Biparental immune priming in the pipefish *Syngnathus typhle*

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**A B S T R A C T**

The transfer of immunity from parents to offspring (trans-generational immune priming [TGIP]) boosts offspring immune defence 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 defence. To connect TGIP with 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 defence throughout offspring maturation. To disentangle maternal and paternal influences, 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 with 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.

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1. 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 and Fox, 1998). Such parental effects are a key source of trans-generational phenotypic plasticity that allow for immediate responses to novel environmental challenges (Bonduriansky and Day, 2009; Boulinier and Stasiewski (2008)). 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 (Grindstaff et al., 2006; Hasselquist and Nilsson, 2009; Sadd et al., 2005).

The maternal transfer of immunological active components like antibodies via blood, milk, or egg is of major importance during early life stages (Boulinier and Stasiewski, 2008; Brambell, 1970a,b; Ehrlich, 1892), when the adaptive immune system is still subject to maturation (Lindholm et al., 2006; Rossiter, 1996). 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 and 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 (Grindstaff et al., 2003; Hasselquist and Nilsson, 2005; Jiménez de Oya et al., 2011), but can also be found in invertebrates, where it is achieved by other mechanisms than antibody-mediated immunity (Freitak et al., 2009; Freitak et al., 2014; Little et al., 2003; Moret, 2006; Roth et al., 2010; Sadd et al., 2005; 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 and Day, 2009). However, lately the potential of parental transfer of epigenetic traits influencing the offspring phenotype has been discovered (Jablonska and Lamb, 2014, 2015; Jiang et al., 2013). Epigenetics concern stimuli-triggered changes in gene expression that do not involve an alteration of the nucleotide sequence, but are heritable across

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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 (Jablonska and Lamb, 2014, 2015; Szyf, 2015; Youngson and Whitelaw, 2008). Here, RNA molecules (Rassoulzadegan and Cuzin, 2015; Rassoulzadegan et al., 2006), DNA-methylation (Gi and Liu, 2015; Jiang et al., 2013) and histone modifications (Campos et al., 2014; Gaydos et al., 2014; Jones, 2015; Ragunathan et al., 2015; Vilcinskas, 2016, this issue), factors associated with the regulation of gene expression, can be trans-generationally inherited and manifest as stable epigenetic marks in the following generation (Jablonska and Lamb, 2015; Kappeler and Meany, 2010). 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 has only been 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) and invertebrates (Vilcinskas, 2016, this issue). However, the question which mechanisms are responsible for mediating immune priming effects that are based on epigenetic changes in vertebrates is still unresolved.

If epigenetic factors are involved or if males overcome the mechanistic barrier of limited sperm size, TGIP may not be limited to females in a beetle, biparental TGP 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 and 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 with 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., 2012b). Here, females deposit their eggs into a protective brood-pouch of the males, in which they are fertilised and nurse 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., 2012b) suggested that sex-role reversal may open the door for biparental influences on offspring immunity.

In the present study we 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.

We hypothesised that (I) 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 hypothesised that (II) 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 that (III) the paternal immune priming effect would cease upon maturation, with maternal effects ceasing earlier than paternal effects.

We assessed a gene associated with 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 that (IV) changes in epigenetic traces in the offspring generation would show parental sex-based differences.

Finally, we addressed the costs and benefits of TGIP by monitoring key life history traits (size, mass, condition factor, hepatosomatic index) of the juveniles and evaluated their time to reach sexual maturity. We hypothesised that (V) 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.

2. Materials and methods

2.1. Parental generation (P) treatment

Broad-nosed pipefish S. typhle were caught in the south-western Baltic Sea (54 44′N; 9 53′E, Germany) in spring 2013. They were kept under Baltic summer conditions (salinity of 15 psu, 18 °C, and 14–16 h light) and fed twice a day with mysids.

