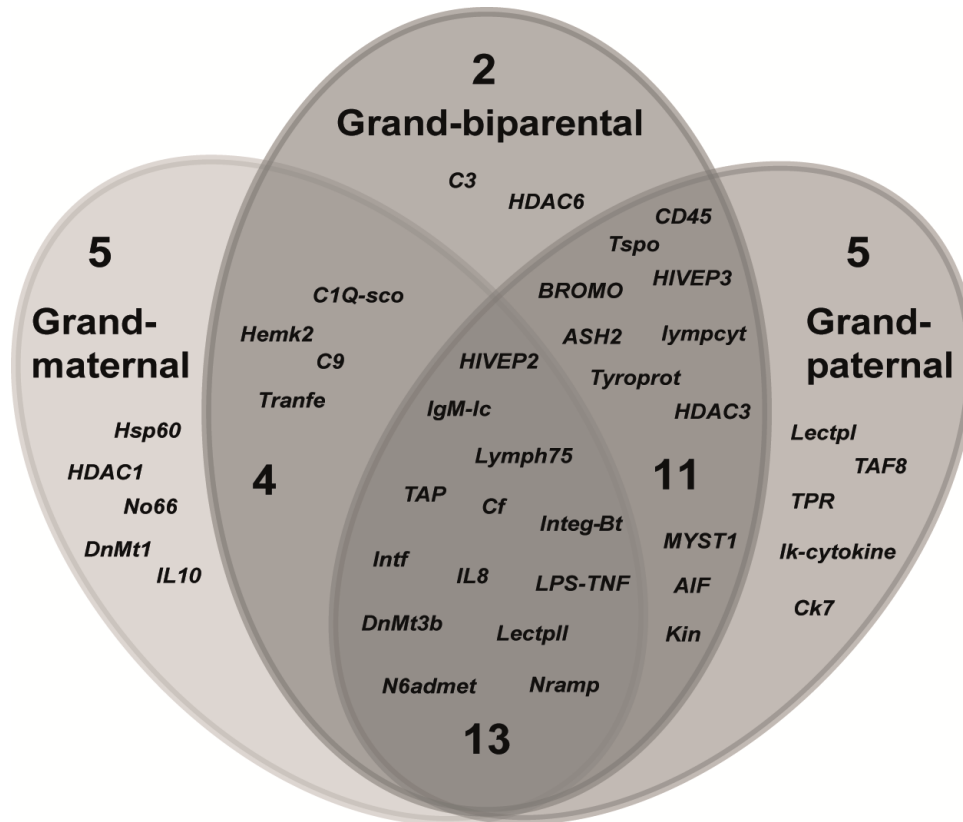


Supplement 5: Principle Component Analysis (PCA) of F0-grandparental and F2-grand-offspring interaction effect for all categories. Interaction effect of F0-grandparental and F2-grand-offspring treatment is depicted via PCA. Grandparental (G.parental; G.control) x F2-offspring (V+/T+; Naive) interaction effect based on fourth root transformed ($-\Delta Ct$ -values) and on an Euclidean distance matrix. PCA-analysis was conducted for S5-A: all target genes (44) and divided in following functional gene categories: S5-B: genes of the innate immune system (13), S5-C: genes of the adaptive immune system (8), S5-D: complement component genes (3), S5-E: methylation/demethylation genes (9), and S5-F: acetylation /deacetylation genes (5). Depicted groups are grandparental control (G.cont) x F2-offspring naïve (Naive), grandparental control (G.cont) x F2-offspring treatment (V+/T+), grandparental treatment (G.parental) x F2-offspring naïve (Naive), grandparental treatment (G.parental) x F2-offspring treatment (V+/T+). Percentage values of variation explained by principle component 1 and principle component 2 is pictured underneath the corresponding axis.

Appendix Chapter III

Supplement 6: Univariate analysis for each target gene and size as life-history trait. FDR corrected (“adjusted”) p-values Benjamini and Hochberg (1995) for the factors F0-grandparents and F2-grand-offspring, the interaction terms F0 x F2 and F0 x family, and size as covariate based on gene expression data. Size was additionally analysed as phenotypic response variable. Significant p-values are marked in bold letters.

