Final Report

Leibniz-Competition

Title: Epigenetic stability and plasticity of social environmental effects (EpiRank)

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Leibniz Institute in charge: Leibniz Institute for Zoo and Wildlife Research (IZW)

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Executive Summary

In mammalian societies, dominance hierarchies often translate into inequalities in access to resources, reproductive performance and survival. Within the EpiRank project, we asked whether such social inequalities were also consistently reflected at the molecular and physiological levels throughout an individual's life. Focusing on a highly social mammal, the spotted hyena (*Crocuta crocuta*), we examined the effect of individual social ranks on DNA methylation, gene expression, gut biomes, metabolite profiles, immune processes, hormones, and Darwinian fitness. To identify rank-specific patterns, we used hundreds of (faecal) samples collected non-invasively as part of a long-term project following thousands of individually known free-ranging female spotted hyenas. Our results indicate that social inequalities were reflected to some extent at the molecular or physiological level in both cubs and adults. More specifically, we identified 149 differentially methylated regions (DMRs) in 44 genes, between high and low-ranking females, and found most DMRs to be hypermethylated in low-ranking ones. RNA-Seq analysis did not reveal significant expression differences between high and low-ranking females, which may be due to the low RNA quality in samples. Social rank had no influence on metabolite profiles, gut biomes and immune effectors, i.e. mucin, neopterin, IgA and IgG. Other preliminary analyses suggest that the effects of social rank on faecal glucocorticoid metabolite concentrations (fGMCs) may interact with other key socio-environmental factors. Regarding life history outcomes, social rank influenced the age at first reproduction, but had no effect on early growth rate, survival to adulthood, litter size, longevity and lifetime reproductive success. Overall, the results were contrary to our expectations, as social rank did not have the clear effect that we expected. As a result, social instability was not studied in greater depth. These seemingly "negative" results may in fact have great "positive" relevance for social inequalities, because they suggest that social status is not strongly programmed at the molecular level, at least in spotted hyenas.

1. Achievement of objectives and milestones

We aimed to determine the role of DNA methylation (work module M2) in converting spotted hyena social trajectories (M1) into gene expression programmes (M3) that influence life history traits and fitness (M12). We implemented this objective using non-invasive methods consisting in tracking the behaviour and life histories of free-ranging female spotted hyenas and collecting mucus and faecal samples during fieldwork (M1), calculating their social ranks and fitness (M1, M12), extracting DNA and RNA from gut epithelium cells for DNA methylation (M2) and gene expression (M3) analyses, and using faecal samples for gut biome compositions (M7), microbiome gene content (M8), metabolomics (M9), immunology (M10) and hormone (M11) analyses. M7-M11 were led by project partners.

For the completion of all work modules, we selected a total number of 363 samples from 217 individuals for laboratory analyses. The same samples were analysed for different work modules, and we obtained a total of 850 measurements. We achieved the milestone of M2 using a DNA capture method to enrich for mammalian CpG methylation, followed by deep-throughput sequencing (MBD-Seq) from a subset of 42 gut epithelium samples. This method allowed us to omit 'M5 Microsatellites', as data provided by MBD-Seq were adequate to determine the genetic background of individuals. For 'M3 Gene expression', RNA-Seq was performed for the same samples, thereby allowing a direct comparison. MBD-Seq and RNA-Seq reads were mapped to a novel genome sequence ('M4 Genomics'), which led to a publication (Westbury et al. 2021). We also prepared a novel custom-made annotation including promoter regions and transcription start sites as regulatory sites for the functional epigenetic analysis. Methylation analysis revealed 149 robust DMRs of which 44 overlapped genes between high and low-ranking females (cubs and adults). These rank-specific signatures were statistically robust and functionally meaningful, and may serve as biomarkers in future (as suggested for another species, Weyrich et al. 2022). RNA-Seq resulted in no significant expression differences between high and low-ranking females but revealed metabolic differences. 'M6 Convergent evolution' in hyenas and rhesus macaques could not be identified, as rank-specific genes did not overlap, which may be due to the

different types of sample material used (gut epithelium cells *vs* blood, Weyrich et al. 2020), or different environments and experiences (free-ranging condition *vs* captivity) (Weyrich et al. 2018, 2019). Rank-specific DNA methylation signatures may also be more likely in other organs, such as brain areas. We published a review on epigenomics and gene regulation in mammalian social systems (Guerrero et al. 2020). The results of these four work modules (M2, M3, M4, M6) are presented and discussed in a manuscript (Vullioud et al. submitted).

For 'M12 Statistical Modelling', a detailed analysis of the effects of social rank on behaviour (den/territory attendance) and multiple measures of performance and Darwinian fitness throughout the lives of female spotted hyenas (i.e. growth rate, survival to the age of one year and adulthood, age at first reproduction, litter size, longevity and lifetime reproductive success) have been published (Benhaiem et al. 2023, Gicquel et al. 2022a, 2022b). These analyses showed that contrary to expectations, female social rank did not influence most performance measures including Darwinian fitness (Gicquel et al. 2022a).

