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Context-dependent DNA methylation variants in marbled crayfish (*Procambarus virginalis*)

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Abstract

The marbled crayfish is a recently discovered freshwater crayfish. It is the only known decapod crustacean that reproduces by obligate apomictic parthenogenesis resulting in a clonal, all-female population. All known marbled crayfish have a single origin, and the species is estimated to have an age of about 25-30 years. Furthermore, it has spread already into several countries globally and shows high adaptability to different environments in the absence of genetic adaptation. Epigenetic mechanisms were suggested to play a role in the rapid adaptation of this species. This thesis aims to identify context-dependent DNA methylation variants in marbled crayfish.

Samples from two tissues and four distinct habitats were analyzed with a capture-based bisulfite sequencing approach to identify methylation variants. The results were validated by deep amplicon sequencing of tissue-specific and location-specific regions and with newly collected samples from the same tissues and locations as before. The location-specific methylation patterns suggest the existence of epigenetic ecotypes and allow the tracing of the origin of marbled crayfish populations by their DNA methylation fingerprint. In a laboratory trial, single rearing conditions were changed to see the influence of specific parameters on the methylation pattern. Within six months, methylation changes could be observed for the group kept at a lower temperature compared to the control group, indicating the adaptation of the methylation pattern caused by an environmental trigger.Furthermore, the laboratory population showed a different methylation pattern compared to the wild populations.

Lastly, the potential of marbled crayfish as an aquaculture livestock was explored, and a framework for an environmentally safe aquaculture was established. Additionally, tracing the origin of marbled crayfish using location-specific methylation patterns was proposed for certifying sustainable and transparent aquaculture practices. These results provide insight into the rapid adaptation in invasive species and provide a proof of concept for environmental origin tracing with DNA methylation fingerprinting.

Zusammenfassung

Der Marmorkrebs ist ein kürzlich entdeckter Flusskrebs, welcher der einzige bekannte Zehnfußkrebs ist, der sich durch obligat apomiktische Parthenogenese fortpflanzt. Dies führt zu einer rein weiblichen Population, deren Ursprung von einem einzigen Tier ausging. Die Art hat ein geschätztes Alter von etwa 25-30 Jahren. Darüber hinaus hat der Marmorkrebs sich bereits weltweit ausgebreitet und zeigt eine hohe Anpassungsfähigkeit an verschiedene Umgebungen ohne die Möglichkeit der genetischen Anpassung. Aus diesem Grund scheinen epigenetische Mechanismen eine Rolle bei der schnellen Anpassung dieser Art zu spielen. Diese Arbeit hat zum Ziel, kontextabhängige DNA-Methylierungsvarianten in Marmorkrebsen zu identifizieren.

Proben aus zwei Geweben und vier verschiedenen Lebensräumen wurden mit einem capture-basierten Bisulfit-Sequenzierungsansatz analysiert, um Methylierungsvarianten zu identifizieren. Diese Ergebnisse wurden durch Amplikonsequenzierung gewebsspezifischer und ortsspezifischer Regionen und neu gewonnen Proben derselben Gewebe und Orte wie zuvor validiert. Beide Experimente zeigten kontextabhängige Methylierungsmuster für verschiedene Gewebe und Orte. Die ortsspezifischen Methylierungsmuster deuten auf die Existenz epigenetischer Ökotypen hin und erlauben es, den Ursprung der Marmorkrebspopulationen anhand ihres Methylierungs-Fingerabdrucks zurückzuverfolgen. In einem Laborversuch wurden einzelne Aufzuchtbedingungen verändert, um den Einfluss spezifischer Parameter auf das Methylierungsmuster zu erkennen. Innerhalb von sechs Monaten konnten bei der Gruppe, die bei niedriger Temperatur gehalten wurde, im Vergleich zur Kontrollgruppe Methylierungsveränderungen beobachtet werden, was auf die Anpassung des Methylierungsmusters durch einen umweltbedingten Auslöser hinweist. Darüber hinaus zeigte die Laborpopulation ein anderes Methylierungsmuster als die Wildpopulationen. Schließlich wurde das Potenzial von Marmorkrebsen als Nutztiere untersucht und ein Rahmen für eine ökologisch sichere Aquakultur vorgeschlagen. Darüber hinaus wurde vorgeschlagen, den Ursprung der Marmorkrebse anhand ortsspezifischer Methylierungsmuster zurückzuverfolgen, um eine nachhaltige und transparente Aquakultur zu zertifizieren. Die Ergebnisse dieser Arbeit geben Einblicke in die schnelle Anpassung invasiver Arten und etablieren ein Konzept für die Herkunftsverfolgung von Nutztieren mit Hilfe von DNA-Methylierungs-Fingerabdrücken.

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List of Abbreviations

°dH	Grad deutscher Härte (degree German water hardness)
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ASV	Angelsportverein
BLAST	Basic Local Alignment Search Tool
bp	base pair
CHG	cytosine-non-guanine-guanine trinucleotide in 5’ to 3’ direction
CHH	cytosine-non-guanine-non-guanine trinucleotide in 5’ to 3’ direction
CpG	cytosine and guanine linked by a phosphate in 5´ to 3´direction
DKFZ	Deutsches Krebsforschungszentrum
DMR	differentially methylated region
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
eDNA	environmental DNA
FCR	feed conversion ratio
Gbp	giga base pair
HPLC	high-performance liquid chromatography
Kbp	kilo base pair
NTU	nephelometric turbidity unit
PCA	principal component analysis
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RRBS	reduced representative bisulfite sequencing
SAM	S-adenyl methionine
SD	standard deviation
SNV	single nucleotide variant
TET	ten-eleven translocation methylcytosine dioxygenase
TPM	transcripts per kilobase million
TSS	transcription start site
TTS	transcription termination site
UTR	untranslated region
WGBS	whole-genome bisulfite sequencing

1 Introduction

1.1 The Marbled crayfish

The recently discovered freshwater crayfish species *Procambarus virginalis* (Lyko, 2017) is the only known freshwater crayfish reproducing by obligate apomictic parthenogenesis. It emerged in the German aquarium trade about 25 years ago (Scholtz et al., 2003) and gained popularity among aquarists due to its remarkable marbled pattern on its carapace (Figure 1). So far, the species and its origin were unknown, and therefore it was preliminarily named “Marmorkrebs”, the German word for marbled crayfish.

The first analysis using microsatellite markers revealed apomictic parthenogenesis as reproduction mode along with a triploid genome, resulting in an all-female population (Martin et al., 2007). While the species was initially classified as a member of the North American Cambaridae family by Scholtz *et al.* (2003), the specific classification took seven more years. The genetic comparison of *Procambarus fallax* with marbled crayfish by two mitochondrial loci showed a high similarity to *P. fallax,* initially suggesting the marbled crayfish to be the parthenogenetic form of *P. fallax* (Martin et al., 2010). Therefore, the preliminary taxonomic name *Procambarus fallax f. virginalis* was given. Because of its reproductive isolation from the parent species and considerable genetic differences, the marbled crayfish now constitutes an independent species (Lyko, 2017; Vogt et al., 2015) with the given name *Procambarus virginalis* (Lyko, 2017). Additionally, genome assembly and analysis of marbled crayfish genomes from distinct populations highlighted a single origin of all known marbled crayfish populations and, therefore, the clonal propagation (Gutekunst et al., 2018).

Figure 1: Picture of wild-caught marbled crayfish with a marbled pattern on the carapace.

1.1.1 Distribution

Through anthropogenic releases, marbled crayfish have been introduced into various freshwater systems, where they have formed numerous stable populations (Chucholl et al., 2012), including Madagascar and various European countries like Germany or Malta (Chucholl and Pfeiffer, 2010; Deidun et al., 2018; Jones et al., 2009). Notably, marbled crayfish form stable populations in various habitats with highly different environmental conditions, such as climate zones, water quality, and water bodies. For example, marbled crayfish occur in lentic as well as lotic water bodies including rivers, lakes, ponds, swamps, rice fields, gravel pits, and drainage ditches (Gutekunst et al., 2018; Jones et al., 2009) and climatic zones from humid to sub-arid (Andriantsoa et al., 2019).