Genes/ Size Residuals DF=162	F0 Grandparents FDR-adjust DF=3	F2 Offspring FDR-adjust DF=2	F0 x F2 FDR-adjust DF=6	F0 x Family FDR-adjust DF=12	Size FDR-adjust DF=1
<i>CD45</i>	<0.001	0.822	0.303	<0.001	0.486
<i>HIVEP2</i>	<0.001	0.646	0.303	<0.001	0.894
<i>HIVEP3</i>	0.008	0.598	0.366	0.082	0.886
<i>IgM-Ic</i>	<0.001	0.573	0.640	<0.001	0.316
<i>Integ-Bt</i>	0.000	0.031	0.198	<0.001	0.316
<i>Lymph75</i>	0.047	0.305	0.303	0.022	0.952
<i>Lympcyt</i>	<0.001	0.596	0.198	<0.001	0.952
<i>TAP</i>	<0.001	0.168	0.303	<0.001	0.922
<i>AIF</i>	0.001	<0.001	0.205	<0.001	0.952
<i>Calrcul</i>	0.058	0.286	0.198	<0.001	0.340
<i>Cf</i>	<0.001	0.822	0.198	<0.001	0.730
<i>IL8</i>	<0.001	0.315	0.418	0.001	0.260
<i>Intf</i>	<0.001	0.035	0.198	0.001	0.952
<i>Kin</i>	<0.001	0.822	0.483	<0.001	0.952
<i>LectptI</i>	0.019	1.000	0.188	<0.001	0.730
<i>LectpII</i>	<0.001	0.646	0.939	<0.001	0.316
<i>Nramp</i>	<0.001	0.933	0.256	<0.001	0.500
<i>Pepdgly</i>	0.444	0.608	0.188	<0.001	0.340
<i>Tranfe</i>	0.002	0.665	0.820	<0.001	0.500
<i>Tspo</i>	<0.001	0.412	0.303	<0.001	0.730
<i>Hsp60</i>	0.003	0.035	0.198	<0.001	0.952
<i>Ck7</i>	0.011	0.365	0.640	0.001	0.747
<i>Ik-cytokine</i>	0.009	0.597	0.303	<0.001	0.886
<i>IL10</i>	0.004	<0.001	0.294	<0.001	0.638
<i>LPS-TNF</i>	<0.001	0.412	0.827	<0.001	0.730
<i>Tyroprot</i>	0.000	0.822	0.303	<0.001	0.425
<i>C1Q-sco</i>	0.008	0.596	0.482	<0.001	0.730
<i>C3</i>	0.035	0.254	0.482	<0.001	0.606
<i>C9</i>	0.007	0.161	0.198	<0.001	0.730
<i>TAF8</i>	0.003	0.412	0.198	<0.001	0.730
<i>ASH2</i>	<0.001	0.714	0.288	<0.001	0.886
<i>TPR</i>	0.035	0.950	0.296	0.002	0.952
<i>BROMO</i>	<0.001	0.368	0.303	<0.001	0.952
<i>MYST1</i>	<0.001	0.046	0.198	<0.001	0.730
<i>HDAC1</i>	0.004	0.048	0.188	<0.001	0.316
<i>HDAC3</i>	<0.001	0.888	0.198	<0.001	0.636
<i>HDAC6</i>	0.029	0.926	0.552	0.100	0.850
<i>JmjC-PHD</i>	0.320	0.714	0.407	0.001	0.886
<i>No66</i>	0.001	0.205	0.198	<0.001	0.747
<i>DnMt1</i>	0.017	0.217	0.198	0.007	0.514
<i>DnMt3a</i>	0.028	0.263	0.399	0.006	0.500
<i>DnMt3b</i>	0.001	0.312	0.198	<0.001	0.747
<i>HemK2</i>	0.001	0.090	0.303	<0.001	0.824
<i>N6admet</i>	<0.001	0.038	0.198	<0.001	0.730
Size	<0.001	0.193	0.939	<0.001	-



Supplement 7: Venn diagram. Overview of target genes showing grand-paternal, grand-maternal and/or grand-biparental treatment effects.

Appendix Chapter III

Supplement 8: Primerlist. Primers sequences for the examined genes used for gene expression measurements. BLASTX hits of other teleost species with e-values $<10^{-20}$ (submission is still in process).