For the experimental setup mature pipefish were either left naïve or injected with 50 μl of 10^8 cells/ml heat-killed bacteria solution according to (Roth et al., 2012b). Two novel bacteria species, Vibrio spp. (Italian isolate, I2K3) (Roth et al., 2012a) and T. maritimum (DSM No. 17995) (Suzuki et al., 2001) were applied to exclude confounding effects with previous pathogen encounters in the wild. Both bacteria types activate the immune system of fish and cause two different diseases: ‘vibriosis’ (Alcaide et al., 2001) and ‘flexibacteriosis’ (Bernardet et al., 1990; Kolugas et al., 2012), respectively. This allowed us to analyse the transmission of bacteria-specific immune memory by mothers and fathers, respectively and potential additive immune priming effects. Mating pairs of immune-challenged individuals were formed in 36 cm x 80 cm aquaria connected to a semi-flow-through system with maternal, paternal or biparental immune challenge in a fully reciprocal mating design: 1. (MatV+PatV+), 2. (MatT+PatV+), 3. (MatNPatV+), 4. (MatNPatV+), 5. (MatT+PatNP), 6. (MatV+PatNP), 7. (MatNPatN), 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) (Fig. S1 in the supplementary online Appendix). A sham exposure with PBS was not implemented as earlier studies had suggested that TGIP is not confounded by the sterile needle injection (Roth et al., 2012b) and sham injection with PBS does not induce immune gene expression (Birrer et al., 2012).

2.2. Filial generation (F1) treatment

All 56 couples mated successfully after the immune challenge and about five weeks later (July 2013), one-week-old offspring (8 days post birth) were exposed to homologous and heterologous heat-killed bacteria (Vibrio (V+) and Tenacibaculum (T+)), or stayed naïve as controls (N). For this purpose, a needle was dipped into a solution of 10^9 cells/ml heat-killed bacteria and used to prick the juveniles intraperitoneally. Subsequently, these were kept in separate tanks for 20 h. After this incubation time, the juveniles' body
standard lengths were measured, and whole-body samples were used for RNA extraction. For the analysis we selected only families with a minimum clutch size of 15 juveniles, with 4 replicates per parental treatment (28 families in all). From each of the 28 families, 15 offspring were used and distributed over the three treatments, resulting in 5 specimens 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 36 cm × 80 cm aquaria connected to a semi-flow-through circulation system using three tank replicates per parental treatment and a density of 20 pipefish per tank. To compare TGIP between different developmental stages, four-month-old juveniles (not fully sexually mature) from the 7 different parental treatment groups were injected with 20 µl 10⁶ cells/ml heat-killed Vibrio (V+) or Tenacibaculum (T+) bacteria (intraperitoneally) or stayed naïve (N), using 3 replicates per offspring treatment per sampling event. In total, 9 individuals of the 7 parental treatment groups were randomly collected out of the tanks at two consecutive sampling events, resulting in a total number of 126 sampled juveniles. After incubation (20 h), body standard length and body mass were measured before the fish were sacrificed. The liver was weighed and the hepatosomatic index (HSI = ratio of liver mass [g] relative to body mass [g]) was 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 gain 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).

Remaining F1-offspring (approx. 280 pipefish) were left immunologically untreated and, to maintain an equal density ratio (20 pipefish per tank), 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.

2.3. Quantification of gene expression

We quantified the mRNA-level of 44 pre-selected target genes of previous studies (Birrer et al., 2012; Roth et al., 2012b) using quantitative real time polymerase chain reaction (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 functional categories: (i) innate immune system (immediate and non-specific immune defence upon infection, i.e. phagocytosis), (ii) adaptive immune system (specific antibody-mediated immune defence), (iii) innate and adaptive immune genes (genes connected to both pathways), (iv) complement system (complements the antibody and phagocytic cell-mediated immune response), and (v) epigenetic modulators (DNA methylation, histone de/methylation, histone de/acetylation) (Table S6). Primer efficiencies were tested, with all primer pairs attaining standard curves with slopes of log quantity vs. threshold cycle (Ct) between −3.6 and −3.1 and efficiencies of 90–100% (Table S7).