Another key objective of the project was to study the consequences of changes in social rank. This objective could not be pursued because we did not find a clear effect of social rank on the variables considered.

We followed our financial plan, which covered most of the anticipated costs for all work modules. Part of the budget was saved due to decreased costs (sequencing costs), the resignation of the recruited doctoral student, and open access (OA) publications which were supported by OA grants. Furthermore, due to COVID-19 restrictions, conferences were cancelled and we organised one network meeting online. We re-directed these cost savings to fund additional services and the collection of additional samples from the field site.

2. Activities and obstacles

Each member of the project has contributed to carrying out the tasks set out in the proposal. With our collaborators we aimed to study whether social ranks affect physiological and immune processes (M7-M11). We were able to achieve most of these objectives, although not all the results have yet been published. The project suffered a few delays and we encountered some difficulties when working with the main sample material used in the project. Although faecal samples can be collected non-invasively and repeatedly from the same individuals, unlike other biological samples such as blood or tissue, they require validation of their use or additional purification and quality control steps, which prolonged some laboratory procedures and analyses (for M2, M3, M9, M10). The COVID-19 restrictions also delayed laboratory work (for M10 and M11) because of space capacity limitations and other restrictions (e.g. sickness of personnel). During the course of the project, the Leibniz-IZW underwent three evaluations, and successfully applied for a "large strategic extraordinary item of expenditure" (STB), process which involved the two PIs and several of the IZW-internal co-applicants. As senior postdocs both PIs also simultaneously established their own research groups, acquired third party funding and supervised students up to the doctoral level. Other time-consuming activities involving one or the other PI included teaching duties, the management of the field station, and a change of research institute.

Whole genome sequencing of the spotted hyena (M4) did not yield a sufficient number of reads after the generation of Chromium 10x libraries prepared from three fresh samples of spotted hyena. This delayed the bioinformatic analyses of data for M2 and M3 for which the genome was essential. One year later than planned we received a spotted hyena genome. A further delay in bioinformatic analysis was caused by the long-term sickness (1.5 years) of the bioinformatician of the Department of Evolutionary Genetics (Leibniz-IZW) and the difficulty of finding a temporary replacement.

We had recruited a doctoral student for 'M7 Intestinal biome compositions'/'M8 Microbiome gene content'. This candidate decided to quit academia and left the project, which delayed the analysis for these two work modules. For M7, we used multi-amplicon sequencing with a stringent quality assessment for the bacterial and eukaryotic community profiling. Preliminary explorations based on ordination analysis indicate that the gut microbiome of spotted hyenas

is not influenced by social rank. For M8, we are currently using sequencing data for bacterial DNA analysis. The PI of 'M9 Metabolomics' generously offered to finance the costs of more samples than originally envisaged. We used a targeted GC-MS metabolomics assay and multivariate analyses. For 'M10 Immunology' we used general linear models to test for the effect of social rank on mucin, neopterin, IgA and IgG levels measured in faecal samples using validated assays. These analyses revealed that social rank had no influence on these immune effectors. We also completed 'M11 Hormones' and measured faecal glucocorticoid metabolite concentrations (fGMCs) using an enzyme immunoassay previously validated for this species. Preliminary analyses focusing on adult females suggest that the effect of social rank on fGMCs may be context dependent.

Overall, the project generated numerous results that we will continue to explore in future. While a number of studies in ecology have investigated the relationships between environmental conditions and DNA methylation patterns in free-ranging wildlife populations, to our knowledge our project was the first investigating this topic using non-invasively collected sample material. More generally, the identification of 'epigenetic signatures' in faecal samples could be used as an approach to studying non-invasive biological markers, which could be useful for health diagnosis and of interest to researchers and wildlife conservationists.

3. Results and successes

Publications: Both PIs published 19 articles in peer-reviewed scientific journals, and were first or senior authors on 13 of them. Our review on epigenomic and gene regulation mechanisms in mammalian social systems (Guerrero et al. 2020) was already cited 12 times. One EpiRank manuscript is submitted and others are currently in preparation.

Scientific events: Both PIs and their students gave presentations at conferences, workshops and invited seminars (n = 19 presentations, e.g. ESEB, iDiV). The PIs also organised an international conference (> 250 participants, WRC2019) and gave lectures at the MSc level (Potsdam University).

Qualification work: One BSc, 10 MSc and two doctoral students supervised by the PIs received their titles at seven universities in Germany and abroad. Several MSc students and doctoral students supervised by the PIs achieved their degrees with excellent marks during the course of the project. One MSc student received a prize for the best MSc thesis of the year at her University. Three doctoral projects involving the PIs are currently ongoing.