Gene	Primer: Forward (5'-3')	Primer: Reverse (5'-3')	Amplicon length (bp)	BLASTx
CD45	CGTCACGTCAAGAGGAAAGAC	AATGGACCACGACTGGAGGA	160	ACI46145.1
HIVEP2	CCATACAGGTGAAAGACCTTATCC	TTCTGTATGTTCTCAACGTCTTC	190	XP_696438.4
HIVEP3	CGATGACACTCCAGGTAGAATAG	TTAACCTCTGTCAAGTGGTGCTAC	111	XP_008321814.1
IgM-Ic	GTGACCCTCTTCCCTCCTTC	CACCTACATCAGCCTCTTCCAG	166	BAB91000.1
Integ-Bt	ACAAGGATGCGTCCAGTGT	GCGTAGGTGTAGTAGAACCCAGCAG	193	XP_008305870.1
Lymph75	GCGCGGATATCCTAACCAT	CATGAGTGCCATCGTACCAC	150	XP_005724198.1
lympcyt	CATACACACTCATGGTGCTTCAC	TGATAGTTGATGATGTCACAGACG	136	XP_007577143.1
TAP	GAGGACTCCATGGTGATGTTG	GCGGGTAAGTCTCTTCAATTCTC	137	XP_004572711.1
AIF	GGCTTTTCTGACCGATGAAC	CTCCTCCTGCCACTTCTGAC	204	XP_007559540.1
Calrcul	TGCACGTCACTTTAACTACAAGG	GCCAGACTCAACCTTCTTATTGTC	100	XP_004068493.1
Cf	TTACAGAGCGGCCTCACC	TCCAGATGCAAAAAGCAGGTC	176	XP_006626829.1
IL8	GCGACTACAACCCACTGC	GACAGACTCGTTCACCACTCAC	172	AGR27883.1
Intf	ATCTTCTCCATCAAGGCCAGAG	GCATATACAAGGACCATAACGAAG	144	NP_001028804.1
Kin	CGACAACAACCCAGACTCAG	TGTGGGTCTTTGGTGCTG	226	XP_005447761.1
Lectptl	ATCCGCTCGAATCAGTTCA	AGGCCGTGTGCAACATTC	90	AAQ56012.1
Lectpll	CGCCTCAATCAGTTCACGA	CGAGAGCGTCTGCAACATT	91	AAQ56013.1
Nramp	GAGTGGTCTGGGCTGTTTC	CAGTAGCACTCGTGCGAAAC	164	XP_009098790.1
Pepdgly	GGTTTCAACGTGCCAATCAAT	CACAGGCAGACGGTCAACTAC	100	ACJ10072.1
Tranfe	AATGCGCCACTGTAGTCATAGTAA	ATTAAGATGGACGTGTGAAGTG	182	XP_006786562.1
Tspo	TGGGGTATGGCTCCTATCTG	CCGGTTAGAAGCACGATCTC	173	ACQ58389.1
Hsp60	GTACGGTCATCATCGAGCAG	AGCACTGTGGCAGTGGTG	152	AAV40980.1
Ck7	TCAGATCATTGCCACCATAAAG	GGGCAGGTACAGATGTCTTGTT	142	ADE58987.1
Ik-cytokine	GCCGAGTGTTACCCTGCTAC	CAGCGCCTCCTTGTGTT	162	XP_004557045.1
IL10	TTCTGACTGCACAGTTGCT	TCTTCGATTGTCTGGTCCGAG	188	XP_004069312.1
LPS-TNF	GTAGAGTATAAGCCAGGCCTGAAG	AGTCGTGGACACAGAATGGTATC	100	ADZ99105.1
Tyroprot	GAACGACAGTTGCTCTCCACT	CTGAACCACTGTTGTAGTGAATC	210	XP_008283842.1
C1Q-sco	GTGATCATAGAGGTCGTCATTGAG	CTTCAAGATGAGTTGTCACATGGT	150	XP_008426360.1
C3	AGACCCCAACATGAAGCAGT	CTCTCTCAATAGGCTCCATGC	191	ADU33222.1
C9	CAGCCCATTTATACGCTGGT	AGCCATTCCTCAAAGAGGT	197	AFU81223.1
TAF8	ACATTCCGTGAACCAGTCTCAG	ATCAATGGGAAAGAGGTGATGT	152	ACN10220.1
ASH2	CACTCTGGGCTTCTCATTGT	CTCTTCTCGGCTTTGTCCAC	145	XP_003439681.1
TPR	GAAGTAGACCATTCCCAGACCA	AAGCATTATCGGCATACCAGAG	92	XP_006795785.1
BROMO	GAGCCAGTAGGTTCTTGAGCAT	AAGCAGAGTCAGATCAGGAAGG	184	XP_008283083.1
MYST1	ATCCTCACCGAAGTCAACAAG	GCTCGTAACTGAAGGCGATG	158	AAI55278.1
HDAC1	GTAATCGCTGGTGTCTGGTTG	CACGCTGTTGGACCTATGAAA	149	XP_004566614.1
HDAC3	CGTATGCTGAGGTTGAAGCA	CAGAGTTACAGGCAGCTATTCCA	134	XP_003977098.1
HDAC6	ATGTGAGTGAGGTGGGCATAG	CGAGTTCAATCCAAGTCTCGTT	111	XP_006810086.1
JmjC-PHD	CACATTGGTTTGTGCGATAGA	CCAGACCTCCTCCATCAACTC	88	XP_008318334.1
NO66	GAACTCCACATCCTCCTCCA	TACCGTCTCCTCCTACCAGA	97	XP_005935471.1
DnMt1	CTTCAACTCTCGCACCTACTCA	AGTTCCTCACATTCTCCAGCA	111	XP_006631651.1
DnMt3a	CCATTTCGATCTGGTGATAGGAG	GTTCCCTCATAAAGACCCTTCC	84	XP_008932658.1
DnMt3b	GTGCTTCTGGGATGGACAAG	GAGTTGGAGCGTGTGGTGAT	101	XP_003962984.1
HemK2	CAAGACAGCATCACGCAACT	TTGAACAGAGCACATCCACT	105	XP_004066645.1
N6admet	AGATGAAGTCGTCGCCATAGAT	GCAACACGATTAGCTGAGGAG	182	NP_080802.1
DDP-gly	CTAGCCTCCTTGATGCACTGT	AGATGATGAGATGGTCTGACAGG	199	XP_008411280.1
G6PD	ATCTTCACTCCACTCCTTCATCA	GTAAGTGCCTTCATAGCGGAATC	132	XP_007566410.1
Ribop	AAGAGAAGAAGGAGGAGCAGGT	CACGAAGGTGTCTGTTGAAAG	107	KFP09164.1
Ubi	CGTGAAGACATTGACGGGTA	GCAGCACCAGATGAAGAGTG	196	AEP96154.1