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 months post birth) was performed with an RNeasy96 Universal-Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. Extraction yields were measured by means of a spectrophotometer (NanoDrop ND-1000; Peqlab, Erlangen, Germany) 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 BioMark HD system (Fluidigm, South San Francisco, CA, USA) 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 µl TaqMan PreAmp Master Mix (Applied Biosystems, Waltham, MA, USA) and 1.25 µl cDNA per sample. The mixture was pre-amplified (10 min at 95 °C, 14 cycles: 5 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 µl x 2 Ssofast-EvaGreen Supermix with Low ROX (Bio-Rad Laboratories, Hercules, CA, USA) with 0.35 µl 20 x DNA Binding Dye Sample & Assay Loading Reagent (Fluidigm) and 3.15 µl pre-amplified PCR products. An assay mix was prepared by combining 0.7 µl of 50 [µM] primer pair mix, 3.5 µl Assay Loading Reagent (Fluidigm), and 3.15 µl low EDTA-TE buffer. Finally, 5 µl 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 the manufacturer’s (Fluidigm) instructions. The samples were distributed randomly across chips and each of these included no template controls (NTC), controls for gDNA contamination (-Rt) and standards and two technical replicates per sample and gene.

2.4. 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 and Mangelsdorf, 2003). The combination of the housekeeping genes ubiquitin (Ubi) and ribosome protein (Ribop) showed the highest stability (geNorm M > 0.85) (Hellemands et al., 2007) and their geometric mean was used to quantify relative gene expression of each target gene by calculating −ΔCt-values. All plots and statistical tests were performed in R v 3.2.2 (R Core Team, 2015) and PRIMER v6 (Clarke and Gorley, 2006). Multivariate statistics were used to infer differences in the entire expression pattern of 29 immune genes and 15 genes associated with epigenetic regulation (Table S6).

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). Thus, we assessed the effect of seven parental treatment combinations on gene expression (29 immune genes), life history parameters (body size, body mass, CF, and 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 × 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 analyzed maternal, paternal and biparental effects on genes associated with 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 −ΔCt-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 PRIMER6 (Clarke, 1993; Clarke and Gorley, 2006) 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 principal 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 (Chessel et al., 2004; Dolédec and Chessel, 1987; Thioulouse et al., 1995). 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 principal components. The length of the arrow is directly 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 of normality for each response variable were graphically examined and tested with the Shapiro–Wilk and Levine tests after Box–Cox transformation using Jmp9 (SAS Institute Inc., Cary, NC, USA). A linear model was fitted using the fixed factors 'F0-parents' and 'F1-offspring', the non-independent factor 'family' or '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.

3. Results
3.1. Maternal, paternal and biparental immune priming effects in one-week- vs. four-month-old juveniles
3.1.1. One-week-old F1-juveniles – immune gene expression
29 candidate genes connected to pathways of the innate immune system, the adaptive immune system and the complement component system were significantly affected upon the parental bacteria treatments (PERMANOVA-immune: F6,377 = 12.44, p < 0.001; see Fig. 1A and Table S1 in the supplementary online Appendix). The PCA revealed both a difference between the two applied parental bacteria treatments (Vibrio and Tenacibaculum) and a parental sex-based effect (Fig. 1A and Table S2). F1-offspring of mothers and fathers challenged with Vibrio or Tenacibaculum displayed a significantly differential expression profile along the second principal component (PC2: 18.9% of total variation) clustering according to the specific bacteria species (ANOSIM-immune: MatV+ vs. Mat+, p = 0.001; PatV+ vs. Pat+, p = 0.001; Fig. 1A and Table S2). However, both biparental treatment groups clusters together with the maternal-Tenacibaculum and as well as the paternal-Tenacibaculum treatments, which included their respective single parental Tenacibaculum treatment (Fig. 1A).