1.1.2 Marbled crayfish for human consumption and the commercial potential of freshwater crayfish

The introduction and first detection of marbled crayfish in Madagascar around 2005 raised substantial ecological concerns and reservations about this alien species (Hanamura et al., 2009; Jones et al., 2007, 2009). However, Madagascar is one of the world's poorest countries (World Bank, 2020), and a limited intake of dietary protein is, therefore, a significant problem. Freshwater crayfish are a rich source of protein, and thus the marbled crayfish was quickly used as protein resource. Farmers in Madagascar harvested them from rivers and lakes and started to distribute them intentionally over the country. Today, it is sold in large quantities on markets and counts as one of the most important and affordable protein sources in Madagascar (Andriantsoa et al., 2020). This raises the question if marbled crayfish can potentially be farmed in other countries as well.

Freshwater crayfish are consumed in most countries in the world, but from more than 670 known crayfish species (Crandall and De Grave, 2017), commercial aquaculture production is mainly pursued with the red swamp crayfish, *Procambarus clarkii*, a species that is native to Mexico and the USA (Loureiro et al., 2015). The dominance of this species in crayfish culture results from the historical eradication of the native European crayfish species by the crayfish plague in the middle of the 19th century

(Goldberg et al., 2007a) and the introduction of the red swamp crayfish to various countries in the world (Holdich et al., 2009; Wang et al., 2018). China became the leading producer and consumer of *P. clarkii*, with more than 720,000 tons in 2015 (Wang et al., 2018). Globally, freshwater crayfish are gaining popularity as a livestock for aquaculture with an annual value of more than 10 billion US dollars (FAO Fisheries and Aquaculture Department, 2018).

Marbled crayfish are increasingly considered for commercial aquaculture (Jurmalietis et al., 2019). They are closely related to *P. clarkii* and can tolerate a variety of habitats. The spread and use of marbled crayfish as a protein source are exemplified in Madagascar as described above. Crayfish meat is sold at local markets all over the country, and farming is mainly pursued on rice fields or by harvesting wild populations from lakes and rivers (Andriantsoa et al., 2019, 2020; Gutekunst et al., 2018). Nevertheless, the introduction and distribution of marbled crayfish raise ecological concerns, as it is considered an invasive species with a unique reproduction mode. Therefore, suitable and ecologically safe methods for aquaculture of those animals need to be developed.

The commercial potential of crayfish and crustaceans is not only limited to the meat, as shells of crustaceans are a rich source of Chitin (Kaur and Dhillon, 2015). Chitin is a polysaccharide, forming the exoskeleton of crustacean and insects. By extracting chitin and synthesizing derivatives from it, an important raw material is generated for the chemical industry. It is already used for many products, such as wound bandages, filter materials, and biodegradable plastics (Park and Kim, 2010). Shrimp and crab shell waste currently represent the main source for chitin production (Younes & Rinaudo, 2015), but the raw material demand is higher than the current production value (fmi Future Market Insights, 2017).

1.1.3 Genetic features

The marbled crayfish also represents an attractive model organism for genetic and epigenetic research. Therefore, the genome was characterized by Gutekunst *et al.* (2018). Genome sequencing and assembly have revealed a 3.5 Gbp genome with over 21,000 predicted genes (Gutekunst et al., 2018). Notably, the same study also demonstrated clonality within the global population. A comparative analysis of polymorphisms between genomes from 10 individual marbled crayfish populations, the

marbled crayfish reference genome, and a *P. fallax* animal revealed high similarities among the marbled crayfish samples and a more distant genomic relationship to *P. fallax*, confirming the clonality of marbled crayfish. Only 416 single nucleotide variants (SNVs) were detected among the 11 marbled crayfish. Notably, a total of only four non-synonymous SNVs was found, illustrating the low genetic complexity (Gutekunst et al., 2018) and raising the question of how phenotypic adaptation to diverse habitats is mediated.

1.2 Epigenetic modifications

Epigenetic adaptation could play an important role in the marbled crayfish for adapting to different environments.

Conrad Hal Waddington first mentioned the field of epigenetics in his publication “The Epigenotype” from 1942, where the term epigenotype was introduced. He defined the epigenotype as a complex development process lying between genotype and phenotype (Waddington, 1942). Since then, many definitions were adapted while gaining more and more information about epigenetic mechanisms. Nowadays, epigenetics is widely understood as the study of heritable changes in gene expression profiles independent of alterations in the nucleotide sequence (Fisher, 2002; Goldberg et al., 2007b). Best illustrated are epigenetic mechanisms in establishing cellular identity by lineage-specific expression patterns, which lead to cell differentiation and are maintained through replication and cell division (Fisher, 2002).

In contrast to genetic alterations, epigenetic alterations occur much faster (Rando and Verstrepen, 2007) and are reversible (Ramchandani et al., 1999). Furthermore, the epigenome can be modulated by the environment, giving the possibility of a dynamic response or phenotypic plasticity by changing the expression according to environmental triggers (Bollati and Baccarelli, 2010; Duncan et al., 2014; Lyko et al., 2010). The epigenetic machinery contains a wide variety of molecular modulation possibilities. However, so far, the epigenetic research is mainly focused on histone modifications, non-coding RNAs, RNA editing, and modifications of nucleic acids, such as DNA methylation, which is the best-understood mechanism (Bernstein et al., 2007; Goldberg et al., 2007a; Gott and Emeson, 2000).

1.2.1 Detection and analysis of 5-methylcytosine

DNA methylation in the 5-methylcytosine context (5mC) is the biochemical modification of cytosine by the addition of a methyl group to the fifth carbon of the nucleotide. This modification was first described in 1925 by Johnson and Coghill, but as they could not reproduce their results, the existence of methylcytosine was only accepted after Hotchkiss showed an “epi-cytosine” in his publication in 1948 (Hotchkiss, 1948). In 1975 Riggs proposed methylcytosine as an epigenetic mark that might be involved in X-chromosome inactivation (Riggs, 1975), and Holliday and Pugh mentioned a function in gene regulation during development (Holliday and Pugh, 1975).

Methylation of cytosines is well conserved and can be detected in all domains of life (Breiling and Lyko, 2015; Feng et al., 2010; Zemach et al., 2010). In animals, DNA methylation occurs mainly in a CpG context (except mammalian embryonic stem cells and mammalian oocytes (Ramsahoye et al., 2000; Tomizawa et al., 2011)), meaning methylation can occur on those cytosines that are followed by guanine ordered in 5´ to 3´direction (Jones, 2012). In plants, cytosine methylation can also be related to a non-CpG context like CHG or CHH (H relates to adenine, thymine or cytosine) (Law and Jacobsen, 2010). The functional analysis of DNA methylation in various studies suggested significant roles in biological processes like gene regulation, chromatin remodeling, cell differentiation, and development (Lorincz et al., 2004; Smith and Meissner, 2013). Roles in environmental adaptation were also suggested but remain less well understood (Feil and Fraga, 2012; Hu and Barrett, 2017; Verhoeven et al., 2016)

The development of detection methods strongly supported the analysis of DNA methylation. The first reproducible detection of 5-methylcytosine was based on paper chromatography in 1948 (Hotchkiss, 1948). Chromatographical methods advanced over the years, and, later, mass spectrometry analysis was established (Kuo et al., 1980; Razin and Cedar, 1977; Razin and Sedat, 1977; Wyatt, 1950). All these methods are limited in the way that only global methylation ratios are detected. A major change in detecting 5-methylcytosine and an enormous step towards understanding methylation mechanisms was achieved when bisulfite sequencing was first introduced in 1992 (Frommer et al., 1992). This method allowed the detection of methyl groups with a single-base resolution. By treating the DNA with bisulfite, the conversion of unmethylated cytosines to uracil occurs. Methylated cytosines are not affected by the

treatment. Amplification of the DNA with polymerase-chain-reaction (PCR) leads to a conversion of uracil to thymine. Sequencing of this bisulfite-treated and PCR amplified DNA allows the detection of methylated cytosines by comparison with an untreated reference sequence. Unmethylated cytosines appear as mismatches in the sequence comparison as they are sequenced as thymine, while methylated cytosines match the reference sequence. Still, the method had some limitations as sequencing itself was not as advanced as it is today, and therefore only clones with specific regions could be analyzed.