Supplement 9: Supplementary analysis for size and family effect in PERMANOVA model based on 44 target genes. Multivariate PERMANOVA analysis to assess the effect of size and size*grandparental interaction effect as well as family defined over strata as random term on relative gene expression data ($-\Delta\text{Ct}$ -values) based on an Euclidean distance matrix with p-values obtained by 999 permutations (significance code: 0***,0.001**,0.01*).

Size effects		Model: Genes~Size					
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	Sig
Size	1	90.6	90.556	1.84690	0.00988	0.18691	
Residuals	185	9070.9	49.032	0.99012	1		
Total	186	9161.5					
Grandparents*Size		Model: Genes~F0-grandparents*Size					
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	Sig
F0-grandparents	3	893.6	297.881	6.6444	0.09754	0.000999	***
Size	1	40.2	40.236	0.8975	0.00439	0.495504	
F0*Size	3	202.7	67.569	1.5072	0.02213	0.097932	
Residuals	179	8024.9	44.832		0.87594		
Total	186	9161.5			1		
Family effect		Model: Genes~F0-grandparents*F2-offspring+Size, strata = Family					
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	Sig
F0-grandparents	3	893.6	297.881	6.8059	0.09754	0.000999	***
F2-offspring	2	260.2	130.107	2.9727	0.02840	0.000999	***
Size	1	47.9	47.934	1.0952	0.00523	0.717283	
F0*F2	6	344.1	57.350	1.3103	0.03756	0.006993	**
Residuals	174	7615.6	43.768		0.83126		
Total	186	9161.5			1		

Appendix Chapter IV

Supplement 1: Names and function of 22 target genes and 2 housekeeping genes assessed for gene expression measurements.

