doi:10.1111/evo.13452

Effects of parental care on resource allocation into immune defense and buccal microbiota in mouthbrooding cichlid fishes*

Isabel S. Keller,[1,2] Till Bayer,[1] Walter Salzburger,[3] and Olivia Roth[1]

[1]*Geomar, Helmholtz Centre for Ocean Research, Düsternbrooker Weg 20, 24105 Kiel, Germany*

[2]*E-mail: itanger@geomar.de*

[3]*Zoological Institute, University of Basel, Vesalgasse 1, 4051 Basel, Switzerland*

Received March 10, 2017
Accepted February 6, 2018

Sexual dimorphism is founded upon a resource allocation trade-off between investments in reproduction versus other life-history traits including the immune system. In species with conventional parental care roles, theory predicts that males maximize their lifetime reproductive success by allocating resources toward sexual selection, while females achieve this through prolonging their lifespan. Here, we examine the interrelation between sexual dimorphism and parental care strategies in closely related maternal and biparental mouthbrooding cichlid fishes from East African Lake Tanganyika. We measured cellular immune parameters, examined the relative expression of 28 immune system and life history-related candidate genes and analyzed the microbiota composition in the buccal cavity. According to our predictions, maternal mouthbrooders are more sexually dimorphic in immune parameters than biparental mouthbrooders, which has possibly arisen through a differential resource allocation into parental care versus secondary sexual traits. Biparental mouthbrooders, on the other hand, which share the costs of parental care, feature an upregulated adaptive immune response and stronger antiviral properties, while their inflammation response is reduced. Overall, our results suggest a differential resource allocation trade-off between the two modes of parental investment.

KEY WORDS: **Buccal microbiota, cichlids, mouthbrooding, parental investment, sexual dimorphism, sexual immune dimorphism.**

Sexual selection is one of the strongest evolutionary forces acting on sexually reproducing individuals (Stearns 1987) and is ultimately rooted in anisogamy (Schärer et al. 2012). With the provisioning of large eggs and small sperm, the investment per reproductive unit is larger in females compared to males (Kokko and Jennions 2008), leading to distinct selection regimes in the two sexes (Trivers 1972; Lessells 1998). To maximize lifetime reproductive success (LRS), males need to invest into attracting mating partners, for example via secondary sexual signals, while females should invest into prolonged longevity (Bateman 1948; Trivers 1972; Clutton-Brock 1991). As a consequence, females have, in general, a more efficient immune defense (Kurtz et al. 2000; Siva-Jothy 2000; Rolff 2002; Roth and Kurtz 2008). This sexual immune dimorphism is primarily regulated by female longevity and male intrasexual selection (Zuk and Stoehr 2002).

Parental investment into the offspring after fertilization (i.e., postzygotic parental care) may enhance sex-specific sexual selection via shifts in the operational sex ratio and variability in male mating success (Gonzalez-Voyer et al. 2008; Kokko and Jennions 2008). In most species, postzygotic parental care is a pure female endeavour, while males desert after fertilization (Royle et al. 2012). In teleost fishes, however, male parental care is commonly observed, either in the form of biparental or paternal care (Reynolds et al. 2002; Mank et al. 2005). In species with paternal care, males might be restricted in LRS through parental care and thus become the limiting sex, whereas females have to invest in secondary sexual signals to attract mates (Vincent 1991). Thus, sexual dimorphism resulting from parental care is expected to be small or even partially inversed in such taxa (Aisenberg and Peretti 2011; Roth et al. 2011). In species with biparental care, inter- and intrasexual selection is typically weak, decreasing the need

*This article corresponds to Spagopoulou, F., and M. P. K. Blom. 2018. Digest: Life history evolution in Darwin's dream ponds. Evolution. https://doi.org/10.1111/evo.13473.

for investment into expensive secondary sexual signals, therefore possibly attenuating sexual dimorphism (Gonzalez-Voyer et al. 2008).

In addition, during times of parental care, resources to be allocated to other life-history traits are limited, leading to a resource allocation trade-off between reproduction and other life-history traits including the immune system (Stearns 1989; French et al. 2007; Bourgeon et al. 2009; Reavey et al. 2014; Keller et al. 2017).

The immune system in fish consists of several immunological barriers (Tort et al. 2003). The first such barrier for pathogens that attempt to invade the fish' body are mucosal surfaces such as gills, skin, the buccal cavity, or the gut (Salinas 2015). A variety of different immunological components from both the adaptive and the innate immune system are thus expressed in the fish' mucus to fight the adherence of pathogenic microorganisms (Gomez et al. 2013). At the same time, the colonization of commensal microorganisms is permitted, or even facilitated (Maynard et al. 2012; Llewellyn et al. 2014). Excessive investment into parental care entailing a resource allocation trade-off could thus negatively influence the ability of the immune system to tolerate and shape a healthy microbial community (Bailey et al. 2011; Levin et al. 2016).

Examining the interrelation between sexual dimorphism and parental investment is vital to the understanding of mating system and sex role evolution. So far, studies addressing these relationships were of theoretical nature (Gonzalez-Voyer et al. 2008; Kokko and Jennions 2008; Lehtonen and Meyer 2011), or mainly focused on the interaction between size/morphology or behavioral traits (e.g., schooling, foraging, predator avoidance behavior (reviewed in (Magurran 2000)), while the immune system has largely been ignored. This is striking, given that the connection between sexual selection and immune dimorphism is well established (Rolff 2002; McKean and Nunney 2005; Roth et al. 2011).

In the present study, we examined the interrelation between sex-specific parental investment, sexual dimorphism in immune response and buccal microbiota composition in a set of closely related maternal and biparental moutbrooding cichlid fishes from Lake Tanganyika in East Africa. Cichlids are well-known for their unique taxonomic and eco-morphological diversity (Kocher 2004; Salzburger et al. 2014) but also show an outstanding variety of mating behaviors and parental investment strategies. One of the most enigmatic parental investment strategies is mouthbrooding (MB). During MB, eggs and fry are guarded in the buccal cavity, in close contact with the parental mucosa, for up to 4–6 weeks (Keenleyside 1991; Grone et al. 2012). MB, which has evolved multiple times in cichlids (Goodwin et al. 1998), may be provided by either one parent alone (maternal mouthbrooding, MMB; paternal mouthbrooding) or by both parents sequentially (biparental mouthbrooding; BPMB) (Keenleyside 1991). In any case, MB is costly, as carrying the offspring in the buccal cavity induces energy expenditure, while limiting possibilities for food uptake, and is accompanied by a higher predation and infection risk for the caregiving parent(s) (Ghalambor et al. 2004; Trumbo 2007; Knowles et al. 2009; Reardon and Chapman 2010; Keller et al. 2017).

We first hypothesized that sexual dimorphism depends on the sex-specific investment into parental care and predicted that sexual dimorphism is reduced when both sexes care for their young in an equal way (BPMB). MMB species, on the other hand, were expected to show a more pronounced sexual dimorphism. In line with this, we predict sex-specific investment into parental care to influence the crosstalk between the immune system and the buccal microbiota resulting in a more similar buccal microbiota composition between males and females in BPMB as compared to MMB species. To our knowledge, we here provide the first examination of the buccal mucosa microbial communities in MB cichlids. In our second hypothesis, we focused on the immune system and tested the prediction that the parental care system (MMB vs BPMB) determines a species' immunological activity. In MMB species, females should allocate resources toward parental investment, whereas males are expected to invest into secondary sexual signals, deducting—in both sexes—resources from the immune defense system. In BPMB, on the other hand, more resources should be available for life-history traits such as immune defense, as both sexes share the costs associated with parental investment and the need for investment into sexual signaling is reduced. We therefore expected BPMB species to generally feature a higher metabolite expression and a higher immunological activity, possibly selecting for the colonization and establishment of a more diverse buccal microbiota compared to MMB species. The existence of both MB modes within a single adaptive radiation and lake, as well as the extreme investment into parental care through MB, allows us to disentangle parental investment and strictly sex-specific life-history strategies to study the influence of parental investment on sexual dimorphism. To this end, we measured cellular immune parameters; performed comparative gene expression assays on immune, metabolism, and sex hormone genes; and examined the composition and diversity of the buccal mucosa microbiota. This permits us to pursue the dynamics of sexual dimorphism between two parental care strategies to gain insights into how parental investment governs resource allocation.

Materials and methods

SAMPLE COLLECTION

To examine brooding-mode related and sex-dependent differences in immunological activity and in buccal mucosa microbiota, we sampled a total of 279 specimens (for sample sizes see Table S1) belonging to seven cichlid species from Lake

Tanganyika in East Africa, with either maternal (*Astatotilapia burtoni*, *Tropheus moorii*, *Simochromis babaulti*, and *Interochromis lookii*) or biparental (*Eretmodus cyanostictus*, *Xenotilapia spiloptera*, and *Perrissodus microlepis*) MB mode. All specimens included in this study were collected by the authors, A. Indermaur, M. Grimm, or local divers Kedric and Adam in July 2013 and 2014 near Kalambo Lodge in Zambia (GPS coordinates: 8°37′23.6″S 31°12′01.6″E) using snorkelling or scuba-diving, and under study permits nrs. 000954 and 001994 issued by the Republic of Zambia. Non-MB adult fishes were kept alive in aerated ponds for a maximum of 6 h after catch. After killing by pithing, we recorded standard and total length, weight, and sex of each specimen. Then, swaps of the buccal mucosa were taken and placed in 70% ethanol for subsequent DNA extraction and microbiota analysis; gills were dissected and stored in in RNAlater (Qiagen) for gene expression analysis; blood, spleen, and head kidney were immediately processed for cellular immune parameter analyses in the field.