With the development of new sequencing technologies and high-throughput sequencing, the methylation analysis could be expanded to whole-genome bisulfite sequencing (WGBS) in 2008 (Cokus et al., 2008). Since then, WGBS is considered a gold standard in methylome analysis.

Nevertheless, other assays based on bisulfite treatment have been developed to lower the cost for sequencing or providing the ability to analyze methylation without a reference genome. Methods like DNA methylation microarrays or reduced representation bisulfite sequencing (RRBS) were established and are still being used (Meissner, 2005; Schumacher, 2006; Weber et al., 2005).

1.2.2 The DNA methylation machinery in animals

The DNA methylation machinery in animals is controlled by two enzyme families called DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) enzymes. While DNMTs are adding methyl groups to cytosines, using S-adenyl methionine (SAM) as a substrate, TETs are responsible for the active demethylation (Figure 2A) (Ambrosi et al., 2017).

DNMTs usually show N-terminal regulatory domains and C-terminal catalytic domains (Figure 2B) (Lyko, 2018). Five catalytic domains are highly conserved across species, while additional domains show conservation to a lesser degree (Pósfai et al., 1989). The DNMT family is highly conserved among species. Nevertheless, the number of paralogues varies among organisms (Figure 2C) (Lyko, 2018). Interestingly, loss of DNMTs is correlated with the absence of DNA cytosine methylation, as seen in *Caenorhabditis elegans* (Wenzel et al., 2011), showing the role of DNMT1 and DNMT3 for the addition and maintenance of DNA methylation.

DNMT3 is responsible for the initial methylation of unmethylated DNA and is therefore described as *de novo* methyltransferase, whereas DNMT1 is maintaining DNA methylation during replication (Figure 2A). Maintenance is needed whenever a cell has replicated, as only the original strand is carrying the original methylation marks. In animals, methylation is symmetric, so it occurs on both strands of the CpG dinucleotide (Bird, 1980; Feng et al., 2010; Zemach et al., 2010). As such, new methylation marks need to be added to the newly synthesized strand. DNMT1 recognizes hemimethylated DNA and methylates the new strand accordingly (Goll and Bestor, 2005; Law and Jacobsen, 2010; Lyko, 2018). At least one copy of either DNMT1 or DNMT3 is conserved in animals and plants with 5-methylcytosine, illustrating a strong conversation of the DNMT family (Lyko, 2018; Zemach and Zilberman, 2010).

DNMT2 is also well conserved (Figure 2C) but does not play a role in DNA methylation. It is mediating tRNA methylation and therefore is not considered a classical DNA methyltransferase. DNMT2 can methylate cytosine 38 in $tRNA^{Asp}$, $tRNA^{Gly}$, and $tRNA^{Val}$ (Goll et al., 2006; Kaiser et al., 2017; Legrand et al., 2017).

Antagonists to the DNMT family are the enzymes of the TET family. They catalyze the stepwise demethylation of the DNA by oxidizing 5-methylcytosine to 5-hydroxymethylcytosine and further to 5-formylcytosine and 5-carboxycytosine (Ito et al., 2011). Active demethylation prevents the genome from hypermethylation and plays a crucial role in shaping gene expression patterns.

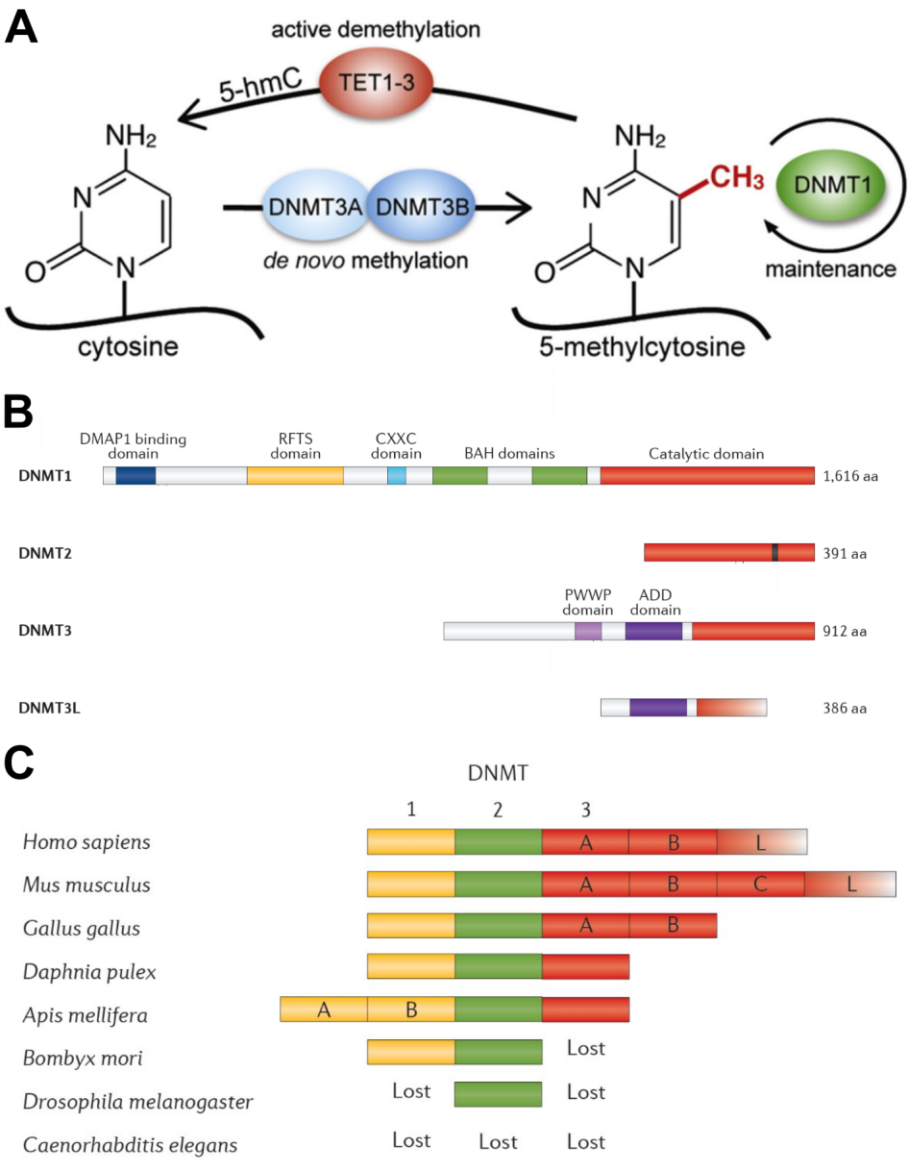

Figure 2: The DNA methylation machinery in animals. (A) DNA methylation pathway. DNA methylation predominantly occurs in a 5-methylcytosine context. The *de novo* DNMTs DNMT3A and DNMT3B introduce new methylation marks, while DNMT1 maintains the methylation patterns during replication. Removal of methyl groups is pursued by TET1–3 proteins that can convert 5-methylcytosine to its oxidized derivative 5-hydroxymethylcytosine (5hmC) and further to 5-formylcytosine and 5-carboxylcytosine (not shown in the figure). (B) Catalytic domains in DNMTs. Conserved domains are represented in different colors. The catalytic domain (red) is conserved in all DNMTs. The number of amino acids (aa) represents the human homologs. DNMT3A is displayed for DNMT3. DNMT3L, the catalytically inactive DNMT3 variant, lacks the N-terminal domain (including the Pro-Trp-Trp-Pro (PWWP) domain) and the C-terminal part of the catalytic. (C) Conserved paralogues of the DNMT family in animals. DNMT1 is shown in yellow, DNMT2, and DNMT3 are displayed in green and red, respectively. Figures adapted from (A) Ambrosi et al. (2017); (B) and (C) from Lyko et al. (2018).

1.2.3 The DNA methylation landscape in animals

The DNA methylation machinery is well conserved in all domains of life, but the methylation landscape is varying considerably among genomes from different species.