Gene	Gene name	Function	Category
<i>IgM-Ic</i>	Immunoglobulin light chain	Recognition of antigen or pathogen	Adaptive
<i>Integ-Bt</i>	Integrin-beta 1	Cell signaling and adhesion of Ig, primary IgM antibody response	Adaptive
<i>Cf</i>	Coagulation factor II	Blood clotting	Innate
<i>LectptI</i>	Lectin protein type I	Pathogen recognition receptor (C-type lectin type I)	Innate
<i>LectptII</i>	Lectin protein type II	Pathogen recognition receptor (C-type lectin type II)	Innate
<i>LectptIII</i>	Mannose binding lectin	Pathogen recognition receptor (Mannose specific)	Innate
<i>Pepdgly</i>	Peptidoglycan recognition protein II	Recognition of peptidoglycan of pathogen	Innate
<i>Ck7</i>	Chemokine 7	Chemotaxis for leukocytes, monocytes, neutrophils, blood cells	Innate & Adaptive
<i>ASH2</i>	Histone methyltransferase (ASH2)	Histone methyltransferase (SET1-Ash2 complex)	Gene activation
<i>TPR</i>	Lysine-specific demethylase 6A	Histone demethylation (H3K27me3)	Gene activation
<i>BROMO</i>	Histone-acetyltransferase (KAT2A)	Histone acetylation (KAT2A-Bromodomain)	Gene activation
<i>MYST1</i>	Histone-acetyltransferase (HAT1)	Histone acetylation (MYST1-domain)	Gene activation
<i>HDAC1</i>	Histone deacetylase 1-like	Histone deacetylation (hydrolysis of N(6)-acetyl-lysine residues)	Gene silencing
<i>HDAC3</i>	Histone deacetylase 3-like	Histone deacetylation (hydrolysis of N(6)-acetyl-lysine residues)	Gene silencing
<i>HDAC6</i>	Histone deacetylase 6-like	Histone deacetylation (deacetylation of lysine residues of core)	Gene silencing
<i>JmjC-PHD</i>	Lysine-specific-demethylase 5B	Histone demethylation (demethylates'Lys-4'(H3K4me))	Gene silencing
<i>NO66</i>	Lysine-specific-histone demethylase NO66	Histone demethylation (demethylates'Lys-4'(H3K4me) (H3K36me))	Gene silencing
<i>DnMt1</i>	DNA-Methyltransferase 1	DNA methyltransferase (C5-methylcytosine)-maintenance methylation	Gene silencing
<i>DnMt3a</i>	DNA-Methyltransferase 3a	DNA methyltransferase (C5-methylcytosine)-de novo methylation	Gene silencing
<i>DnMt3b</i>	DNA-Methyltransferase 3b	DNA methyltransferase (C5-methylcytosine)-de novo methylation	Gene silencing
<i>HemK2</i>	HemK-methyltransferase family member 2	DNA methyltransferase (N6-methyladenine)	Unknown
<i>SIN3A</i>	SIN transcription regulator family memberA	Transcription factor (Repressing)	Gene silencing
<i>Ribop</i>	Ribosomal protein	Translation process	Housekeeping
<i>Ubi</i>	Ubiquitin	Labels regulatory proteins for degradation	Housekeeping

Appendix Chapter IV

Query location : Contig1 101 to 370 (+)
Database location : ENSGACP00000014192 2 to 92 (+)
Genomic location : groupXV 9785261 to 9785533 (-)