CELLULAR IMMUNE PARAMETER ANALYSIS

To determine possible differences in cell population composition and cell activity between sexes and MB modes, we subjected blood, spleen, and head kidney extractions to flow cytometric analyses in the field. To this end, we used a BD Accuri C6 Flow Cytometer® following the protocol described in Roth et al. (2011) with the modifications used for cichlids as described by Diepeveen et al. (2013). We examined blood from the caudal vein as proxy for peripheral immune reactions; head kidney, as main lymphocyte proliferation organ; and spleen, as blood filtration and pathogen neutralization organ. For cell population analysis, cells were measured in up to 10,000 counts per sample on slow flow rate. Lymphocytes and monocytes were then distinguished according to scatter plots (FSC; cell size and SSC; cell complexity). For cell activity analysis, cells were measured to 20,000 counts on medium flow rate. Dividing and resting cells were discriminated on the basis of the emission of red fluorescence (low Propidium Iodide emission: resting stage G_0/G_1; twofold Propidium Iodide emission: dividing stage G_2/S-phase). Flow cytometric measurements were analysed using predefined gating with manual adjustments for each species in the BD Accuri C6 Software (Version 1.0.264.21) (See Protocol S1 for more detailed protocol and sample gating).

IMMUNE GENE EXPRESSION ANALYSIS

To examine relative differences in gene expression in immune-, metabolite, and hormone-related genes between species as well as according to MB mode and sex, we built a gene expression assay containing 32 genes on the Fluidigm-BioMarkTM system (Biomark HD/Fluidigm Corporation, San Francisco, CA). Candidate genes were selected to represent parts of all important immunological pathways. For primer design, we used a set of pipefish and cichlid candidate genes taken from Diepeveen et al. (2013) and Beemelmanns and Roth (2016) and blasted them against available cichlid transcriptome resources from GenBank (Baldo et al. 2011; Brawand et al. 2014). The available cichlid sequences were then assembled using CodonCode Aligner (Version 3.7.1), resulting in one cichlid multiple species alignment per gene. Conserved sequence regions were subsequently uploaded in the web-based software PRIMER3 (Version 4.0.0, (Koressaar and Remm 2007; Untergasser et al. 2012)) for primer picking. All primers were tested for specificity against all target species using pooled cDNA from all species in a quantitative real-time PCR experiment with 5 × HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne). Primer pairs ($N = 32$) were selected, with a threshold of efficiencies of above 90%, and standard curves with slopes of log quality versus threshold cycle (C_t) between –3.5 and 3.2 (see Table S2).

For RNA extractions from the immunologically active gill tissue (up to 15 specimens per species and sex) we used the RNeasy 96 Universal Tissue Kit (Qiagen) following the manufacturers protocol (Birrer et al. 2012; Beemelmanns and Roth 2016). RNA yield was measured by spectrometry (NanoDrop ND-1000; peQLab); 200 ng/μL were then used for reverse transcription reactions with the QuantiTect® Reverse-Transcription Kit (Qiagen). From the samples with sufficient RNA yield, we determined relative gene expression of 32 genes with the Fluidigm-BioMarkTM system based on 96.96 dynamic arrays (GE-Chip). Each chip run included three technical replicates, a negative control (HPLC H_2O), and a –RT control to test for residual gDNA (see protocol S2 for detailed information).

MICROBIOTA ANALYSIS OF THE BUCCAL MUCOSA

Mucosa swabs of the buccal cavity were stored in ethanol at around 4°C until arrival in the laboratory at GEOMAR, Germany, and then frozen at –20°C before further use. For in-depth analysis, we randomly selected a subset of 10 samples per sex and species. Ethanol was evaporated at 60°C before DNA extraction using DNeasy 96 blood and tissue kit (Qiagen) following the manufacturers protocol. DNA was then stored at –20°C. We are aware that this method could lead to an underrepresentation of Gram-positive bacteria (Yuan et al. 2012) in all treatments, which would however not affect the comparison of buccal microbiota between parental care strategies, as bacterial phyla sampled would be consistent for all treatments.

For amplification and sequencing of the V4 domain of bacterial 16S rRNA, we used the PCR primers F151 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al. 2011), adding index, barcode, pad, and linker sequence as described in Kozich et al. (2013) to each primer (for primers used see Table S3). After amplification (1 × 30 s; 98°C; 30 × (9 s; 98°C,

15 s; 55°C, 20 s; 72°C); 1 × 10 min; 72°C) PCR products were visualized by gel electrophoresis and purified with the MinElute 96 PCR purification Kit (Qiagen) following the manufacturers protocol. Between 20 and 40 ng of each sample were pooled per plate and PCR products were cleaned using the Nucleospin Gel & PCR clean up (Marcherey-Nagel). After elution from the gel the purified product was pooled in equimolar amounts (~40 ng/μL) to one final pool. Following the protocol of Kozich et al. (2013), 16S rRNA amplicons were sequenced on a MiSeq sequencer (Illumina). All sequence data is accessible in the NCBISequence Read Archive under BioProject PRJNA430528. Sequence raw reads were quality filtered and assembled using MOTHUR version 1.16.1 (Schloss et al. 2009). After merging, reads were aligned against the SILVA alignment database (release 119) and sequences not covering the v4 region (SILVA alignment position 1968–11550) were removed (Pruesse et al. 2007). A preclustering step (allowing 2 bp difference) was applied to reduce sequencing noise: chimeric sequences were removed using the MOTHUR implementation UCHIME (Edgar et al. 2011). Taxonomic classification was performed against the RDP database (Cole et al. 2014) using the CLASSIFY.SEQS function with a bootstrap cut-off of 80%. To obtain operational taxonomic units (OTUs), sequences were clustered at a 0.03 distance level. Additionally, the data were subsampled to 5000 reads per sample for further analysis. Sample sizes after processing and subsampling are shown in Table S1.

STATISTICAL ANALYSIS

All data were analyzed and visualised using R (R 3.0.2). The same statistical model, with "sex" and "MB mode" and "species" nested in "MB mode" as fixed factors was used for all analyses. The factor "species" was nested in "MB mode," as species determines MB mode and thus the two factors are nonindependent. Using this model, the interpretation of species-specific effects remains possible while, at the same time, accounting for the nonindependence of the factors "species" and "MB mode". However, we would like to note that this model limits our conclusions to the set of species used and does not allow to draw general inferences about other MB species. Differences in sample numbers between the analyses can be explained by either too low RNA yield for gene expression analysis or failed runs on the flow cytometer during fieldwork. Pagel's λ was calculated for all variables to determine the strength of the phylogenetic signal for each parameter (Pagel 1999; Münkemüller et al. 2012; Cooper et al. 2016; Anderson and Wiens 2017) (R package PHYTOOLS, command phylosig (Revell 2012)).

FLOW CYTOMETRY DATA

Cell population measurements were comprised of lymphocyte and monocyte counts, whereas cell activity measurements indicate the amount of cells in the resting (G_0/G_1 phase of the cell cycle) versus the dividing stage (G_2/S phase of the cell cycle) (for final sample sizes, see Table S1). Normal distribution and variance homogeneity was achieved using Box-Cox transformation. Interaction of the factors "sex," "brooding mode," and "species" nested in brooding mode were analyzed in a nested ANOVA (aov(x~sex × MB_mode + species%in%MB_mode); post hoc tests of significant results were done using TukeyHSD tests. For visualization purposes, all samples were divided by the mean over all males from maternal MB species, which sets males from maternal brooding species as control.

GENE EXPRESSION DATA

Mean C_t, standard deviation (SD), and coefficient of variance (CV) were calculated for each sample triplicate. Single values with a CV lower than 4% were replaced by the mean value over all samples and treatments per gene. If more than half of the values per gene were below 4%, the whole gene was excluded. According to this threshold, two genes (ras-related protein and dicentracine) were removed from the dataset. Average expression stability was evaluated using geNorm of the program BIOGAZELLE GBASE+ version 3.0 (Vandesompele et al. 2002; Mestdagh et al. 2009). The two genes with the most constant expression over all tested samples were taken as reference genes (*F2RL1* and *ADNPB*). To standardize relative gene expression assays, the mean of the two reference genes was subtracted from the mean C_t value of the gene of interest, i.e. $-\Delta C_t$ values were used for statistical analysis (as in (Beemelmanns and Roth 2016)). In total, we thus obtained $-\Delta C_t$ values for 28 genes.

A Box-Cox transformation was used to accomplish both normal distribution and variance homogeneity, if necessary. To disentangle the effects of sex and MB mode on gene expression, we used the same model as for the flow cytometric analysis in a multivariate approach. Genes were grouped into six nonmutually exclusive categories: (i) all genes combined (28 genes); (ii) adaptive immune system genes (11); (iii) innate immune system genes (10); (iv) all immune system genes combined (21), (v) sex-related genes (3); and (vi) metabolism genes (4). A nested PERMANOVA (adonis(x~ sex × MB_mode + species % in % MB _mode, method = "euclidean," permuations = 1000)) was run over the data. ANOVA (aov(x~sex × BM + species%in%MB_mode)) and Tukey HSD tests were performed on all genes in which significant effects were detected in the PERMANOVA and the ANOVA (Beemelmanns and Roth 2016). Gene expression patterns across all genes were visualized with a heatmap (R package MNF, command aheatmap (Gaujoux and Seoighe 2010)). Additionally, interactive effects of sex and MB mode are additionally shown as bar charts with $-\Delta\Delta C_t$ values: the mean ΔC_t value of males from maternal brooding species was subtracted from each sample. Genes affected by MB mode are depicted as $-\Delta C_t$ values.