While in animals, methylation is exclusively in a CpG context (except mammalian embryonic stem cells and mammalian oocytes) (Bird, 1980; Ramsahoye et al., 2000; Tomizawa et al., 2011), in plants, non-CpG methylation (in the context of CHG and CHH, respectively) can be observed (Zemach and Zilberman, 2010). In general, methylation levels in a CpG context show a bimodal distribution in animals (Raddatz et al., 2013; Wang et al., 2014; Zemach and Zilberman, 2010). An exception is *Bombyx mori*, which shows a unimodal methylation distribution (Xiang et al., 2010). This could be caused by the lack of DMNT3 (Figure 1C) and, therefore, the methylation by DNMT1 only (Lyko, 2018). Also, methylation patterns can differ between species. Ubiquitous methylation patterns can be observed in vertebrate genomes, where 70-80 % of all CpGs are methylated (Breiling and Lyko, 2015; Li and Zhang, 2014; Schübeler, 2015). The majority of CpGs are methylated with an exception for enhancers and CpG islands. CpG islands are CpG-rich regions, which are mostly unmethylated and often associated with promoters, suggesting a functional role in gene regulation (Schübeler, 2015) (Figure 3).

Overall, methylation levels in invertebrates are lower than in vertebrates and are mainly targeted to specific genetic features like gene bodies or repeats (Figure 3) (Feng et al., 2010; Schübeler, 2015; Zemach and Zilberman, 2010). Two subclasses can be defined: mosaic methylation for genomes with intermediate levels of methylation like it was observed in *Ciona intestinalis* (23.6 % methylation) or oysters (15 % methylation (Suzuki et al., 2013; Wang et al., 2020) and sporadic methylation for lowly methylated genomes as represented in *Apis mellifera* or *Dinoponera quadriceps* (Lyko et al., 2010; Patalano et al., 2015). Consistent with the loss of DNMT1 and DNMT3 or complete loss of all DNMTs, organisms like *Caenorhabditis elegans* and *Drosophila melanogaster* (Figure 2C) (Raddatz et al., 2013; Simpson et al., 1986) show no detectable cytosine methylation.

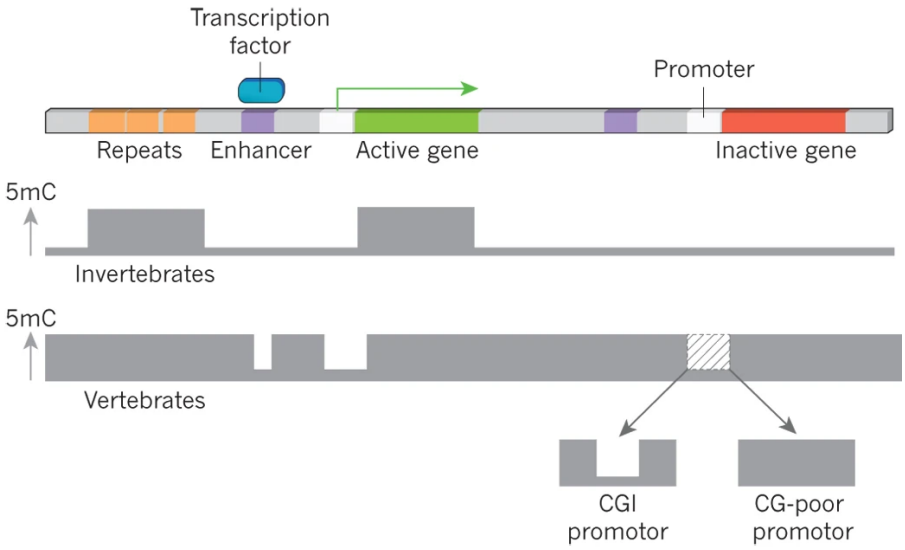

Figure 3: DNA methylation landscape in animals. The representative genomic region includes an active and inactive gene with promoter and enhancer regions. The bar´s height indicates the relative proportion of DNA methylation (5-methylcytosine, 5mC) observed in each region. CpG islands (CGIs), which often overlap with promoter regions, generally remain unmethylated, whereas CG-poor promoters are methylated when not active. Figure adapted from Schübeler (2015).

The difference between global methylation in vertebrates and targeted methylation in invertebrates suggests a difference in methylation patterns of genomic features like gene bodies. In vertebrates, gene bodies are similarly highly methylated as the genome with a substantial decrease of methylation in enhancer regions, transcription starting sites (TSS), and CpG rich regions (Figure 3) (Schübeler, 2015). Invertebrate methylation is mainly targeted to gene bodies or repeats, and therefore methylation levels increase in active gene bodies and decrease up- and downstream of it (Schübeler, 2015). Within gene bodies, two types of patterns can be observed. For mosaic methylated genes, the methylation levels increase after the TSS until it reaches a plateau, which is stable among the gene body and decreases at the transcription termination site (TTS) (Feng et al., 2010; Zemach et al., 2010). The methylation level in sporadically methylated genes is high right after the TSS and before the TTS and decreases to a certain level within the gene body (Zemach et al., 2010).

DNA methylation of gene bodies is evolutionarily strongly conserved, but the understanding of the function of methylation in non-vertebrates remains to be investigated. Functional roles in the stabilization of chromatin structures (Jones, 2012), chromatin remodeling (Lorincz et al., 2004), and alternative splicing of RNA (Lyko et al., 2010) are often discussed. Furthermore, methylation in non-vertebrates targets housekeep-

ing genes and plays a role in suppressing transcriptional noise or cryptic intragenic promotors, by methylation of coding regions (Neri et al., 2017).

1.2.4 Methylation variation as an environmental response

The ability of epigenetic marks to adapt quickly, when compared to genetic changes, repeatedly raises the question of whether epigenetic modifications play a role in the rapid adaptation of organisms to environmental changes. The comparison of DNA methylation or chromatin modification variation to DNA sequence variation in human cohorts showed that genetic variations could explain most DNA methylation changes. A genetic mutation causes methylation variation, such that transcription factors or transcription factor binding are influenced by the genetic variation, leading to differential methylation of the transcription factor binding site (Schübeler, 2015). Knowledge about the crosstalk between genetic variation and epigenetic changes is essential.

The ability to explore purely epigenetic changes, which are not driven by genetic variations, is crucial for gaining knowledge about the role of DNA methylation in development, disease, and adaptation. A potential model to study the influence of epigenetics in diseases is detecting methylation differences in identical twins, as identical genomes eliminate the genetic variation. Comparisons of disease-discordant identical twins revealed a small number of differentially methylated regions in several studies (Bell and Spector, 2012). Gene ontology analysis of the affected genes also identified genes with a role according to the individual disease traits. However, those studies are limited by small sample numbers, replications, and limitation to one-to-one comparisons (Bell and Spector, 2012).

A possibility to identify the role of epigenetics in adaptation is the comparison of epigenetic marks from distinct populations of the same species. Best suited are invasive species, as they adapt to environmental changes rapidly. Thus, epigenetic mechanisms seem to be better suited to promote this adaptation than genetic variations. Indeed, many studies have analyzed epigenetic modifications in plants and animals in this regard.

The often-used plant species *Arabidopsis thaliana* was variably methylated in different ecotypes (Becker et al., 2011). *Arabidopsis* is globally distributed and is characterized by distant phenotypes (Atwell et al., 2010), making the study of epimutations an excit-

ing topic. The study of Becker et al. (2011) revealed methylation variation between distinct lineages separated for 30 generations that arose from the same ancestral lineage. However, later comparison of genetic variances to epigenetic variances explained most of the methylation changes by genetic polymorphisms (Dubin et al., 2015), which are frequent in this species (Alonso-Blanco et al., 2016), and thus define a large number of genetic ecotypes in *Arabidopsis* rather than epigenetic ecotypes (Ferrero-Serrano and Assmann, 2019).

Several key studies in animals also tempt to link epigenetic variation to adaptivity. For example, Le Luyer *et al.* (2017) found methylation differences in salmon reared in artificial hatcheries and released to the wild compared to those born in the wild. These methylation differences might explain the known fitness differences between the two environments. The analysis also included investigations on genetic polymorphisms, one of the most substantial confounding factors for analyzing methylation variation.