Alignment score : 288
E-value : 9.2e-26
Alignment length : 91
Percentage identity: 61.54

```
>group:BROADS1:groupXV:9784961:9785833:-1
ATTTTGCATGATTTCAATGCCTCACTGCTCCTCACTGTTTTAAGTTCTCCTGACACAGAA
AGTCGTAACAGTTTTTCATGAACGCACACTGAAAACCTGCACCAGGATGATGTGGTCAATAA
TCCTCTTGGTGGCTGTTCTGTGCCACGGTGAGACATTTTGAAAACCTATCTGACAGATGTT
TTTGTCTTGTACTATTATAATTTAAACCGCTGCTCTTTATTGTGAGACATGTGTCAATTT
CTTATCAATGTTTATATTTTCAATTTCTTATTTTTCAGACACTTCTGCAAACCAATTCCTGACT
CAGACAGACCCTTCCAAGGCCGTCGGTCTCAGAGGGACTGTGACCATCAGCGCCAAGGGG
AGTTCAGATATCGGTCCTGATCTCAGTTGGTACCTTCAGAAACCTGGACAGGCTCCAAA
CTTCTCATTTACGACTCATCAACTCGTTCTCTGGAACCTCCTCCTCGATTGAGTGGCAGT
CGTTCTGGTTCTGACTACACTTTAACCATCAGTGGTGTGGAGGCTGAAGATGTTGGCGAT
TACTACTGTCTTGGTTACCATGGAGTTGGAGAGTTTACACACAGTGATTTAGAGCCGTACAA
AAACCTCCTTCACTTTGATAGCAGTGAAATCGGCCTGAACGTGGACCTGAGGGTTGGTGG
AGAGACTGTGGTGGAGAAAACATGGTCCAGTGAACCAACACGAGAGTCATCAAGCAGAA
CCACAATGAATATGAATAAAACAGAAGCACACTCACAGACACTCTTATATATGAACTAAA
ATGAAATGTTTATTCTTGGCAGTTCAGTCAGTCTCTCTATGATTTATTAGAGGAGGTTAG
TTTCTTGTGGACCATAACTTCTAATGTATATA
```

```
>Contig Syngnathus typhle immunoglobulin light chain
AACACCCCTAGCTCTACGAGTCAGGATGTGGTCTTCCGCACCTTGCCTTGTCTTTGGGC
TCTGCTGATTCCTGGATCTTCTGCCCAGAATTTTCTGAGGCAGACGGACAGACTCATGTC
TGTCTCTCTCGGAGGAACCGTGAAGATCAGGGCCACAGGGAGTCAAAACATTGGTAGCTA
TCTCAGTTGGTACCAAGTGAACCTGGACAGGCTCCCAAACCTGCTCATCTACGACGCAAC
ATCAAAATTCTCAGACGCGCCATCTMGATTGACGCGCACTCGATCCGGCTCTGACTACAC
TCTGACCATCAGWGRTGTCCAGAGAGATGACCTGGCCGAATACTACTGTTTCGCCTACTA
CGGCGGTGAGGAGTGSAGTTTTCGCTGAAGAAGGAACCAAACCTCATCCTTGCGGGTGAGGT
GCATCGGCCACCGTGACCCTCTTCCCTCCTTCCAAGGAGCAGCTGCAAACAGAAAAGGC
CACAGCCGTGTGCGTGGCCAAAGGGCGCCCCCAAACCTTGGACCCTGGGTTGGAAGGTTGA
TGGCGCCCCCGCTACCTCGGGGGTATCGCACAGCTCCCTGGAAGAGGCTGATGTAGGTGG
GCTTTACAGCTGGACCAGCACCTTGAGCCTCTCACCGGACCAGTGGCGCAAGGCCAGTC
AGTGAGCTGCGAGGCCAGCCTAACCGGGCAAACCCCTGTCACGCAAACACTGCCGAACCC
AGGACAATGCTCAGAGTAGAGCCCGAACCCGTGTCTGTGTCTTTGCGACGTTGAAGCATT
GAGAAATAAAAATGTCTGAGTATCACCATCTGTCTCTGTAGCAGCYGYTTGGRAGCAAAC
TTGGTAACACGACAAATAAACGTCTCATCAAGCACATGTATATTGTGACAGGTAGTCTGG
AG
```

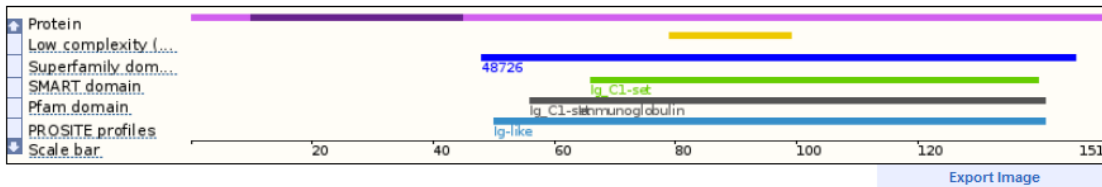
THIS STYLE: Location of selected alignment