Hence, the biparental treatments were more similar to the maternal and parental Tenacibaculum treatments in comparison to the parental Vibrio treatment. Particularly, the paternal Tenacibaculum treatment was not significantly different from the biparental treatment comprising the respective paternal Tenacibaculum treatment (ANOSIM-immune: Pat+ vs. Bi-(MatV+Pat+), p > 0.05; Fig. 1A and Table S2).

3.1.2. 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: F6,98 = 3.21, p < 0.001; Fig. 2A and Table S1). In the multivariate PCA analysis we identified a difference between the two parental bacteria treatments (Vibrio and Tenacibaculum) independent of the parental sex effect clustered in opposed directions along the first principal component (PC1: 27.8% of the total variation) (ANOSIM-immune: MatV+ vs. Mat+, p = 0.001; PatV+ vs. Pat+, p < 0.028; Fig. 2A and Table S3). Juveniles from both paternal treatments were not significantly different from those of the respective biparental treatments (ANOSIM-immune: Pat+ vs. Bi-(MatV+Pat+), p > 0.05; PatV+ vs. Bi-(Mat+PatV+), p > 0.05; Fig. 2A and Table S3) indicating a long-lasting paternal immune priming effect.

3.1.3. 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 h: F6,97 = 7.29, p < 0.001; Table S1) and blood (PERMANOVA-cells blood: F6,97 = 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 the paternal Vibrio treatment (ANOVA-LM-ratio h: F2,97 = 6.4, p < 0.001, TukeyHSD: C < MatV+, C < PatV+; Fig. 4A and Table S5). In contrast, solely the paternal Tenacibaculum treatment elevated the L/M-ratio in F1-offspring (TukeyHSD: C = Mat+, C < Pat+; Fig. 4A and Table S5). When parents received maternal-Vibrio and paternal-Tenacibaculum treatment in a biparental combination, the L/M-ratio was significantly raised in contrast to the control group (TukeyHSD: C < Bi-(MatV+Pat+); Fig. 4 and Table S5) 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 positively influenced upon the paternal Vibrio treatment only (ANOVA-LM-ratio blood/F0-parents: F2,97 = 5.15, p < 0.001, TukeyHSD: C < PatV+; Fig. 4B and Table S5) and further revealed a Vibrio-specific offspring treatment effect (ANOVA-LM-ratio blood/F1-offspring: F2,73 = 7.89, p < 0.001, TukeyHSD: F1-N < F1-V+; Table S5).

3.2. Interaction of maternal, paternal and biparental immune priming effects and bacteria specificity
3.2.1. One-week-old F1-juveniles – immune gene expression
Based on the 29 immune genes a significant interaction between the F0-parental and the F1-offspring treatment in the one-week-old juveniles was detected (PERMANOVA-immune: F12,377 = 1.75, p < 0.001; Fig. 1B and Table S1). The applied pairwise comparison of interaction terms showed that the biparental immune priming effect was significantly influenced by the paternal Tenacibaculum immune challenge. Tenacibaculum-exposed F1-offspring of the biparental treatment Bi-(MatV+PatV+) were not significantly different from Tenacibaculum-challenged offspring of the single parental Tenacibaculum treatment (ANOVA-immune: Bi-(MatV+PatV+)/F1-T+ vs. Pat+ vs. F1-T+, p > 0.05; Table S2). With parental Vibrio treatment the two groups revealed a significantly different immune gene expression pattern (ANOSIM-immune: Bi-(MatV+PatV+)/F1-V+ vs. PatV+/F1-V+, p = 0.004; Table S2).

In contrast, both maternal treatment interaction terms were significantly different from both biparental treatment interactions (ANOVA-immune: Bi-(MatV+PatV+)/F1-V+ vs. MatV+/F1-V+, p = 0.002; Bi-(Mat+PatV+)/F1-T+ vs. Mat+T+/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 principal component (52.8% of total variation) displaying a clustering of the parental bacteria treatments apart from the parental con-
control (PERMANOVA-immune: $F_{2,377} = 18.36, p < 0.001$; Fig. 1B and Table S1). 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 parental 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-control/F1-bac) (Fig. 1B).