In the brown anole lizard (*Anolis sagrei*), a different methylation pattern was observed when lizards were exposed to a different habitat for four days, and differentially methylated cytosines were found in genes responsible for signal transduction, immune response, and circadian rhythm (Hu et al., 2019). Another notable study for the functional analysis of methylation variation was performed by Gore *et al.* (2018). They used cavefish (*Astyanax mexicanus*), which are losing eye function over time. Known genetic variations do not drive this process, but it was observed that promotor hypermethylation might repress eye-specific genes and promote eye defects. Injection of a DNA methylation inhibitor could partially restore eye development, illustrating the role of methylation in this context.

For invasive species, it is crucial to have high plasticity to respond to environmental changes rapidly. This is often not explainable by genetic variation, as this mechanism cannot evolve as rapidly as required (Carneiro and Lyko, 2020). Therefore, several studies were performed in invasive species to explore possible epigenetic mechanisms. Examples are the mussel *Xenostrobus secures*, where global hypomethylation was observed in a recently evolved population, was suggested to promote higher phenotypic plasticity (Ardura et al., 2017), or the whitefly (*Bemisia tabaci*) where the knockdown of DNMT1 resulted in a higher sensitivity to thermal changes (Dai et al., 2018). However, investigation of methylation patterns was not performed, and many

claims in the field of adaptation in invasive species are based on indirect detection of methylation.

1.2.5 Marbled crayfish epigenetics

The marbled crayfish represents an invasive species with a monoclonal genome, thus providing a unique opportunity to study rapid adaptation by epigenetic mechanisms. To study epigenetic mechanisms in the marbled crayfish, the epigenetic machinery and landscape were analyzed. In the genome of marbled crayfish (Gutekunst et al., 2018), homologs of DNA methyltransferases DNMT1 and DNMT3 were identified (Gatzmann et al., 2018). Additionally, a single copy of a TET hydroxymethylase was present in the genome (Gatzmann et al., 2018). Indications for the presence of DNA methylation had already been provided in 2008 by Vogt et al. (Vogt et al., 2008), but the full characterization of the methylome was performed by Falckenhayn in 2016 using whole-genome bisulfite sequencing (Falckenhayn, 2016). This analysis revealed a symmetric CpG specific methylation with a bimodal distribution. As in many invertebrates, the methylation landscape is characterized by a mosaic pattern targeted to gene bodies (Figure 4A), showing a bimodal distribution. Genes were either highly or lowly methylated. More specifically, genes identified as housekeeping genes revealed enrichment in DNA methylation of gene bodies compared to methylation levels over all genes (Figure 4B & C). Exploring different tissues and samples from various animals and one embryonic stage established a relatively stable and mainly tissue invariant methylation pattern (Figure 4B & C) (Gatzmann et al., 2018).

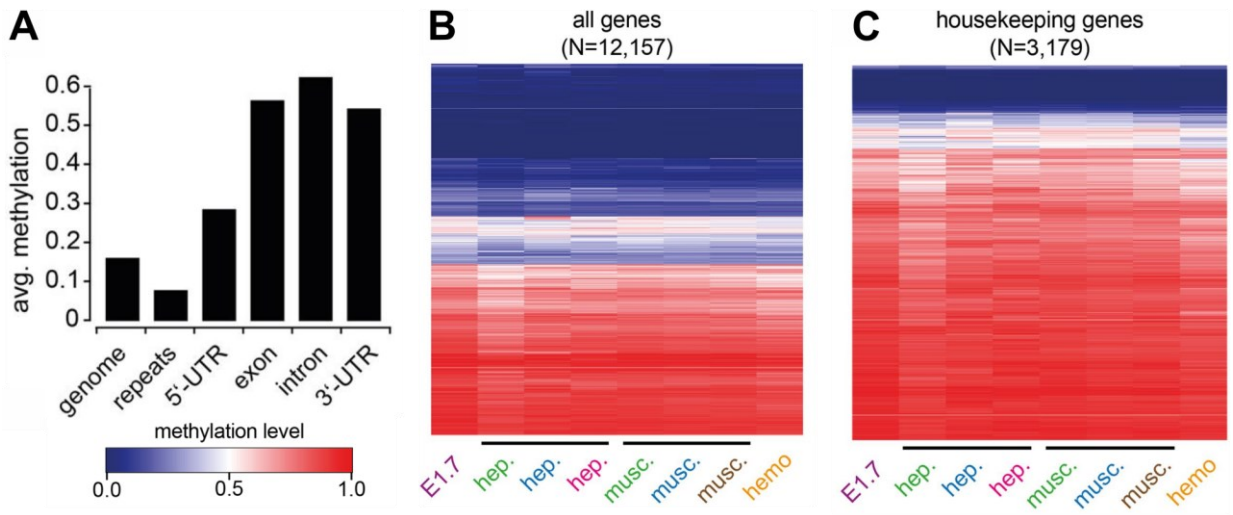

Figure 4: Characterization of the marbled crayfish methylome. (A) Methylation levels of the genome and predicted gene features. (B and C) Analysis of gene body methylation patterns in all genes and housekeeping genes from different developmental stages, tissues, and animals. Methylation levels are indicated on a scale from 0 (blue) to 1 (red). E1.7: stage 1.7 embryos, hep.: hepatopancreas, musc.: abdominal musculature, hemo: hemocytes. Colors denote individual animals. (B) The heatmap shows average gene body methylation levels in eight independent samples (columns). (C) Parallel analysis of housekeeping. Figure adapted from Gatzmann (2018).

A detailed comparison of 8 whole-genome bisulfite sequencing datasets from different animals, different tissues, and different developmental stages also indicated a potentially smaller group of genes that showed more variable methylation levels (Gatzmann et al., 2018). This was confirmed by systematic analyses of methylation variance (Figure 5). A variance cutoff of >0.006 identified 846 genes (Figure 5A), 149 of which were consistently methylated or unmethylated (mean ratio >0.8 or <0.2, respectively) and excluded from further analysis, thus defining a core set of 697 variably methylated genes (Figure 5B). Metric multidimensional analysis based on the methylation levels of these genes separated hepatopancreas samples from abdominal muscle samples (Figure 5C). This finding suggested the presence of previously unrecognized tissue-specific methylation patterns.

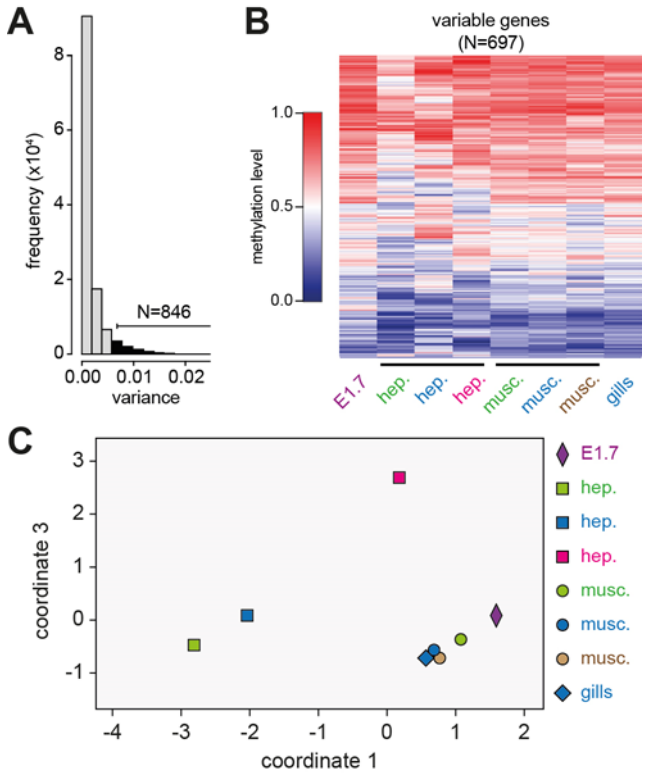

Figure 5: Identification and characterization of variably methylated genes. (A) Density plot of methylation variance for the 12,244 genes with sufficient coverage in all eight samples. 846 genes showed a methylation variance of >0.06. (B) Comparative analysis of variably methylated genes. The heatmap shows average gene body methylation levels for the 697 variably methylated genes with a mean ratio of >0.2 and <0.8 in eight independent samples (columns). Methylation levels are indicated on a scale from 0 (blue) to 1 (red). (C) Metric multidimensional scaling analysis is based on the methylation levels of the 697 variably methylated genes. Figure adapted from Gatzmann (2019).