Third-party funding: The PIs applied for and successfully acquired third-party funding for two postdocs (DFG, BMBF) and a doctoral position (BMBF), open access publications (n = 4; Leibniz, Projekt DEAL), an outreach project (ESEB), a conference (DFG), symposiums and workshops (n = 5, BMBF).

Knowledge transfer: The PIs were involved in numerous activities to disseminate the outcomes of the research. These include designing a new website to present the Serengeti hyena research project, preparing, translating and distributing leaflets on the project, presenting the project to the public during the Leibniz-IZW's open day, authoring and translating a science comic into several languages, conceptualising an exhibition "Epigeneum" on epigenetics at Rostock zoological garden, as well as giving interviews and presentations at public events.

4. Equal opportunities, career development and internationalisation

Our project put a strong emphasis on supporting the careers of young scientists including BSc, MSc and doctoral candidates and was committed to implement measures to promote diversity and equal opportunity. The project was led by two women and the sex ratio was balanced when considering all scientists, technical assistants and students associated with the project (20 women, 17 men). One of the PIs received funding from the iDiv Female Scientist Career Fund, which aims to strengthen the networking of female scientists and

provides coaching to enhance their skills. The other PI and another project member coordinate the structured doctoral training programme of the Leibniz-IZW, which aims to support and guide doctoral students, particularly females through specific workshops and courses. All students were supervised by numerous scientists (e.g. in thesis advisory committees) to increase networking opportunities and ensure a wide scientific perspective. The EpiRank project involved researchers (at all levels) with diverse nationalities (British, Canadian, Chilean, French, German, Hungarian, Israeli, Mexican, Portuguese and Swiss).

5. Structures and collaboration

The activities and progress of all project members were coordinated and evaluated from the start by the PIs via monthly meetings among the Leibniz-IZW-based project members, seven networking meetings involving all or some international collaboration partners, and frequent email exchange and phone calls, all documented by detailed minutes shared among the project partners. The meetings were used to discuss the current status of the project and re-evaluate methodological procedures originally proposed in the proposal. They led to some cost-saving changes, in particular regarding the sequencing and the subsequent DNA methylation analysis. Two external collaboration partners were invited to give seminar talks at the Leibniz-IZW, which resulted in a new collaboration with colleagues at the Leibniz-IZW. The project has expanded with the arrival of new collaborative partners and students, which has enabled us to work in greater depth on the various work modules. The EpiRank project stimulated new collaborations with international scientists outside the consortium, based, e.g., at the CEFE-CNRS in Montpellier, France; iDiv; Universities in Halle and Tübingen in Germany, and University of South Florida, USA.

6. Quality assurance

Our project does not involve any experiments on animals. Spotted hyenas were observed in their natural environment and all data and samples were collected using non-invasive methods described in field research permits and following ethical guidelines. Laboratory technical approaches were performed with blinded samples and standard quality control procedures were performed throughout. We included investigations of many technical aspects, and validated those immune assays still needed to be validated. To establish the direct effect of DNA methylation on the expression of genes, we used aliquots of the same samples, i.e. gut epithelium samples of a subset of samples. Bioinformatic data were strictly quality-checked and filtered throughout their computation. Since the start of the project we published our scientific articles in open access peer-reviewed journals, or made them publicly available. We intend to continue in this way for future publications. Our data, bioinformatic and statistical analysis scripts were and will further be submitted to online databases (e.g. NCBI, figshare) and repositories (e.g. GitHub) to enable reproducibility. All standard quality measures, such as laboratory documentation in lab books or documentation of R scripts, are being implemented, following the official guidelines developed by the Leibniz-IZW for storing and analysing data.

7. Additional resources

Most ‘in-kind’ services received in the context of the project were personnel resources. The project was supported by many students (from BSc to doctoral students) and laboratory technicians for sample preparation, laboratory, bioinformatic or statistical analyses. In addition, in terms of material resources, the Leibniz-IZW and the Helmholtz Center Munich financed consumables and additional analyses.

8. Outlook

We aim to write and submit at least three additional manuscripts, currently in preparation, to peer-reviewed journals and to continue to present the project results at scientific conferences

(e.g. ETH, iDiv). The project generated a large amount of data which has yet to be exploited, and which will provide the basis to answer further exciting questions, beyond those outlined in the proposal. These include an assessment of the role of individual characteristics, such as reproduction status, age, personality or the experience of traumatic events on the epigenome, or determining the influence of clan membership on intestinal biome signatures to study routes of transmission of gut microorganisms. Our results allowed us to evaluate the use of DNA methylation patterns as potential rank-specific signatures, as we use samples from females with social ranks across the entire linear dominance hierarchy. Our entirely non-invasive method may be used by other projects to assess the ranks of unknown female spotted hyenas.

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