### 3.2.2. Four-month-old F1-juveniles – gene expression and immune cell counts

In four-month-old offspring there was no interaction between F1-paternal treatment and F0-offspring treatment on the 29 immune genes (PERMANOVA-immune: $F_{2,377} = 0.83, p > 0.05$; Fig. 2B and Table S1). The lymphocyte/monocyte ratio in the head kidney of four-month-old juveniles revealed a significant F1-paternal treatment and F0-offspring treatment interaction (PERMANOVA-cells hk: $F_{12,97} = 2.75, p < 0.005$; Table S1). The L/M-ratio in the head kidney of F1-offspring with parental *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; Fig. S2 and Table S5).

### 3.3. Maternal, paternal and biparental immune priming effects connected to epigenetic regulation mechanisms

#### 3.3.1. 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: $F_{6,377} = 6.6, p = 0.001$; Fig. 3 and Table S1). 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, $p > 0.05$; Fig. 3A and 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, $p = 0.004$; Fig. 3A and Table S4).

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Fig. 1. Immune gene expression of one-week-old *Syngnathus typhle* F1-juveniles ($N = 420$). (A) PCA plot based on 29 immune genes visualizing the expression profiles of 7 F0-parental treatment groups (parental control (Control), paternal *Vibrio* (PatV+), and *Tenacibaculum* (PatT+), maternal *Vibrio* (MatV+) and *Tenacibaculum* (MatT+), and the biparental treatment groups (Bi-MV+ xPT+ and Bi-MF+ xPV+)). (B) BCA plot based on 29 immune genes displaying the interaction of four F0-parental treatment groups (parental control (Control), paternal (Pat), maternal (Mat), and biparental (Bi)) with F1-offspring treatment (*Vibrio*, *Tenacibaculum* combined in bacterial challenge (Bac) versus no challenge or naive (N)). Colors represent different parental bacterial challenges, with *Vibrio* in red, *Tenacibaculum* in blue and the joint biparental treatments in purple. The corresponding arrow-scatterplots on the right hand side represent the contribution of each variable (immune gene) to the total variability. The contribution of each gene is symbolised by the length of the arrow, which is directly proportional to the contribution of each variable. The scale of the graphs is given by grid size d in the upper right hand corner of each panel. The Eigenvalues are represented by a bar chart in the lower right hand corner, with the two black bars equivalent to the two axes used to draw the PCA or BCA plot and their corresponding scatterplots (A: PC1 = 24.7%, PC2 = 18.0%; B: PC1 = 52.8%, PC2 = 20.1%).

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Fig. 2. Immune gene expression of four-month-old juveniles of S. typhle (N = 126). (A) PCA plot based on 29 immune genes visualizing the expression profiles of 7 F0-parental treatment groups (parental control (Control), paternal Vibrio (PatV+) and Tenacibaculum (PatT+), maternal Vibrio (MatV+) and Tenacibaculum (MatT+), and the biparental treatment groups (Bi-MV+PV+/Bi-MT+TP+)). (B) BCA plot based on 29 immune genes displaying the interaction of four F0-parental treatment groups (parental control (Control), paternal (Pat), maternal (Mat), and biparental (Bi)) with F1-offspring treatment (Vibrio, Tenacibaculum combined in bacterial challenge (Bac) versus no challenge (N)). Colors represent different parental bacterial challenges with Vibrio in red, Tenacibaculum in blue and the joint biparental groups in purple. The corresponding arrow-scanplots on the right hand side represent the contribution of each variable (immune gene) to the total variability. The contribution of each gene is symbolised by the length of the arrow, which is directly proportional to the contribution of each variable. The scale of the graphs is given by grid size d in the upper right hand corner of each panel. The Eigenvalues are represented by a bar chart in the lower right hand corner, with the two black bars equivalent to the two axes used to draw the PCA or BCA plot and their corresponding scanplots (A: PC1 = 27.8%, PC2 = 14.7%; B: PC1 = 41.6%, PC2 = 17.3%).