1.3 Aims of the Ph.D. thesis

The marbled crayfish is a unique freshwater crayfish. While it is a clonal organism with a single origin, it has spread widely and adapts to various habitats. Since genetic variation cannot drive this adaptation, epigenetic mechanisms are likely the cause of this rapid adaptation. DNA methylation is suggested to play a role in environmental adaptation (Verhoeven et al., 2016). However, these variations are often challenging to detect, as genetic variants influence the results and/or the sample number is limiting statistical power (Lea et al., 2017).

This thesis aims to identify context-dependent DNA methylation patterns in marbled crayfish. Therefore, five different habitats with wild German marbled crayfish populations will be characterized to identify habitats with highly different environmental conditions. Furthermore, methylation patterns of samples from two of the German habitats

will be compared with samples from two Malagasy habitats using a capture-based methylation assay. This assay is designed to cover a subset of 361 genes (after filtering), which will be characterized to get insight into the gene and methylation structure of this subset. Additionally, the results from the capture-based methylation analysis will be validated to confirm the results technically and biologically. Lastly, a concept for the aquaculture of marbled crayfish will be developed to suggest environmentally safe and sustainable aquaculture of a potentially invasive species.

2 Material and Methods

The material and methods section lists and describes the material and methods used in this thesis. This chapter gives an overview of the field and laboratory work and the computational tools and methods used to analyze the results.

2.1 Material

2.1.1 Field equipment

- Buckets (L x W x H: 37.6 cm x 26 cm x 18.9 cm, Bauhaus AG)
- Caliper 15 cm
- Dispo Ladle SteriPlast HDPE, sterile, 500mm (bürkle)
- Hand nets 6.5 cm x 7.5 cm (AniOne)
- Luer Lock Cap, Male/Female (Uhs)
- Lumix GX80 (Panasonic)
- Multiparameter HI 991301 (Hanna Instruments)
- Permanent marker
- Plastic bags clear
- Sample bag (Fisherbrand)
- Sterivex HV 0,45µm Filter Unit (Millipore)
- Syringe, sterile Luer- Lock™ (BD)
- Test bottles 250 ml (Raiffeisen)
- Traps 30 cm x 60 cm
- Whirl-Pak® 1242 ml Stand-Up Bag (Merck)
- Whirl-Pak® sample bag (Nasco)

2.1.2 Laboratory equipment

2.1.2.1 Devices

- ALPHA 2-4 LDplus freeze dryer (Christ)

- Analytic balance CP64 (Sartorius)
- Centrifuge 5425 (Eppendorf)
- Centrifuge 5804R (Eppendorf)
- Covaris Focused-ultrasonicator E220 (Covaris)
- DynaMag-96 Side Magnet (Thermo Fisher Scientific)
- Incubator Med line (Liebherr)
- Incubator Multitron (Infors AG)
- Incubator shaker Innova 4200 (New Brunswick Scientific Company, Inc.)
- NanoDrop 2000 (Thermo Scientific)
- Qubit 4.0 Fluorometer (Invitrogen)
- TapeStation 2200 (Agilent)
- Thermocycler, DNA Engine (BioRad)
- Thermomix TM5 (Vorwerk)
- Thermomixer (Eppendorf)
- TissueRuptor (Qiagen)
- Transluminator U:Genius^3 (Syngene)
- Vacuum concentrator RC1010 (Jouan)
- Weighing EK-200i digital scale (A&D)

2.1.2.2 Reaction kits

- Agencourt AMPure XP Kit (Beckman Coulter Genomics)
- DNeasy Blood & Tissue Kit (Qiagen)
- Dynabeads MyOne Streptavidin T1 (Thermo Fisher Scientific)
- EZ-DNA Methylation-Gold Kit (Zymo Research)
- KAPA HiFi HotStart ReadyMix PCR Kit (Roche)
- Nextera XT Index Kit v2 Set A (Illumina)
- Pyromark PCR Kit (Qiagen)
- QIAquick Gel Extraction Kit (Qiagen)
- QIAquick PCR Purification Kit (Qiagen)
- Qubit reagents (Invitrogen)
- SURE competent cells (Agilent)
- SureSelect Custom designed baits (Agilent)

- SureSelectXT Methyl-Seq Library Preparation Kit for targeted methylation sequencing (Agilent)
- Tape Station Kits (Agilent)
- TOPO-TA ® Cloning Kit (Invitrogen)

2.1.2.3 Chemicals and buffers

- Acetic Acid (Merck)
- Agarose Standard (Carl Roth)
- DMSO (Sigma-Aldrich)
- dNTPs 100 mM (VWR)
- EDTA (GERBU Biotechnik Gmbh)
- Ethanol (Sigma-Aldrich)
- Fire-Taq Blue (Steinbrenner)
- Isopropanol (Sigma-Aldrich)
- Mangan(II)-chloride Tetrahydrate (Carl Roth)
- PBS 1x (Gibco Life Technologies)
- Pre-Lyses Buffer: 10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 10 mM NaCl
- Primer (Sigma-Aldrich)
- Proteinase K (Ambion)
- ReadyMix PCR buffer (Thermo Fisher Scientific)
- RNase A 50 mg/ml (Invitrogen)
- SDS (Roth)
- Sodium Chloride (Sigma-Aldrich)
- TAE 1x Buffer: 40 mM Tris pH 7.6, 20 mM acetic acid, 1 mM EDTA
- Taq DNA polymerase (PrimaAmp)
- Taq-Polymerase ThermoPrime Plus DNA Polymerase (Thermo Fisher Scientific)
- Tris (Sigma-Aldrich)
- UltraPure Distilled Water (Invitrogen)

2.1.2.4 Consumables

- 8-strips PCR Microtubes 0.2ml (4titude)

- 96-well plates (Thermo Fisher Scientific)
- Buckets (L x W x H: 25.6 cm x 18.1 cm x 13.6 cm)
- D1000 ScreenTape (Agilent)
- Falcon tubes 15ml (Greiner)
- Falcon tubes 50 ml (Greiner)
- Genomic ScreenTape (Agilent)
- High sensitivity D100 Screen Tape (Agilent)
- Microcentrifuge tubes 1.5ml (Eppendorf)
- MicroTUBE Crimp-Cap AFA Fiber 6x16mm (Covaris)
- Optical Cap 8x (Agilent)
- Optical Tube 8x (Agilent)
- QubitTM Assay tubes (Qubit)

2.1.3 Computational tools

- BisAMP (Bormann et al., 2019)
- BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi)
- FinchTV (version 1.4.0)
- QGIS (version 3.6.0 with GRASS 7.6.0)
- R Studio (version 3.6.1)

2.2 Methods

2.2.1 Habitat characterization and distribution of marbled crayfish in Germany

In order to study the habitat diversity of marbled crayfish in Germany, it was necessary to identify and characterize stable wild populations. Therefore, a literature search for publications and newspaper articles was conducted, and information from local fishers and fishing clubs was obtained.

2.2.2 Sample collection for all experimental parts

In July and August 2017, field trips to the locations with potential marbled crayfish populations were conducted, and samples were collected. Sampling for bead-based capture assays was carried out in August 2017 for Reilinger See, October 2017 for Singliser See, and as mentioned in Andriantsoa et al., 2019, from October 2017 to March 2018 in Madagascar (Andriantsoa et al., 2019). Sampling for the validation experiment was carried out from March to May 2019 in Germany and Madagascar.

2.2.2.1 Sampling method

Five lakes in Germany (Krumme Lanke, Singliser See, Reilinger See, Baggersee Epple, and Moosweiher, Figure 6) were explored, and in all of them, marbled crayfish were found and collected. Prior to collection, water bodies were explored to find suitable spots to enter the water during the daytime. Several spots depending on the accessibility of the lake, were defined as collection sites. Animals were collected during the night by hand nets. At Reilinger See, the local fishing club supported catching the animals by traps. Two traps were put in the lake overnight, and the crayfish collection was done the next morning (Reilinger See, ASV Reilingen). The water depth of collection sites and traps were noted. During the daytime, no marbled crayfish were found.