Table 1. Nested ANOVA results of immune cell parameter: significant *P* values ($P < 0.05$) are marked in bold letters.

	Lymphocyte/monocyte					Active/inactive cells				
Blood	Df	SS	MS	*F* value	Pr(>*F*)	Df	SS	MS	*F* value	Pr(>*F*)
Sex	1	2.15×10^{-8}	2.15×10^{-8}	1.96	0.163	1	4.17×10^{-9}	4.17×10^{-9}	5.42	**0.021**
MB mode	1	2.02×10^{-9}	2.02×10^{-9}	0.18	0.668	1	2.46×10^{-9}	2.46×10^{-9}	3.19	0.076
Sex × MB mode	1	1.07×10^{-9}	1.07×10^{-9}	0.10	0.755	1	3.12×10^{-9}	3.12×10^{-9}	4.05	**0.046**
Species in MB mode	5	2.03×10^{-7}	4.05×10^{-8}	3.69	**0.003**	5	1.50×10^{-8}	3.00×10^{-9}	3.90	**0.002**
Residuals	233	2.56×10^{-6}	1.10×10^{-8}			171	1.32×10^{-7}	7.70×10^{-10}		
Spleen										
	Df	SS	MS	*F* value	Pr(>*F*)	Df	SS	MS	*F* value	Pr(>*F*)
Sex	1	1.23×10^{-7}	1.23×10^{-7}	7.64	**0.0062**	1	1.73×10^{-8}	1.73×10^{-8}	3.40	0.067
MB mode	1	5.83×10^{-7}	5.83×10^{-7}	36.16	**<0.001**	1	2.00×10^{-11}	2.35×10^{-11}	0.00	0.946
Sex × MB mode	1	1.37×10^{-8}	1.37×10^{-8}	0.85	0.3582	1	1.30×10^{-8}	1.30×10^{-8}	2.56	0.111
Species in MB mode	5	1.78×10^{-6}	3.55×10^{-7}	22.05	**<0.001**	5	3.13×10^{-8}	6.26×10^{-9}	1.23	0.296
Residuals	234	3.77×10^{-6}	1.61×10^{-8}			189	9.61×10^{-7}	5.09×10^{-9}		
Head kidney										
	Df	SS	MS	*F* value	Pr(>*F*)	Df	SS	MS	*F* value	Pr(>*F*)
Sex	1	6.22×10^{-8}	6.22×10^{-8}	2.83	0.094	1	2.14×10^{-8}	2.14×10^{-8}	1.33	0.250
MB mode	1	1.02×10^{-8}	1.02×10^{-8}	0.46	0.496	1	5.00×10^{-8}	5.00×10^{-8}	3.10	0.080
Sex × MB mode	1	3.21×10^{-8}	3.21×10^{-8}	1.46	0.228	1	3.20×10^{-9}	3.18×10^{-9}	0.20	0.658
Species in MB mode	5	1.86×10^{-6}	3.71×10^{-7}	16.89	**<0.001**	5	1.43×10^{-7}	2.86×10^{-8}	1.77	0.120
Residuals	235	5.16×10^{-6}	2.20×10^{-8}			199	3.21×10^{-6}	1.61×10^{-8}		

MIRCOBIOTA ANALYSIS OF THE BUCCAL CAVITY

To examine β-diversity of the microbial community structure in the buccal cavity of MB cichlids, we calculated a Bray Curtis dissimilarity matrix and performed a nested PERMANOVA with "sex" and "MB mode" as factors and "species" nested in MB mode (adonis(x∼ sex × MB_mode + species % in % MB_mode, method = "bray," permutations = 10,000)) for all samples and the 50 most frequent OTUs. Further resolution on the OTUs driving such an effect was achieved with a multilevel pattern analysis with a significance level of 0.05 (R package INDICSPECIES, command indval (Dufrêne and Legendre 1997)) was applied on the 50 most frequent OTUs. For visualization, a principle component analysis (PCA) was run on the 50 most frequent OTUs. Inverse Simpson index (1/D) was calculated for samples with more than 5000 reads in MOTHUR (Version 1.39.0) as a proxy for α-diversity. The inverse Simpson index was log transformed to achieve normality and variance homogeneity before running a nested ANOVA (aov (x ∼ sex × MB_mode + species %in% MB_mode) to investigate differences between the sample groups (sex, MB_mode, and species nested in MB_mode).

Results

CELLULAR IMMUNE COMPONENTS

Cell activity analyses were based on the information about the cell-cycle stage of adaptive immune cells. In blood, the proportion of active to inactive immune cells was higher in females of MMB species compared to females of BPMB species and to males of both MMB and BPMB species (Nested ANOVA sex × MB_mode; $F_{5,171} = 4.0486$, $P < 0.05$). There was also an overall sex effect: females had in general a higher proportion of active adaptive immune cells in the blood (Nested ANOVA sex; $F_{1,171} = 5.4206$, $P < 0.05$). No effects were found in head kidney and spleen. The nested factor (species in MB_mode) was significant in all of the above analyses (see Table 1; Fig. 1A). Immune cell population composition was affected by MB mode and sex in spleen tissue: BPMB species had a higher ratio of innate to adaptive immune cells than MMB species (Nested ANOVA MB_mode; $F_{1,234} = 36.1613$, $P < 0.0001$), indicating a generally higher amount of innate immune cells in BPMB species. Females, independent of MB mode, had a lower ratio of innate to adaptive immune cells than males (nested ANOVA sex; $F_{1,234} = 7.6391$, $P < 0.01$). Blood and the head kidney cellular immune parameters were neither affected by MB mode nor by sex and no interactive effect of MB mode and sex was found (see Table 1; Fig. 2A). No strong phylogenetic signal was detected in either of the examined variables (Pagel's $\lambda << 1$; Table S4).

GENE EXPRESSION

The main effect MB mode and the interaction of MB mode and sex significantly influenced gene expression in five gene categories (all except metabolism genes), according to the nested

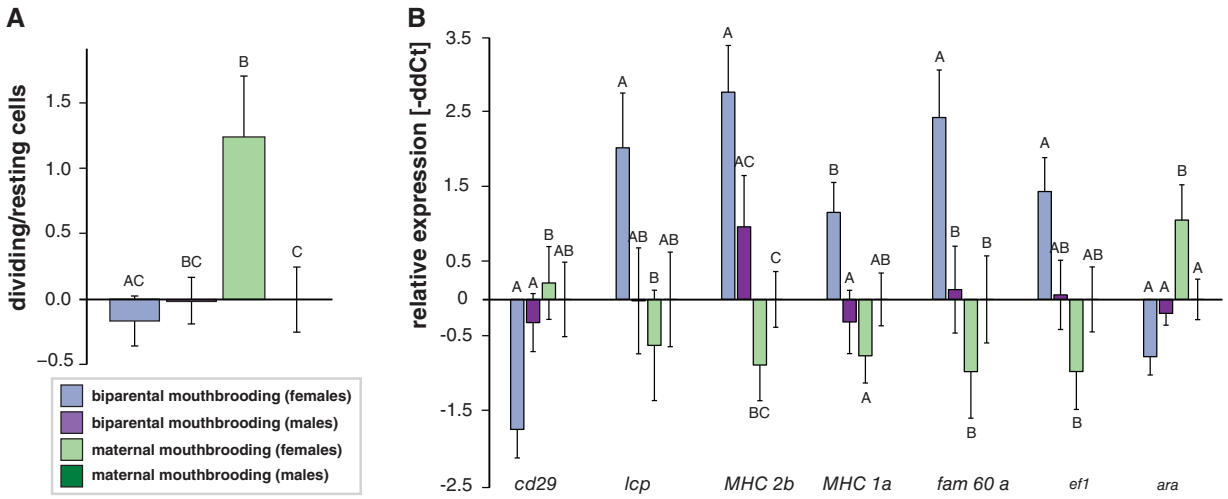

Figure 1. Interactive effects of sex and brooding mode on cell activity and candidate gene expression: Both graphs show group means and standard errors. Lettering denotes significant differences. BPMB species are greenish (females light green, males dark green) and MMB species are violet (females light and males dark). Only genes affected by the interaction of sex and brooding modes are shown. (A) Proportion of active to inactive adaptive immune cells of the blood. All samples are displayed relative to males of MMB species. (B) Relative gene expression of all interaction effects. $-\Delta\Delta C_t$ values relative to male maternal mean gene expression.

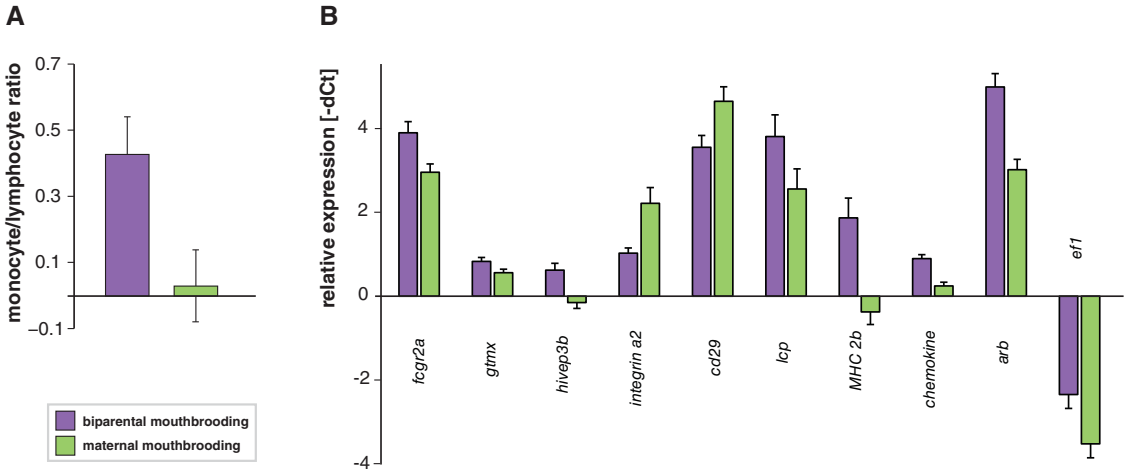

Figure 2. Effects of brooding mode on cell population and candidate gene expression: Both graphs show group means and standard errors. Only significant results are shown. BPMB species are in green, MMB species in violet. (A) Proportion of innate to adaptive immune cells of the spleen. (B) Relative gene expression of all brooding mode effects. $-\Delta C_t$ values show relative expression.