3.3.2. Four-month-old F1-juveniles – expression of epigenetic regulation genes

In contrast 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: F6,93 = 6.6, p = 0.001, Table S1; ANOSIM-epigen: Pat vs. Control, p = 0.001; Mat vs. Control, p = 0.006; Bi vs. Control, p = 0.001; Fig. 3B and Table S4). This is additionally supported in the graphical visualisation of the PCA, in which parental treatments are found in distinct clusters along the first principal component (45.8% of total variation). No significant differences between maternal, paternal and biparental treatment could be identified (Fig. 3B and Table S4).

3.3.4. Costs of immune priming – life history traits

3.3.1. 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: F2,378 = 30.04, p < 0.001, C < MatV+, C < MatT+, C < PatV+, C < PatT+; Fig. S3A and Table S5). F1-offspring from mothers challenged with bacteria even revealed a larger body size than offspring with paternal treatment (TukeyHSD: PatV+ < MatV+, PatT+ < MatT+; Fig. S3A and Table 7). In addition, F1-offspring of mothers with Vibrio exposure were bigger in size than offspring of mothers with Tenacibaculum treatment (TukeyHSD: MatT+ < MatV+; Fig. S3A and Table S5).

3.3.4. Four-month-old F1-juveniles – life history (size/mass/CF/HSI)

In a multivariate approach, body size, mass, CF and HSI of four-month-old juvenile pipefish were significantly influenced by the parental immune challenge (PERMANOVA-life-size/mass/CF/HSI: F6,98 = 2.56, p < 0.004; Table S1). However, body length and mass of four-month-old juveniles were only increased 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 than the control group (ANOVA-size: F6,98 = 2.86, p = 0.013, TukeyHSD: C < MatV+; Table S5 and Fig. S3B; ANOVA-mass: F6,98 = 4.43, p = 0.001, TukeyHSD: C < MatV+, PatT+ < MatV+, C < Bi- (MatV+PatT+); Table S5 and Fig. S3C). Further, the paternal Tenacibaculum treatment positively influenced the HSI of F1-offspring, which had a larger liver (1.24 ± 0.24 mg) than offspring of the parental control treatment (ANOVA-HSI: F6,74 = 2.39, p = 0.034, TukeyHSD: C < T+; Table S1 and S5 and Fig. S3D).
3.4.3. Six-month-old F1-offspring – life history (sexual maturity)

Upon a parental immune challenge, juvenile males developed a brood pouch (i.e. sexual maturity) one month (average 38.6 ± 2 days) later than offspring of unexposed parents (ANOVA-maturity males: F3,168 = 158.8, p < 0.001, TukeyHSD: C < Mat, C < Pat, C < B; Fig. S4 and Table S5). If both parents received the bacterial treatment, males reached sexual maturity even later (6 ± 1 days) (TukeyHSD: Mat < Bi, Pat < Bi; Fig. S4 and Table S5).

4. Discussion

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

We explored the synergistic effects of maternal and paternal immune priming by applying two different bacteria species (Vibrio spp. and T. maritimum). Contrary to our expectations, the biparental effects pretty much compensated each other and did not pile up to an additive impact. In contrast, biparental immune priming with the same bacteria phyotypes for both parents induced synergistic beneficial effects (Roth et al., 2012b). This discrepancy may have arisen due to strong bacteria-specific immune priming effects (Beemelmanns and Roth, 2016, unpublished data). 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 siblings with the respective single maternal and single paternal Tenacibaculum treatments. In contrast, the fact that the offspring treatment group with single parental Vibrio treatments clustered far apart from the control group and in an opposite direction to the parental Tenacibaculum groups points to an antagonistic Vibrio-mediated paternal 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, independent of the applied bacteria species. Hence, offspring of fathers who had been challenged with Vibrio or Tenacibaculum bacteria showed a similar immune gene expression profile as offspring of the corresponding biparental treatment combinations, while the maternal groups were significantly different from the respective biparental groups. Neither was any synergistic parental immune priming effect detected in four-month-old juveniles, but we did find 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 as compared to the control.
4.2. 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 immune gene expression profile that could not be differentiated from that of F1-offspring of parents exposed to biparental treatment, also including 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 impact of the paternal treatment on immune gene expression than that of the maternal treatment for both one-week-old and four-month-old juveniles.