Figure 6: Map of Germany, showing the sample locations Krumme Lanke, Singliser See Reilinger See, Baggersee Epple, and Moosweiher indicated by red dots.

2.2.2.2 Characterization of the ecosystems

Abiotic factors from each of the five sampling sites were analyzed. The water body was categorized into a trophic level. The collection site's sediment was identified, and basic water parameters (temperature, pH, conductivity, total dissolved solids) were taken. To test those parameters, a multiparameter device (Multiparameter HI 991301, Hanna Instruments) was used at a depth of 10 cm below the water surface. For detailed water analysis, 250 ml of water from three locations (Krumme Lanke, Singliser See, Reilinger See) was collected at a later timepoint, stored at room temperature during the field trip, then at 4 °C in the laboratory until sent to Raiffeisen Laborservice (Omont, Germany), for the analysis of 25 parameters (see Appendix for detailed reports). For Moosweiher and Baggersee Epple, reports from the Landesamt für Umwelt, Messung und Naturschutz Baden-Württemberg were used to obtain information about the water quality of the lakes.

Biotic features of each water body were also characterized. This includes the vegetation in the water and at the shore and the fauna in the water. Additional crayfish spe-

cies were collected using traps (Reilinger See) or hand catching. To obtain a better overview of the fauna, fishing clubs were asked, or governmental reports were studied.

The following habitats were explored:

A) Krumme Lanke is a lake of 15 ha in size and about 8 meters deep. It is located in the south-west of Berlin and is connected to a broader water system of lakes and channels close to the river Havel. The lake is eutrophic with various fish like eels, bass, roach, jackfish, and wels catfish and at least two different crayfish species (*Procambarus virginalis* and *Faxonius limosus*). Parts of the shore are not accessible as a fence protects them. The lake is surrounded by deciduous trees, which often project into the water. Shallow areas at the shore with muddy and sandy ground, bordered by dead leaves and woods, were chosen to search for marbled crayfish, as two dead *Faxonius limosus* and one dead marbled crayfish could be detected in these areas, but only at one spot, live marbled crayfish could be collected.
B) Singliser See is an oligotrophic, 75 ha, and about 30-meter-deep lake close to Borken in the north of Hessen. It is a former coal mining site, which was flooded for renaturation. The lake is surrounded by small water channels, another lake, and a medium-sized river called Schwalm. The lake is characterized by blue to turquoise water. The coloration of the water results from the lack of water plants and fish in the lake. The ground and shores are sandy; some parts of the shore have pebbled areas. Deciduous trees surround the lake, and the shore is partially covered with reed.
C) Close to Heidelberg in the village Reilingen, the 9 ha and 16-meter-deep lake Reilinger See is located. It is a eutrophic lake with lots of different fish like eels, bass, and carps maintained and fished by the local fishery club ASV Reilingen e.V. Additionally, mussels, turtles, and crayfish can be found, probably because of unauthorized anthropogenic releases. The lake is surrounded by a few trees, mainly large cottonwoods, and bushes. Parts of the shore are covered by reed. The lake has no direct connection to any other water body, and there is no lake close to Reilinger See. The ground is sandy and muddy with lots of leaves and branches of trees in the water, offering many places to hide for crayfish. The lake froze-over completely in cold winters, sometimes even for weeks. This also happened in Feb-

ruary and March 2018, when the lake was covered by ice for four weeks. At Reilinger See, we got help from the local fishing club, which put traps in the water.

D) The Baggersee Epple was built in a gravel pit in the municipality Kirchentellinsfurt close to Tübingen. The lake has a direct connection to another lake and the Neckar by a stream called Schlierbach. Meadows and woods surround the lake, which has about 25 ha and a maximum depth of five meters. The water body is mesotrophic and different species of fish could be detected.

E) Lake Moosweiher is the lake where the first stable wild population of marbled crayfish in Germany was described (Chucholl and Pfeiffer, 2010). The lake is located in the north of Freiburg, south-west on Germany. It is a small mesotrophic lake of 7.6 ha with a maximum depth of 8 meters (Chucholl and Pfeiffer, 2010), at the border of the city encompassed by trees and small meadows. During the catching, large fish and moraines could be observed. Additionally, there is a known presence of *Faxonius limosus* in the lake (Chucholl and Pfeiffer, 2010). There is no connection to any other water body.

2.2.3 Marbled crayfish handling and morphological identification

All collected crayfish were morphologically identified. The carapace was checked for a distinct marbled pattern. Additionally, the sex of the animal was determined. If other crayfish species were observed during the catch, they were identified morphologically as well.

All caught animals were measured using a 15 cm caliper and dissected. Crayfish were rinsed with 70% ethanol, dried with a tissue towel, and decapitated. Hepatopancreas and abdominal muscle tissues were taken immediately and stored separately in a 1.5 ml tube filled with absolute ethanol. Samples were stored at -80 °C until used for DNA extraction for genetic authentication or methylation analysis.

2.2.4 Genetic authentication

To confirm the crayfish identity, genotyping was performed in three animals per collection site. A fragment of Cytochrome b was used as a marker, using a marbled crayfish from the Heidelberg strain (Gutekunst et al., 2018) as a reference. *Faxonius limosus*, a

crayfish species which is co-existing with marbled crayfish, was used as a negative control. It was found in 2 lakes during the collection period alongside marbled crayfish.

2.2.4.1 DNA extraction

Genomic DNA was extracted from 100 mg abdominal muscle tissue using the following protocol. The tissue was transferred to a 15 ml falcon tube and homogenized in lysis buffer containing 4.5 ml pre-lysis buffer (10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 10 mM NaCl), 250 µl SDS (20%), 25 µl proteinase K (20 mg/ml) and 4 µl RNase A (50 mg/ml) using a Tissue Ruptor (Qiagen). The homogenized tissue was then incubated at 37°C overnight without shaking. Then, 2.5 ml NaCl 5M was added to the sample, mix thoroughly, and centrifuged at 8200 g for 15 min at 4°C. The clear aqueous phase was then transferred to a new 15 ml falcon tube, and 5.6 ml Isopropanol was added, followed by mixing and centrifugation for 10 min at room temperature and 8200g. After centrifugation, the supernatant was removed carefully, and the pellet was washed with 1 ml ethanol 70% and transferred to a 1.5 ml Eppendorf tube. To ensure not to lose any DNA, an additional centrifugation step was performed with 20,000g for 5 min at room temperature. The ethanol was removed, and the pellet was air-dried for 5-10 min. Finally, the pellet was resuspended in 20-50 µl DNase-free water depending on the size.

The quality and quantity of the DNA were checked either on the TapeStation or 0.7 % agarose gel and NanoDrop. Samples were directly used for further analysis or stored at -20 °C until further needed.

2.2.4.2 Polymerase chain reaction (PCR)

For genetic authentication of the collected marbled crayfish, a region of the mitochondrial Cytochrome b (see Appendix for primer list) was amplified with the following conditions.

Each PCR reaction mix contained 2.5 µl 10x Reaction Buffer (Steinbrenner), 1 µl dNTPs (10 mmol, VWR), 1 µl of each primer (forwards and reverse, Sigma-Aldrich), 0.5 µl Fire-Taq blue polymerase (1 U/µl, Steinbrenner) and 14 µl UltraPure water (Invitrogen). 5 µl DNA was added to the reaction mix to a final volume of 25 µl. For the DNA amplification, a Bio-Rad Peltier Thermal Cycler was used with the following program. Initial denaturation with 96 °C for 3 min, followed by 30 cycles of denaturation at

96 °C for 30 s, annealing at 57°C for 30 s and elongation at 72 °C for 30 s. A final elongation step at 72 °C for 3 min was performed. Samples were then cooled down to 4 °C until the PCR run was stopped. Products were put on ice or at 4 °C until further use.

5 µl of the PCR products were checked with agarose gel electrophorese on a 1% agarose gel. If amplicons in the expected size of 274 bp were obtained, the remaining 20 µl were cleaned using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instruction. The purified PCR products were then cloned according to the manufacturer's protocol in SURE competent cells (Agilent) using the TOPO TA Cloning Kit (Invitrogen). Cleaned plasmids were subsequently sent to GATC (Eurofins) for sequencing (Sanger sequencing).