PERMANOVA. Metabolism genes were the only category exclusively affected by the interaction of MB mode and sex but not by the main effects sex or MB mode. Sex had no effect on the expression of the genes examined here (Table 2; Fig. 3). Univariate analyses (ANOVA) revealed that interactive effects of MB mode and sex affected four of the 11 genes belonging to the adaptive immune response (*MHC1a*, *cd29*, *MHC2b*, *lcp*) (Table S5; Fig. 1B). Females from BPMB species upregulated *lcp* expression (promoting T-cell development and activation) compared to females of MMB species. *MHC1a* (presentation of antigens to cytotoxic T cells) was upregulated in females of BPMB species compared to BPMB males and females of MMB. *Cd29* (*integrin beta 1*, which builds heterodimers with *integrin alpha 2* enhancing adhesion of T cells (Boisvert et al. 2010) showed the opposite pattern to *MHC1a*, that is *cd29* was upregulated in both sexes of MMB species compared to females of BPMB. *MHC2b* expression was higher in BPMB and MMB males and in BPMB females than in MMB females. Differences in adaptive immune gene expression between MB modes were found in eight genes. Five genes (*fcgr2a*, *hivep3b*, *gtmx*, *MHC1a*, and *MHC2b*) were upregulated in BPMB species, whereas three genes (*cd29*, *integrin alpha 2*, *MHC1*) were upregulated in MMB species (Fig. 2B).

Regarding the innate immune genes, we found an interactive effect of MB mode and sex on the expression of one gene, *fam60a*,

Table 2. Nested PERMANOVA results of candidate gene expression: PERMANOVA to assess effects of sex, MB mode the interaction and the nested factor "species" on the relative expression of candidate genes (ΔC_t values).

PERMANOVA									
	Model	**Sex**		**MB mode**		**Sex * MB mode**		**Species in MB mode**	
Gene categories	*R2*	*F. Model*	*Pr (>F)*	*F. Model*	*Pr (>F)*	*F. Model*	*Pr (>F)*	*F. Model*	*Pr (>F)*
All genes	0.76	0.49	0.780	5.49	**0.003**	3.47	**0.019**	8.34	**0.001**
Immune system	0.78	0.55	0.653	4.96	**0.010**	3.42	**0.026**	7.30	**0.001**
Adaptive IS	0.75	0.74	0.551	7.08	**0.002**	4.04	**0.011**	8.46	**0.001**
Innate IS	0.81	0.37	0.784	2.99	**0.050**	2.85	**0.047**	6.24	**0.001**
Metabolism genes	0.76	0.25	0.803	3.18	0.063	4.12	**0.023**	8.52	**0.001**
Sex-related genes	0.57	0.95	0.410	17.53	**0.001**	3.19	**0.043**	19.57	**0.001**
Df Residuals/Model	159								
Df Total	167								

Significant results ($P < 0.05$) are marked in bold letters. Results of the univariate posthoc analysis (ANOVA) can be found in Table S5.

which was upregulated in BPMB females (Fig. 1B). An effect of brooding mode was detected in two genes (*fam60a*, *chemokine*), which are upregulated in BPMB species (Fig. 2B). In the sex-related genes, MMB females showed a higher expression in *ara* than the other three groups examined (Fig. 1B). Probably driven by this, also the MMB species combined had a higher expression of *ara* compared to BPMB species. *Arb* showed higher expression levels in BPMB species (Fig. 2B). One of the four metabolism related genes, *ef1,* showed a differential expression pattern, with a higher level of gene expression in BPMB females compared to MMB females (Fig. 1B), resulting in a higher expression of *ef1* in BPMB species overall (Fig. 2B). A summary of all results is provided in Tables 2 and S5. A phylogenetic signal was detected in four (*arb*, *chemokine*, *hivep3b,* and *hsp60*) out of the 28 genes (Table S4).

MICROBIOTA COMMUNITY OF THE BUCCAL MUCOSA

The microbial community of the buccal mucosa over all samples consisted of 7014 OTUs belonging to 750 genera in 28 phyla. The four most abundant phyla, *Proteobacteria* (63.5%), *Bacterioidetes* (14%), *Firmicutes* (9%), and *Actinobacteria* (8%), cover 94.5% of all sequences found. The most abundant genus is *Acinetobacter* (20%) followed by *Halomonas* (7%), *Reinheimera* (6%), *Aeribacillus* (4%), *Aeromonas* (4%), *Bacteriodetes* (4%), *Pseudomonas* (3%), and *Flavobacterium* (3%), which, together, account for 51% of the genus diversity in the buccal mucosa microbial community (Fig. S1 and S2).

The microbiota composition (β-diversity) of all sampled OTUs in the buccal mucosa differs between the two MB modes (nested PERMANOVA MB_mode; $F_{1,97} = 9.25$, $P < 0.0001$). Neither sex nor the interaction between sex and MB mode had an effect on the microbial community (nested PERMANOVA sex; $F_{1,97} = 1.34$, $P = 0.16$/MB_mode × sex; $F_{1,97} = 1.09$, $P = 0.32$). The significance of the nested factor (nested PERMANOVA: species in MB mode, $F_{5,97} = 0.18$, $P < 0.0001$) indicates that variance is being introduced by the nested factor "species." By removal of rare OTUs and performing the analysis with the 50 most frequent OTUs, the same pattern (differences between MB modes and high variance introduced by the nested factor species) was revealed (Fig. 4). Such species-specific variance in buccal mucosa microbiome is to be expected through the differential habitat and dietary preferences of the sampled species (Muschick et al. 2012; Larsen et al. 2013). The observed differences in the 50 most frequent bacterial OTUs in the cichlids' buccal mucosa can be explained by either brooding mode (nested PERMANOVA MB_mode; $F_{1,97} = 10.69$, $P < 0.0001$) or the nested factor "species" (nested PERMANOVA: species in MB_mode, $F_{5,97} = 4.86$, $P < 0.0001$). No sex effects or interactive effects of MB mode and sex were detected (nested PERMANOVA sex; $F_{1,97} = 1.34$, $P = 0.14$/MB_mode × sex; $F_{1,97} = 1.00$, $P = 0.41$). Indicator species analysis of the 50 most frequent OTUs showed a total of 11 OTUs to be driving the differences between MMB and BPMB (Table 3). Five OTUs (*unclassified Cryomorphaceae*, *Fluvicola*, *Erythrobacter*, *unclassified Bacteria*, *Dyadobacter*) were associated with biparental brooding species, whereas eight OTUs (*Aeromonas*, *Aeromicrobioum, unclassified Enterobacteriaceae*, *unclassified Acidobacteria*, *Shewanella*, *unclassified Microbacteriaceae*, *Empedobacter*, *Sphingobacterium*) were associated with MMB species (Fig. 4). There were no differences in microbial community richness (α-diversity, inverse Simpson index) between sexes, MB mode or the interaction between these two factors (nested ANOVA sex: $F_{1,89} = 0.211$, $P > 0.647$; brooding mode: $F_{1,89} = 3.805$, $P > 0.054$; sex × brooding mode: $F_{1,89} = 14.66$, $P > 0.411$). As already indicated in the