THIS STYLE: Location of other alignments

THIS STYLE: Location of Exons

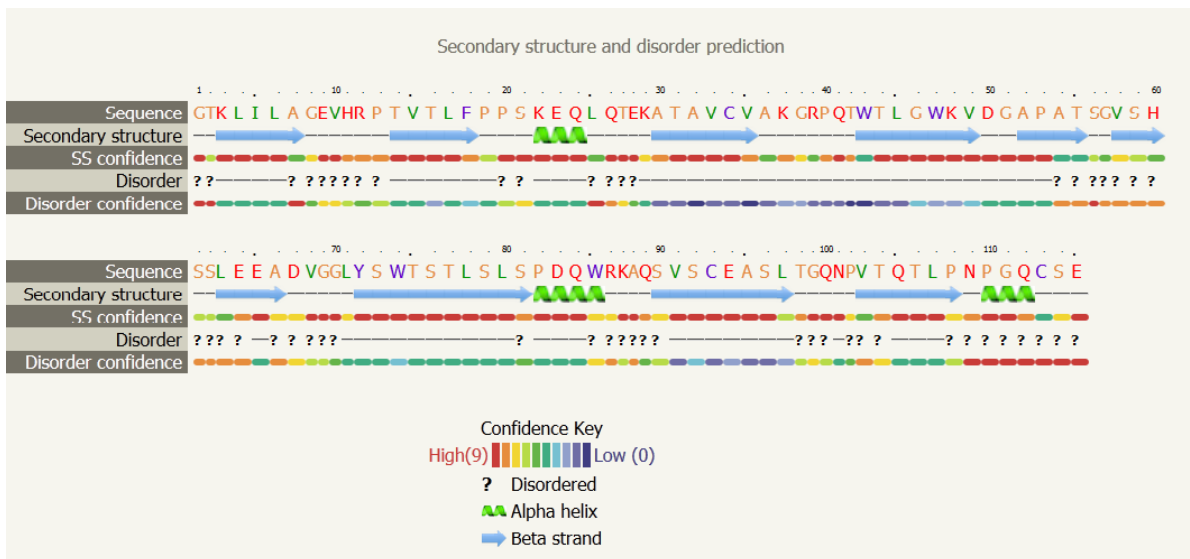
Supplement 2: Alignment of *S. typhle* Ig-Lc with Stickleback (*Gasterosteus aculeatus*) genome to find potential coding regions (marked in red) using *ENSEMBLE* database.

Appendix Chapter IV



Statistics
 Ave. residue weight: 107.617 g/mol
 Charge: -3.0
 Isoelectric point: 4.8184
 Molecular weight: 16,250.20 g/mol
 Number of residues: 151 aa

Supplement 3: Summary of potential protein domains of *S. typhle* Ig-Lc sequence using *ENSEMBLE* database.



Supplement 4: Summary of predicted protein structure of *S. typhle* Ig-Lc using *Phyre*² software.

Curriculum vitae

Anne Beemelmans

GEOMAR | Helmholtz Centre for Ocean Research, Düsternbrooker Weg 20, 24105 Kiel, Germany |
Phone: +49 431 600-4566 | Email: abeemelmans@geomar.de

Personal

Name Anne Beemelmans
Date of birth 12.09.1985
Place of birth Würselen/Germany

Formal Education

- 09/2011 – 04/2016 **PhD, IMPRS (International Max Planck Research School) for Evolutionary Biology**
PhD thesis completed in the working group of Prof. Dr. Thorsten Reusch Helmholtz Centre for Ocean Research Kiel (GEOMAR), Department for Marine Ecology, Subgroup Evolutionary Ecology of Marine Fishes

PhD thesis: “Bi-parental immune priming in the pipefish *Syngnathus typhle*”
- 09/2007 - 03/2011 **Diploma of Biology**, Heinrich-Heine University, Düsseldorf, Germany
Diploma thesis completed in the lab of Prof. Dr. Chris Bridges Institute for Metabolic Physiology, Subgroup Aquatic Ecophysiology