F1-offspring of fathers exposed to *Vibrio* or *Tenacibaculum* bacteria induced their immune cell proliferation in the head kidney, the major immune organ of teleosts, earlier than those of 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 the case of the maternal treatment, only the 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 with various *Vibrio* phylotypes (Roth et al., 2012a). During the present 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 strong similarities or due to immunological cross-reactivity. In contrast, 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, however, 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. Since the fathers are closely connected to the progeny during male pregnancy they probably 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; Bobe and Labbé, 2010; Swain et al., 2008). In the sex-role reversed pipefish *S. typhle* females oviposit their eggs into the male’s brood pouch where they are fertilised 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; Kvarnemo et al., 2011; Ripley and Foran, 2006; Ripley and Foran, 2009). Consequently, males and females have different possibilities to transfer non-genetic components to stimulate the offspring’s immunity. In females, a deposition into the egg yolk is most likely, while males may transfer substances through the sperm fluid and the placenta-like structure in the brood pouch. Males and females seem to have evolved different mechanisms to invest into the immune defence of their progeny.

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

Parental effects are of short duration (Lindholm et al., 2006). Yet, in line with previous findings (Roth et al., 2012b) 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), paternal effects were found 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 the 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.

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4.4. Can changes in epigenetic regulation genes be 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 according to 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 via 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 bacterial challenge. During development and maturation, DNA-methylation patterns and epigenetic marks are under constant change and highly flexible (Monk et al., 1987; Razin and Shemer, 1995). A consistent pattern of epigenetic traces can thus likely only be established after maturation when programming of the parental DNA methylome is complete (Ci and Liu, 2015). Recent data even suggest that long-lasting parental immune priming effects can be maintained via epigenetic marks and are preserved until the second generation (Beemelmanns and Roth, 2016, unpublished data). In contrast 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 (Beemelmanns and Roth, 2016, unpublished data) are mediated through inherited maternal and paternal epigenetic marks.

4.5. Are there any allocation trade-offs and costs associated with maternal, paternal and biparental immune priming?

The maintenance of induced immunity is energetically costly and, therefore, enhanced levels of immune defence in primed offspring imply trade-offs with other fitness-related traits (Lochmiller and Deerenberg, 2000; Schmid-Hempel, 2011; Zanchi et al., 2011). In line with this, TGIP exhibited costs in terms of prolonged to sexual maturity. F1-males of the biparental treatment needed significantly longer to produce 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, this 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 the current clutch due to the possible risk of not surviving the next reproductive event (Landis et al., 2015).

5. Conclusion

Our study highlights the difference between maternal and paternal immune priming in pipefish. It supports our predictions of asymmetric parental investment into offspring immunity and of 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 against current pathogens should be favoured. While maternal TGIP had a strong impact on young juveniles, it diminished during the course of offspring maturation and came with low specificity. Even though maternal effects were weaker and of lower stability, prolonged reproduction time was caused equally by both parents. However, these detrimental effects may be compensated by a 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 bacterial species, the specific type of bacterium determined the strength and degree of biparental immune priming. This led to a compensated biparental impact instead of synergistic additive biparental effects as has been shown in a previous study that used identical pathogens for both parents (Roth et al., 2012b). 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-Carduño et al., 2014). Our results indicate that TGIP has important consequences on the evolution of life history, host–pathogen coevolution and parental investment strategies. We found the first evidence for the involvement of epigenetic regulation processes like DNA-methylation and histone acetylation, which both parents affected more with increasing 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 patterns 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 contributions should be examined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.zool.2016.06.002.

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