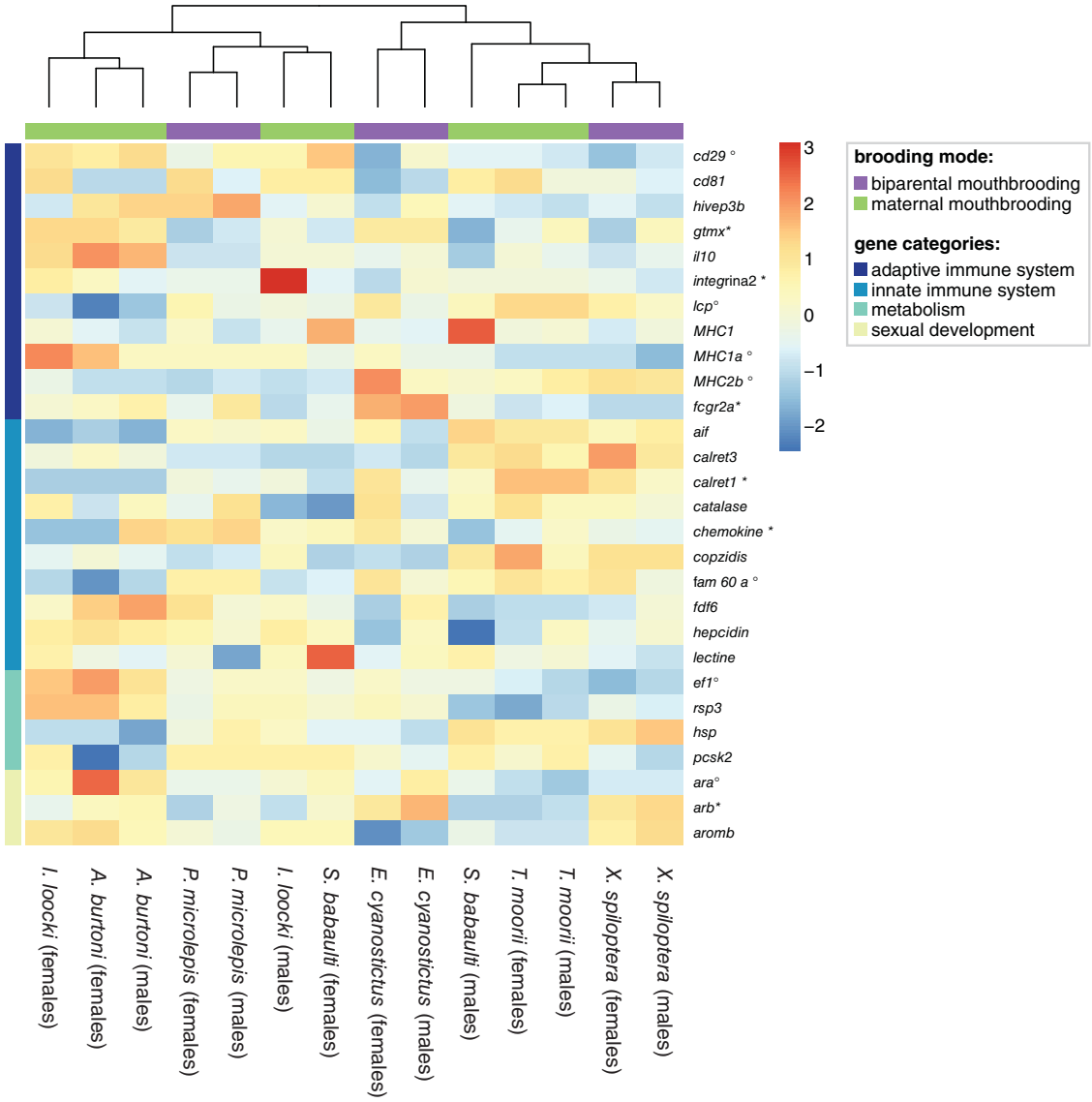

Figure 3. Heat map of gene expression data: Means over species and sex (f. for females and m. for males) were calculated and hierarchically clustered. Z-scores per gene depict expression strength. Color code of the columns depict brooding mode (BPMB (violet) or MMB (green)). Color code of the rows depict gene grouping. Genes marked with * have a brooding mode dependent expression; genes marked with ° have a sex and brooding mode-dependent expression.

β-diversity analysis, the factor "species" nested in MB mode, can be accounted for introducing variance in the model (nested ANOVA species in brooding mode: $F_{5,89} = 6.282$, $P < 0.001$). A phylogenetic signal was detected in four of the 50 most frequent OTUs (*Erythrobacter*, *Empedobacter*, *Cryamorphacea*, and *unclassified Bacteria*) (Pagel's λ ~0.9, Table Ss4).

Discussion

In this study, we investigated the relationship between sex-specific parental investment and sexual immune dimorphism, and its effects on the buccal microbiota in two different parental care modes, BPMB and MMB, in seven species of cichlid fishes from Lake Tanganyika. To this end, we measured immune cell parameters, the expression of candidate genes, and examined buccal mucosa microbiota composition and diversity to assess the influence of maternal and biparental care on sexual immune dimorphism in MB cichlids.

MATERNAL MOUTHBROODING CICHLIDS ARE MORE SEXUALLY DIMORPHIC THAN BIPARENTAL MOUTHBROODERS

We first examined the hypothesis that the intensified maternal investment will increase sexual dimorphism in MMB species,

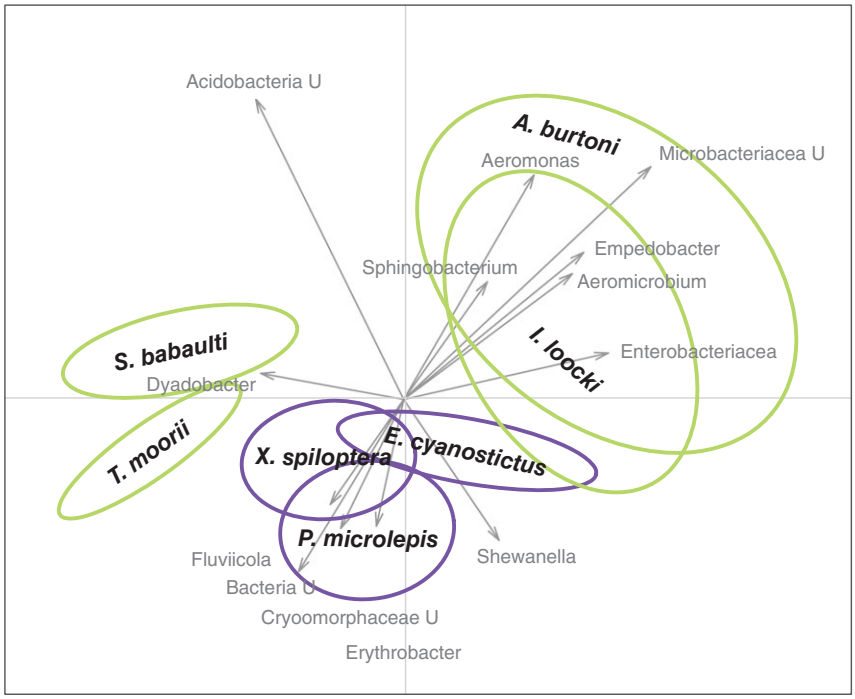

Figure 4. Principal component analysis (PCA) of the most frequent (top 50) OTUs in the buccal mucosa of PBMB and MMB species: MMB species are depicted in green, BPMB species are shown in violet. Arrows show canonical values of indicator species. Percentage of variance explained principle component 1 is 15.2% and for principle component 2 11.0%.

Table 3. Results from the multilevel pattern analysis of the top 50 OTUs: OTU numbers sorted according to their frequency from top to bottom within BPMB and MMB.

BPMB	OTU	Phylum	Genus	A	B	GOF	*P* value
	Otu00029	Bacteroidetes	Cryomorphaceae U	0.91	0.75	0.83	0.001***
	Otu00030	Bacteroidetes	Fluviicola	0.94	0.75	0.84	0.001***
	Otu00039	Proteobacteria	Erythrobacter	0.95	0.63	0.77	0.002**
	Otu00053	Bacteroidetes	Dyadobacter	0.68	0.7	0.688	0.005**
	Otu00050	Bacteria U	Bacteria U	0.95	0.90	0.93	0.001***
MMB	Otu00005	Proteobacteria	Aeromonas	0.95	0.95	0.95	0.001***
	Otu00008	Actinobacteria	Aeromicrobium	0.99	0.3103	0.555	0.042*
	Otu00011	Proteobacteria	Enterobacteriaceae U	0.82	0.74	0.78	0.002**
	Otu00033	Acidobacteria	Acidobacteria U	0.85	0.74	0.80	0.001***
	Otu00037	Proteobacteria	Shewanella	0.97	0.76	0.86	0.001***
	Otu00038	Actinobacteria	Microbacteriaceae U	0.89	0.93	0.91	0.001***
	Otu00041	Bacteroidetes	Empedobacter	1.00	0.28	0.53	0.005**
	Otu00044	Bacteroidetes	Sphingobacterium	0.92	0.83	0.87	0.001***

Phylum and genus of indicator species are listed. "A values" (specificity) shows the percentage of occurrence of the OTU within one MB mode. "B values" (fidelity) values show probability of finding the OTU within this MB mode. GOF is the goodness of fit of this model. Only MB mode-specific OTUs are shown.

while the shared parental investment displayed by BPMB species will attenuate sexual dimorphism. We indeed found that females of MMB species have an increased immune cell activity in blood compared to males, while no sexual immune dimorphism was found in BPMB species. We also detected that females of MMB species have a higher proportion of adaptive immune cells, supporting the hypothesis that parental investment drives sexual immune dimorphism. Overall, and independent of MB mode, females featured a larger proportion of adaptive immune cells compared to males. Interestingly, the cellular immunological analyses and the gene expression assays revealed different results with respect to the activity of the adaptive immune system in different tissues: MMB females showed an elevated proliferation rate of adaptive immune cells in the blood, whereas BPMB species

showed an upregulation of adaptive immune genes in the spleen. This implies that the two organs have distinct functions that are most likely explained by varying immunological activities.

MMB males and females differed in the expression of *ara*, which was upregulated in females, thereby contradicting our hypothesis that androgens should generally be upregulated in males for the establishment of secondary sexual signals. On the other hand, androgens are known to act as immune suppressors (Slater et al. 1995) and may thus negatively impact the expression of immune genes in females of MMB species. In cichlids, the expression of *ara* has been proposed to be tissue- and species-specific, yet independent from sex (Böhne et al. 2014) and possibly subjected to neo-functionalization after duplication (Lorin et al. 2015). Two genes, *MHC 1 alpha* and *fam60a*, differentiated in their expression between BPMB males and females. *MHC1 alpha* is, among other functions, responsible for presenting antigens from intracellular pathogens to cytotoxic T cells, thus inducing the elimination of the infected cells (Nakanishi et al. 2002). A downregulation of its expression could potentially indicate a lower capacity to react toward intracellular pathogens in BPMB species. *Fam60a* inhibits the expression of the TGF beta signaling pathway, which reduces the inflammatory response (Muñoz et al. 2012; Smith et al. 2012). The observed sexual immune dimorphism in *fam60a* in BPMB species contradicts the pattern of hierarchical clustering over all candidate genes in which species with BPMB cluster together. These differential patterns could be explained by high variances in sexual dimorphism among the MMB species (see above). We note that we only examined a small fraction of genes (28) differentially expressed between sexes and MB modes; however, we provide first insights into how parental investment-related sexual dimorphism is reflected in gene expression patterns.