Diploma thesis: “*Broodstock management- survey of molecular biological methods for the identification of species, individuals, and parentages in aquaculture*”
- 07/2008 - 02/2009 **Study abroad**, Queensland University of Technology, Brisbane, Australia
7-month study abroad semester
- 09/2005 - 08/2007 **Pre-Diploma of Biology**, Heinrich-Heine University, Düsseldorf, Germany
- 08/1996 - 07/2005 **A-level**, CMG Gymnasium Übach-Palenberg, Germany

Languages

German	Native speaker	French	Beginner
English	Fluent written and spoken		

Internships

10/2011 - 12/2011	6-week lab rotation in the working group of Dr. Martin Kalbe (Max Planck Institute for Evolutionary Biology Plön, Evolutionary Ecology, Parasitology)
09/2011 - 10/2011	6-week lab rotation in the working group of Prof. Dr. Thorsten Reusch (Helmholtz Centre for Ocean Research Kiel (GEOMAR), Marine Ecology, Evolutionary Ecology of Marine Fishes)
01/2011 - 02/2011	6-week research internship at the Malta Aquaculture Research Centre Fort San Lucjan, Marsaxlokk, Malta – as part of the diploma thesis –

Teaching Experience

01/2012 - present	Supervision of bachelor and master students during their projects (experimental and lab work, data analysis and statistics), GEOMAR Kiel
	Performing seminars about the rtPCR Fluidigm system, GEOMAR Kiel
06/2010 - 08/2010	Organizing bachelor tutorial (“E-learning, soft skills“), HHU Düsseldorf
11/2009 - 11/2009	Student assistant of a basic class for animal physiology, HHU Düsseldorf
06/2008 - 07/2009	Student assistant of a basic class for plant physiology, HHU Düsseldorf

Soft Skills

17.-21.03.2014	Multivariate statistics in R , Geomar, Lutz Becks and Matthias Wegner
27.-28.06.2013	Data visualization in R , MPI for Evolutionary Biology
14.-25.01.2013	R-statistics , CAU Kiel, Hinrich Schulenburg
01.-03.03.2012	rtPCR course , MPI for Evolutionary Biology, David Rogers and Jarek Bryk
16.-20.01.2012	Sequence analysis course , MPI for Evolutionary Biology, Bernd Haubold
12-13.01.2012	Perl course , MPI for Evolutionary Biology, June Wang
10-15.05.2010	Animal welfare and vivisection certificate , HHU Düsseldorf

Conferences and Workshops

2016: Symposium Rapid Reciprocal Adaptation and its Genetic Basis, Münster, Germany

*Poster presentation: "Biparental immune priming in the pipefish *Syngnathus typhle*"*

2015: XV Congress of the European Society for Evolutionary Biology, Lausanne, Switzerland

*Poster presentation: "Grand-parental immune priming effects in the pipefish *Syngnathus typhle*"*

2011-2015: Annual DOKMA-Symposia, GEOMAR Kiel, Germany

Annual talks and organization in 2012

2011-2015: Annual IMPRS-Retreats

Annual talks and organization in 2013

2013: XIV Congress of the European Society for Evolutionary Biology, Lisbon, Portugal

*Talk: "Biparental immune priming in the sex-role reversed pipefish *Syngnathus typhle*"*

2013: 2nd Syngnathid Biology International Symposium, Faro, Portugal

*Talk: "Biparental immune priming in the sex-role reversed pipefish *Syngnathus typhle*" (price for best students talk)*

2012: Workshop of Marine Evolution, AWI Sylt, Germany

*Talk: "Costs and Benefits of immune priming in the sex-role reversed pipefish *Syngnathus typhle*"*

2012: Workshop for Immunology of Fish, CBI Wageningen, Netherlands

*Poster presentation: "Experimental setup to test for biparental immune priming in *Syngnathus typhle*"*

Publications

Roth O, Klein V, **Beemelmans A**, Scharsack JP & Reusch TBH (2012). Male pregnancy and biparental immune priming. *American Naturalist*; 180.6: 802-814.

Beemelmans A and Roth O (2016). Biparental immune priming in the pipefish *Syngnathus typhle*. *Zoology*; *in press*.

Beemelmans A and Roth O (2016). Grand-parental immune priming in the pipefish *Syngnathus typhle*. *in revision*.

Beemelmans A and Roth O (2016). Bacteria-specific immune priming in the pipefish *Syngnathus typhle*. *in revision*.