As opposed to a previous study focusing on sex differences in the gut microbiota of the largemouth bronze gudgeon (Li et al. 2016), we did not find a sex difference or interactive effects "sex" and "MB mode" on the α-diversity of the buccal microbiota. This contradicts our prediction that male and female MMB cichlids should differ in the microbial community diversity in the buccal cavity due to differential investment in the immune system. In any case, our data suggest that sex-specific parental investment, as is the case in MMB cichlids, does not influence microbial diversity in the buccal cavity. The buccal mucosa microbiota show a certain amount of species specificity as implied by the significance of the nested factor ("species in MB mode"), such species specificity has already been found in fish skin microbiota (Larsen et al. 2013). However, diet and habitat alone cannot explain the grouping of cichlid species seen in Figure 4. According to their gut content (see (Muschick et al. 2012) for details), both *T. moori* and *I. looki* should be grouping together with *E. cyanostictus* as all feed on aufwuchs, whereas *S. babaulti* and *A. burtoni* should build one group including *X. spiloptera* (diet: molluscs, plants, sand). *P. microlepis* would be the outgroup ingesting only scales of other fish. Effects of brooding mode on the buccal microbiota key OTUs (Fig. 4) can be masked by such dietary differences among the cichlid species. Still, the grouping of the buccal microbiota key OTUs according to species with the same brooding mode persists as a pattern in the PCA (Fig. 4), which shows the strong effect of brooding mode on the buccal microbiota. We here specifically discussed the interaction of the immune system and the microbiota. However, additional factors may largely influence the microbial community. The mouthbrooding sex should be selected to optimize the buccal microbiota for a successful upbringing of the juveniles, even if this comes at the cost of a suboptimal capability to accomplish other tasks, such as secretion and protection. As a consequence, sexual buccal microbiota dimorphism can be rather expected in MMB cichlids than in BPMB, as only in MMB cichlids tasks differ between sexes. In addition to this, the swapping of juveniles in BPMB cichlids may enhance the exchange of buccal microbiota between sexes, leveling out the differences between males and females. Addressing changes of the buccal mucosa microbiome according to different habitats and food sources in combination with parental care modes and the immune system would be an extremely interesting subject for further studies.

A RESOURCE ALLOCATION TRADE-OFF BETWEEN PARENTAL CARE AND THE IMMUNE SYSTEM

We hypothesized an existing resource allocation trade-off between investing into parental care (in our case MB) versus resource allocation into life-history traits including the immune system (Stearns 1989; Santos and Nakagawa 2012; Carlton et al. 2014; Theis et al. 2017). Accordingly, in BPMB species both males and females were expected to allocate more resources into life-history traits and, thus, to have a more potent immune system, compared to MMB species. This is because in BPMB species both sexes share the costs of parental care, whereas in MMB species females bear the full costs of brood care implying that males should invest more into secondary sexual signals. Our results support this prediction, as BPMB species have a higher ratio of innate to adaptive immune cells in spleen, which is indicative of an improved and increased immediate immune response. Furthermore, BPMB cichlids show an upregulation in eight immune-related genes. Interestingly, five of these genes (*fcgr2a* (antigen receptor and complement activation (Tort et al. 2003)), *hivep3b* (V(D)J recombination and enhancing MHC binding (Diepeveen et al. 2013)), *lcp* (T-cell development and activation (Morley 2012)), *MHC2b* (antigen presentation to CD4+ T cells (Fischer et al. 2006)), *chemokine receptor 9* (T-cell activation (Uehara et al. 2002)) are related to either the development, activation, or binding of T-helper cells, which mediate humoral immune responses. This is in line with the results of our first hypothesis (Fig. 1B), that is

that BPMB females have an elevated capacity of T-cell response. Additionally, BPMB species seem to have a better antiviral activity, as suggested by the observed upregulation of the GTP-binding protein MX (*gtmx*) (Robertsen 2006), and a higher metabolic rate, as suggested by their higher levels of *EF1* expression important for protein elongation (Jürss et al. 1992). Together, these results are suggestive of a less intense resource allocation trade-off between reproduction and other life-history traits in BPMB cichlids.

Only two genes were downregulated in BPMB compared to MMB species, *integrin beta 1* (*cd29*) and *integrin alpha 2*. Both genes encode for components of proinflammatory Th17 T cells membranes, which are, among other functions, responsible for cell migration and mounting an inflammation response (Boisvert et al. 2010). Downregulation of these genes might thus lead to a decreased inflammation response in BPMB species, that is, an immune reaction to nonself-particles. We speculate here that a reduced inflammation response is actually advantageous in the context of BPMB, where eggs (and later larvae) are transferred from one parent to the other: a too strong inflammation response could harm the offspring upon transfer into the other parent's buccal cavity.

Resource allocation away from the immune system to other life-history traits may distort the communication between the microbiota and the immune system, which is a requirement for the establishment of a healthy microbiota (Maynard et al. 2012; Boutin et al. 2013; Llewellyn et al. 2014). On the other hand, microbiota are indispensable for their hosts immune system maturation and protection against invasion by pathogens (Smith et al. 2007; Fraune and Bosch 2010; Pradeu 2011; Sommer and Bäckhed 2013). Through this reciprocal interaction of the immune system and the microbiota a shift in microbial composition (α-diversity and β-diversity) could negatively feedback on the immune reaction (Boutin et al. 2013; Wang et al. 2017). Thus differences in the microbial composition of the buccal cavity between MMB and BPMB could indicate differential allocation of resources to the immune system resulting from higher investment of MMB species in either parental care (females) or secondary sexual signals (males). Indeed, the microbial communities of the three BPMB species, *E. cyanostictus*, *P. microlepis*, and *X. spiloptera*, form a distinct group in the principal component analysis (Fig. 4). The other two groups entail *S. babaulti* and *T. moorii* as well as *A. burtoni* and *I. loockii,* all four species being MMB. While the microbial indicator species (microbial OTUs characteristic to one brooding mode) of the three BPMB species all belong to Gram-negative bacteria, the microbial indicator species of the four MMB species are a mixture of Gram-positive and Gram-negative bacteria. Within the microbial community, both types of bacteria could enhance differential immunological functions as part of the immunological modulation assigned to the microbiota (Martner 2009; Gomez et al. 2013). This differential composition of the buccal cavity microbiota between MMB and BPMB species could thus underline the observed immune gene expression patterns, as the immunological activity of an individual might affect its microbiota and as such be a proxy for differential resource allocation between MMB and BPMB species. Microbiota composition is, in general, highly species specific (Larsen et al. 2013), mainly due to the inherent life styles and habitats. This could entail that species-specific differences in microbiota mask distinct indicator species specific to a certain mode of MB. As opposed to β-diversity, microbial α-diversity did not differ between MMB and BPMB species with respect to species richness, diversity and species distribution.

We would like to note that the main findings reported here are unlikely to be strongly confounded by phylogeny, even though the four MMB species are phylogenetically closer related to one another than to the three BPMB taxa (the MMB species all belong to the Haplochromini, whereas the BPMB species belong to three other tribes—Eretmodini: *E. cyanostictus*; Ectodini: *X. spiloptera*; Perissodini: *P. microlepis*; see (Muschick et al. 2012; Meyer et al. 2015). All species are nevertheless part of the same adaptive radiation within the Lake Tanganyika mouthbrooders (Salzburger et al. 2005). Importantly, principal component analysis of cellular immune parameter (Fig. S3A), gene expression (Fig. S3B), and microbial composition (Fig. 4)) as well as low phylogenetic signals as measured by Pagel's λ do not reflect phylogenetic relationships.

In this study, we attempted to shed light on the vast field of parental investment evolution and associated trade-offs in the immune system using differential parental investment strategies. We detected effects of parental care strategies on sexual immune dimorphism in immune gene expression and microbial communities in MB cichlid fish. So far, the interrelation between sexual immune dimorphism and parental care patterns has not been addressed thoroughly. This is surprising given the connection between immune dimorphism and sex specific life-history traits and thus the potential dependency on parental investment (Houston et al. 2013; Vincent and Gwynne 2014). However, the interdependence of parental investment, sexual selection, and mating system renders it difficult to study and pinpoint reasons of trade-offs detached from the parental care system. We thus suggest that the integration of factors influencing sexual selection, such as for example mating system or operational sex ratio in addition to parental care systems would provide a more holistic picture regarding immune dynamics under differential sexual selection.

Conclusion

In conclusion, sexual immune dimorphism is present in MMB species but not in BPMB species in cellular immune parameter. In addition, females of MMB species generally have a lower

expression of immune-related genes, possibly due to a resource allocation trade-off between investment into the immune system and expenditure during longer phases of parental care. No connection between innate immune system genes, sexual immune dimorphism, and parental investment was found. This implies that the adaptive immune system is more affected by a resource allocation trade-off, possibly because an induced adaptive immune system is more costly than the maintenance of the innate immune system (Boots and Bowers 2004). Additionally, the adaptive immune response is more plastic than the innate one and can also be downregulated upon brooding to not induce an immune reaction toward the own offspring (Warning et al. 2011). Generally, BPMB species show an upregulated adaptive immune response and antiviral properties, while their inflammation response is reduced, possibly as reaction to the exchange of eggs/larvae. Finally, our study paves the way for deeper investigations on the buccal mucosa microbial communities not only in MB cichlids.

AUTHORS CONTRIBUTION

I.S., O.R., and W.S. conducted fieldwork and collected samples. I.S. and O.R. conducted the laboratory work. Data analyzed by I.S. and T.B. O.R. and W.S. contributed materials and analysis tools. I.S., O.R., T.B., and W.S. wrote and edited the manuscript.

ACKNOWLEDGMENTS

We thank M. Poirier, A. Beemelmans, A. Franke, D. Gill, M. Grimm, H. Buhtz, C. Burghard, B. Egger, A. Indermaur, A. Theis, F. Ronco, M. Colombo, F. Meuri, Kedric, and Adam for their support in the laboratory, fieldwork, fish catching, and statistical analysis; the Department of Fisheries, Republic of Zambia, for the research permits; and Vicky Huwiler and her staff at Kalambo Lodge for logistical support during fieldwork. Additionally, we thank Sven Künzel for managing of the Illumina sequencer. This study was supported by grants from the European Research Council (ERC; StG "INTERGENADAPT" and CoG "CICHLID_X") and the Swiss National Science Foundation (SNF) to W.S., and grants from the Volkswagenstiftung and the German Research Foundation (DFG) to O.R.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

DATA ARCHIVING

The doi for our data is https://doi.org/10.1594/PANGAEA.884078.

LITERATURE CITED

Aisenberg, A., and A. V. Peretti. 2011. Sexual dimorphism in immune response, fat reserves and muscle mass in a sex role reversed spider. Zoology 114:272–275.

Anderson, S. R., and J. J. Wiens. 2017. Out of the dark: 350 million years of conservatism and evolution in diel activity patterns in vertebrates. Evolution 71:1944–1959.

Bailey, M. T., S. E. Dowd, J. D. Galley, A. R. Hufnagle, R. G. Allen, and M. Lyte. 2011. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain. Behav. Immun. 25:397–407.

Baldo, L., M. E. Santos, and W. Salzburger. 2011. Comparative transcriptomics of eastern African cichlid fishes shows signs of positive selection and a large contribution of untranslated regions to genetic diversity. Genome Biol. Evol. 3:443–455.

Bateman, A. J. 1948. Intra-sexual selection in Drosophila. Heredity 2:349–368.

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

Birrer, S. C., T. B. H. Reusch, and O. Roth. 2012. Salinity change impairs pipefish immune defence. Fish Shellfish Immunol. 33:1238–1248.

Böhne, A., T. Sengstag, and W. Salzburger. 2014. Comparative transcriptomics in East African cichlids reveals sex- and species-specific expression and new candidates for sex differentiation in fishes. Genome Biol. Evol. 6:2567–2585.

Boisvert, M., N. Chetoui, S. Gendron, and F. Aoudjit. 2010. Alpha2beta1 integrin is the major collagen-binding integrin expressed on human Th17 cells. Eur. J. Immunol. 40:2710–2719.

Boots, M., and R. G. Bowers. 2004. The evolution of resistance through costly acquired immunity. Proc. Biol. Sci. 271:715–23.

Bourgeon, S., Y. Le Maho, and T. Raclot. 2009. Proximate and ultimate mechanisms underlying immunosuppression during the incubation fast in female eiders: roles of triiodothyronine and corticosterone. Gen. Comp. Endocrinol. 163:77–82.

Boutin, S., L. Bernatchez, C. Audet, and N. Deromê. 2013. Network analysis highlights complex interactions between pathogen, host and commensal microbiota. PLoS One 8:1–16.

Brawand, D., C. E. Wagner, Y. I. Li, M. Malinsky, I. Keller, S. Fan, O. Simakov, A. Y. Ng, Z. W. Lim, E. Bezault, et al. 2014. The genomic substrate for adaptive radiation in African cichlid fish. Nature 513:375–381.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, C. A. Lozupone, P. J. Turnbaugh, N. Fierer, and R. Knight. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. USA 108(Suppl):4516–4522.

Carlton, E. D., C. L. Cooper, and G. E. Demas. 2014. Metabolic stressors and signals differentially affect energy allocation between reproduction and immune function. Gen. Comp. Endocrinol. 208:21–29.

Clutton-Brock, T. 1991. The evolution of parental care. Princeton Univ. Press, Princeton.

Cole, J. R., Q. Wang, J. A. Fish, B. Chai, D. M. McGarrell, Y. Sun, C. T. Brown, A. Porras-Alfaro, C. R. Kuske, and J. M. Tiedje. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42:633–642.

Cooper, I. A., J. M. Brown, and T. Getty. 2016. A role for ecology in the evolution of colour variation and sexual dimorphism in Hawaiian damselflies. J. Evol. Biol. 29:418–427.

Diepeveen, E. T., O. Roth, and W. Salzburger. 2013. Immune-related functions of the Hivep gene family in East African cichlid fishes. G3 Genes, Genomes, Genet. 3:2205–2217.

Dufrêne, M., and P. Legendre. 1997. Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecol. Monogr. 67:345–366.

Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200.

Fischer, U., K. Utke, T. Somamoto, B. Köllner, M. Ototake, and T. Nakanishi. 2006. Cytotoxic activities of fish leucocytes. Fish Shellfish Immunol. 20:209–226.

Fraune, S., and T. C. G. Bosch. 2010. Why bacteria matter in animal development and evolution. BioEssays 32:571–580.

French, S. S., D. F. Denardo, and M. C. Moore. 2007. Trade-offs between the reproductive and immune systems: facultative responses to resources or obligate responses to reproduction? Am. Nat. 170:79–89.

Gaujoux, R., and C. Seoighe. 2010. A flexible R package for nonnegative matrix factorization. BMC Bioinformatics 11:367.

Ghalambor, C. K., D. N. Reznick, and J. A. Walker. 2004. Constraints on adaptive evolution: the functional trade-off between reproduction and fast-start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). Am. Nat. 164:38–50.

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

Gonzalez-Voyer, A., J. L. Fitzpatrick, and N. Kolm. 2008. Sexual selection determines parental care patterns in cichlid fishes. Evolution 62:2015–2026.

Goodwin, N. B., S. Balshine-Earn, and J. D. Reynolds. 1998. Evolutionary transitions in parental care in cichlid fish. Proc. R. Soc. B Biol. Sci. 265:2265–2272.

Grone, B. P., R. E. Carpenter, M. Lee, K. P. Maruska, and R. D. Fernald. 2012. Food deprivation explains effects of mouthbrooding on ovaries and steroid hormones, but not brain neuropeptide and receptor mRNAs, in an African cichlid fish. Horm. Behav. 62:18–26.

Houston, A. I., T. Székely, and J. M. McNamara. 2013. The parental investment models of Maynard Smith: a retrospective and prospective view. Anim. Behav. 86:667–674.

Jürss, K., I. Junghan, and R. Bastrop. 1992. the role of elongation-factors in protein-synthesis rate variation in white teleost muscle. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 162:345–350.

Keenleyside, M. H. A. 1991. Parental care. Pp. 191–208 *in* M. H. A. Keenleyside, ed. Cichlid fishes: behaviour, ecology and evolution. Chapman & Hall, Cambridge.

Keller, I. S., W. Salzburger, and O. Roth. 2017. Parental investment matters for maternal and offspring immune defense in the mouthbrooding cichlid *Astatotilapia burtoni*. BMC Evol. Biol. 17:264.

Knowles, S. C. L., S. Nakagawa, and B. C. Sheldon. 2009. Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. Funct. Ecol. 23:405–415.

Kocher, T. D. 2004. Adaptive evolution and explosive speciation: the cichlid fish model. Nat. Rev. Genet. 5:288–298.

Kokko, H., and M. D. Jennions. 2008. Parental investment, sexual selection and sex ratios. J. Evol. Biol. 21:919–948.

Koressaar, T., and M. Remm. 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23:1289–1291.

Kozich, J. J., S. L. Westcott, N. T. Baxter, S. K. Highlander, and P. D. Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. Appl. Environ. Microbiol. 79:5112–5120.

Kurtz, J., A. Wiesner, P. Götz, and K. P. Sauer. 2000. Gender differences and individual variation in the immune system of the scorpionfly *Panorpa vulgaris* (Insecta: Mecoptera). Dev. Comp. Immunol. 24:1–12.

Larsen, A., Z. Tao, S. A. Bullard, and C. R. Arias. 2013. Diversity of the skin microbiota of fishes: evidence for host species specificity. FEMS Microbiol. Ecol. 85:483–494.

Lehtonen, T. K., and A. Meyer. 2011. Heritability and adaptive significance of the number of egg-dummies in the cichlid fish *Astatotilapia burtoni*. Proc. Biol. Sci. 278:2318–2324.

Lessells, C. M. 1998. A theoretical framework for sex-biased parental care. Anim. Behav. 56:395–407.

Levin, I. I., D. M. Zonana, B. K. Fosdick, S. J. Song, R. Knight, R. J. Safran, and I. I. Levin. 2016. Stress response, gut microbial diversity and sexual signals correlate with social interactions. Biol. Lett. 12:pii: 20160352.

Li, X., Q. Yan, E. Ringø, X. Wu, Y. He, and D. Yang. 2016. The influence of weight and gender on intestinal bacterial community of wild large-mouth bronze gudgeon (*Coreius guichenoti*, 1874). BMC Microbiol. 16:191.

Llewellyn, M. S., S. Boutin, S. H. Hoseinifar, and N. Derome. 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. 5:1–1.

Lorin, T., W. Salzburger, and A. Bohne. 2015. Evolutionary fate of the androgen receptor-signaling pathway in ray-finned fishes with a special focus on cichlids. G3 Genes, Genomes, Genet. 5:2275–2283.

Magurran, A. 2000. Sex differences in behaviour as an indirect consequence of mating system. J. Fish Biol. 57:839–857.

Mank, J. E., D. E. L. Promislow, and J. C. Avise. 2005. Phylogenetic perspectives in the evolution of parental care in ray-finned fishes. Evolution 59:1570–1578.

Martner, A. 2009. Regulation of innate and adaptive immune responses by Gram-positive and Gram-negative bacteria. Göteborg University, Sweden.

Maynard, C. L., C. O. Elson, R. D. Hatton, and C. T. Weaver. 2012. Reciprocal interactions of the intestinal microbiota and immune system. Nature 489:231–241.

McKean, K., and L. Nunney. 2005. Bateman's principle and immunity: phenotypically plastic reproductive strategies predict changes in immunological sex differences. Evolution 59:1510–1517.

Mestdagh, P., P. Van Vlierberghe, A. De Weer, D. Muth, F. Westermann, F. Speleman, and J. Vandesompele. 2009. A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol. 10:R64.

Meyer, B. S., M. Matschiner, and W. Salzburger. 2015. A tribal level phylogeny of Lake Tanganyika cichlid fishes based on a genomic multimarker approach. Mol. Phylogenet. Evol. 83:56–71.

Morley, S. C. 2012. The actin-bundling protein L-plastin: a critical regulator of immune cell function. Int. J. Cell Biol. 2012.

Münkemüller, T., S. Lavergne, B. Bzeznik, S. Dray, T. Jombart, K. Schiffers, and W. Thuiller. 2012. How to measure and test phylogenetic signal. Methods Ecol. Evol. 3:743–756.

Muñoz, I. M., T. MacArtney, L. Sanchez-Pulido, C. P. Ponting, S. Rocha, and J. Rouse. 2012. Family with sequence similarity 60A (FAM60A) protein is a cell cycle-fluctuating regulator of the SIN3-HDAC1 histone deacetylase complex. J. Biol. Chem. 287:32346–32353.

Muschick, M., A. Indermaur, and W. Salzburger. 2012. Convergent evolution within an adaptive radiation of cichlid fishes. Curr. Biol. 22:2362–2368.

Nakanishi, T., U. Fischer, J. M. Dijkstra, S. Hasegawa, T. Somamoto, N. Okamoto, and M. Ototake. 2002. Cytotoxic T cell function in fish. Dev. Comp. Immunol. 26:131–139.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. Nature 401:877–884.

Pradeu, T. 2011. A mixed self: the role of symbiosis in development. Biol. Theory 6:80–88.

Pruesse, E., C. Quast, K. Knittel, B. M. Fuchs, W. Ludwig, J. Peplies, and F. O. Glöckner. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35:7188–7196.

Reardon, E. E., and L. J. Chapman. 2010. Hypoxia and energetics of mouth brooding: is parental care a costly affair? Comp. Biochem. Physiol. A Mol. Integr. Physiol. 156:400–406.

Reavey, C. E., N. D. Warnock, H. Vogel, and S. C. Cotter. 2014. Trade-offs between personal immunity and reproduction in the burying beetle, *Nicrophorus vespilloides*. Behav. Ecol. 25:415–423.

Revell, L. J. 2012. phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. 3:217–223.

Reynolds, J. D., N. B. Goodwin, and R. P. Freckleton. 2002. Evolutionary transitions in parental care and live bearing in vertebrates. Philos. Trans. R Soc. London B Biol. Sci. 357:269–281.

Robertsen, B. 2006. The interferon system of teleost fish. Fish Shellfish Immunol. 20:172–191.

Rolff, J. 2002. Bateman's principle and immunity. Proc. R Soc. B Biol. Sci. 269:867–872.

Roth, O., and J. Kurtz. 2008. The stimulation of immune defence accelerates development in the red flour beetle (*Tribolium castaneum*). J. Evol. Biol. 21:1703–1710.

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

Royle, N. J., P. T. Smiseth, and M. Kölliker. 2012. The evolution of parental care. Oxford Univ. Press, Oxford.

Salinas, I. 2015. The mucosal immune system of teleost fish. Biology 4:525–539.

Salzburger, W., T. Mack, E. Verheyen, and A. Meyer. 2005. Out of Tanganyika: genesis, explosive speciation, key-innovations and phylogeography of the haplochromine cichlid fishes. BMC Evol. Biol. 5:17.

Salzburger, W., B. Van Bocxlaer, and A. S. Cohen. 2014. Ecology and evolution of the African Great Lakes and their faunas. Annu. Rev. Ecol. Evol. Syst. 45:519–545.

Santos, E. S. A., and S. Nakagawa. 2012. The costs of parental care: a meta-analysis of the trade-off between parental effort and survival in birds. J. Evol. Biol. 25:1911–1917.

Schärer, L., L. Rowe, and G. Arnqvist. 2012. Anisogamy, chance and the evolution of sex roles. Trends Ecol. Evol. 27:260–264.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75:7537–7541.

Siva-Jothy, M. T. 2000. A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. Proc. Biol. Sci. 267:2523–2527.

Slater, C. H., M. S. Fitzpatrick, and C. B. Schreck. 1995. Androgens and immunocompetence in salmonids: specific binding in and reduced immunocompetence of salmonid lymphocytes exposed to natural and synthetic androgens. Aquaculture 136:363–370.

Smith, K., K. D. McCoy, and A. J. Macpherson. 2007. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin. Immunol. 19:59–69.

Smith, K. T., M. E. Sardiu, S. A. Martin-Brown, C. Seidel, A. Mushegian, R. Egidy, L. Florens, M. P. Washburn, and J. L. Workman. 2012. Human family with sequence similarity 60 member A (FAM60A) protein: a new subunit of the Sin3 deacetylase complex. Mol. Cell. Proteomics 11:1815–1828.

Sommer, F., and F. Bäckhed. 2013. The gut microbiota—masters of host development and physiology. Nat. Rev. Microbiol. 11:227.

Stearns, S. C. 1987. The selection-arena hypothesis. Pp. 337–355 *in* S. C. Stearns, ed. The evolution of sex and its consequences. Birkhäuser Verlag, Basel and Boston.

———. 1989. Trade-offs in life-history evolution. Funct. Ecol. 3:259–268.

Theis, A., O. Roth, F. Cortesi, F. Ronco, W. Salzburger, and B. Egger. 2017. Variation of anal fin egg-spots along an environmental gradient in a haplochromine cichlid fish. Evolution 71:766–777.

Tort, L., J. C. Balasch, and S. Mackenzie. 2003. Fish immune system. A crossroads between innate and adaptive responses. Trends Immunol. 22:277–286.

Trivers, R. L. 1972. Parental investment and sexual selection. Pp. 136–179 *in* B. Campbell, ed. Sexual selection and the descent of man. Aldine Publishing Company, Chicago, Illinois.

Trumbo, S. T. 2007. Defending young biparentally: female risk-taking with and without a male in the burying beetle, *Nicrophorus pustulatus*. Behav. Ecol. Sociobiol. 61:1717–1723. Springer, Berlin, Germany.

Uehara, S., A. Grinberg, J. M. Farber, and P. E. Love. 2002. A role for CCR9 in T lymphocyte development and migration. J. Immunol. 168:2811–2819.

Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B. C. Faircloth, M. Remm, and S. G. Rozen. 2012. Primer3-new capabilities and interfaces. Nucleic Acids Res. 40:1–12.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:RESEARCH0034.

Vincent, A. C. J. 1991. Prospects for sex role reversal in teleost fishes. Netherlands J. Zool. 42:392–399.

Vincent, C. M., and D. T. Gwynne. 2014. Sex-biased immunity is driven by relative differences in reproductive investment sex-biased immunity is driven by relative differences in reproductive investment. Proc. Biol. Sci. 281:pii: 20140333.

Wang, A. R., C. Ran, E. Ringø, and Z. G. Zhou. 2017. Progress in fish gastrointestinal microbiota research. Rev. Aquac. 1–15. https://doi.org/10.1111/raq.12191.

Warning, J. C., S. A. McCracken, and J. M. Morris. 2011. A balancing act: mechanisms by which the fetus avoids rejection by the maternal immune system. Reproduction 141:715–724.

Yuan, S., D. B. Cohen, J. Ravel, Z. Abdo, and L. J. Forney. 2012. Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS One 7:e33865.

Zuk, M., and A. M. Stoehr. 2002. Immune defense and host life history. Am. Nat. 160:11–22.

Associate Editor: B. Koskella
Handling Editor: Mohamed A.F. Noor

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Composition of buccal mucosa microbiota by phylum: relative abundance of the four phyla containing 90% of all bacteria sampled.
Figure S2. Composition of buccal mucosa microbiota by genera: relative abundance of each genus over brooding modes is shown.
Figure S3. Heatmap of top 50 OTUs: Means over species and sexg (f. for females and m. for males) were calculated and hierarchically clustered.
Figure S4. Boxplots of the interactive effects of sex and brooding mode on cell activity and candidate gene expression: Both graphs median and data distribution.
Figure S5. Effects of brooding mode on cell population and candidate gene expression: Both graphs median and data distribution.
Protocol S1. Flow Cytometer protocol and gating: Detailed information about the sampling and preparation of the cell mixtures for both cell population and cell activity analysis and following sample analysis and gating procedure in a BD Accuri C6 Flow cytometer.
Protocol S2. Gene expression analysis: Detailed information about the protocol used for gene expression, including sample treatment and amplification cycles.
Table S1
Table S2
Table S3
Table S4
Table S5