2.2.4.3 Data analysis

The sequencing results from the marbled crayfish of the five investigated locations and the *Faxonius limosus* from Reilinger See were first analyzed in FinchTV version 1.4.0 to assess the quality of each base of the sequence, resulting in a 220 bp amplicon of the cytochrome b region. This part of the amplicon was used to align the sequencing results to the reference sequence of marbled crayfish using the online Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi.). This data analysis follows the workflow described in Gutekunst *et al.* (2018) for the genotyping of marbled crayfish. Only the marker for cytochrome b was used.

2.2.5 Subgenome DNA methylation analysis of marbled crayfish

Whole-genome bisulfite sequencing is today's gold standard to detect DNA methylation sites in the genome with single-base resolution. The method combines bisulfite treatment of the DNA with high-throughput sequencing to determine methylated sites of the genome. The bisulfite treatment causes the deamination of unmethylated cytosines to uracil, which is recognized as thymine in PCR reactions or sequencing. Methylated cytosines are not affected by the bisulfite treatment and remain as cytosines. A comparison of the reference DNA with the bisulfite-treated DNA reads show a mismatch for all unmethylated cytosines as they are called as thymine. All remaining cytosines in the treated sequencing reads were methylated.

2.2.5.1 SureSelect^{XT} Methyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing Assay

Whole-genome bisulfite sequencing (WGBS) is a well-established method to detect all methylated sites of an organism, but the method is still expensive, and the analysis is time-consuming, which limits the number of samples that can be analyzed. To generate statistical power and to be able to detect differences between samples from the same population and among populations, an analysis of a high number of samples is needed. Therefore, a method that is less cost-intensive and faster to analyze is required. Furthermore, not all the genome methylation sites are important for analyzing methylation differences, as the methylation status of some regions is stable among all animals (Gatzmann et al., 2018).

Subgenome methylation analysis allows the sequencing of a small part of the whole genome and is less cost-intensive and faster to analyze. The SureSelect^{XT} Methyl-Seq Target Enrichment System was used to generate subgenome bisulfite-treated libraries for high throughput sequencing on an Illumina platform.

The methylome of marbled crayfish was already published by Gatzmann *et al.* (2018). As described in the introduction, eight independent samples containing different tissues and locations were analyzed by WGBS. A subset of 697 variable methylated genes was identified and used to generate custom beads called “baits” for the SureSelectXT Methyl-Seq analysis.

2.2.5.2 Bait design

The SureSelect^{XT} Methyl-Seq Assay is an in-solid bead-based capture method for subgenome analysis. Baits are biotinylated RNA oligonucleotides which retrieve the desired DNA fragments. The custom-made baits used in the analysis cover whole gene sequences of 697 genes. Each gene is covered in triplicates with this bait-design. Baits have a length of 120 bp and are designed to overlap with an offset of 40 bp. All baits are designed to capture the forward strand (Table 1).

Table 1: Bait design. Example list of baits displaying the probe number, the covered strand and gene, and the probe start and endpoint.

Gene	Probe	Strand	Chrom	Start	End
Pvirginalis_0917	30543	+	chr246	0	120
Pvirginalis_0917	30544	+	chr246	40	160
Pvirginalis_0917	30545	+	chr246	80	200
Pvirginalis_0917	30546	+	chr246	120	240
Pvirginalis_0917	30547	+	chr246	160	280
Pvirginalis_0917	30548	+	chr246	200	320
Pvirginalis_0917	30549	+	chr246	240	360
Pvirginalis_0917	30550	+	chr246	280	400
Pvirginalis_0917	30551	+	chr246	320	440
Pvirginalis_0917	30552	+	chr246	360	480
Pvirginalis_0917	30553	+	chr246	400	520
Pvirginalis_0917	30554	+	chr246	440	560
Pvirginalis_0917	30555	+	chr246	480	600
Pvirginalis_0917	30556	+	chr246	520	640
Pvirginalis_0917	30557	+	chr246	560	680
Pvirginalis_0917	30558	+	chr246	600	720
Pvirginalis_0917	30559	+	chr246	640	760
Pvirginalis_0917	30560	+	chr246	680	800

2.2.5.3 Selection of samples for bead-based methylation analysis and validation

Based on the water parameters results and the habitat characteristics, samples from 2 locations in Germany and 2 locations in Madagascar (Andriantsoa et al., 2019) were selected to analyze them using the bead-based capture assay. The selection was firstly based on water and habitat parameters, focusing on huge differences between the 4 locations, and secondly, based on the availability of samples. These samples were collected by hand catch or with hand nets in August 2017 at Reilinger See, in October 2017 at Singliser See (see Appendix for coordinates), and as mentioned in Andriantsoa *et al*., 2019, from October 2017 to March 2018 in Madagascar. Sampling for the validation experiment (section 2.2.5.8) was carried out from March to May 2019 in Germany and Madagascar.

2.2.5.4 The library preparation protocol for Agilent Sure Select Methyl-Seq Assay.

Library preparation was carried out as described in the SureSelectXT Methyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing Protocol, Version D0, July 2015, and E0, April 2018. Quality control was performed, and sample concentrations were measured on a 2200 TapeStation (Agilent). Multiplexed samples were submitted

to the DKFZ High Throughput Sequencing Core facility (DKFZ, Heidelberg) and sequenced on a HiSeqX ten system (Illumina).

First, DNA from hepatopancreas and abdominal muscle was extracted, like described in section 2.2.4.1. DNA concentration was measured, and either 1µg or 3µg DNA was used for the library preparation following the protocol mas described below.

The first step required DNA shearing, which was done on the focussed-ultrasonicator E220 from Covaris. DNA was fragmented into 150-200 bp, and quality was checked with a D100 DNA ScreenTape on the TapeStation (Agilent).

Subsequently, the fragmented DNA was prepared for hybridization to the custom baits. This includes three steps. First, the end-repair of the DNA fragments, followed by A-tailing of the 3`end and ligation of methylated adapters, creates a pre-capture library. In between each step, the DNA was purified with AMPure XP beads, and the quality was checked on the TapeStation.

To proceed with the hybridization, a minimum of 350 ng pre-prepared library was required. The protocol recommends doing an additional round of pre-library preparation in case of lower amounts. However, a pilot experiment revealed that an amount of 165 ng is sufficient to proceed with the hybridization. Therefore, an additional pre-library preparation was made if the 165 ng could not be reached in the first round.

After the pre-library preparation, samples with at least 165 ng were hybridized to the baits at 65 °C for 16 hours. This step captures the targeted regions of the biotinylated baits. A cleaning step with streptavidin beads was used to remove the remaining DNA and the hybridization buffers. The captured DNA was removed from the streptavidin bound baits, and the subgenome DNA was used for the bisulfite treatment.

The bisulfite conversion was performed with the EZ DNA Methylation-Gold Kit (Zymo Research), following the SureSelect^{XT} protocol's instruction. The bisulfite converted DNA was PCR-amplified on a Bio-Rad Peltier Thermal Cycler to generate sufficient concentrations for sequencing. The cycle number was set to 8 cycles to limit amplification artifacts.

The advantage of the technique is the ability to pool several of samples in one sequencing run and still get enough coverage, as the genome size of the captured library is just much smaller (2 kbp) compared to the whole marbled crayfish genome

(~3,5 Gbp, Gutekunst *et al.*, 2018). To be able to distinguish the samples out of one run, individual indexing is needed. Therefore, the last step of the protocol is the indexing of the samples, which will be combined on one lane of the sequencer. Up to 12 samples with individual single indexes provided in the SureSelectXT Methyl-Seq Library Preparation Kit were pooled. After PCR-amplification, cleaning up with AMPure XP beads and quantification with a high-sensitivity screen tape on the TapeStation, samples were pooled equimolar for a final Volume of 30 µl with 10 nM DNA concentration. Samples were then submitted to the DKFZ High Throughput Sequencing Core facility and sequenced.

2.2.5.5 Establishment of the SureSelect Methyl-Seq assay

The analysis of the sequencing results from the Agilent Sure Select Methyl-Seq Assay was performed by Dr. Geetha Venkatesh and Dr. Günter Raddatz. Read pairs were quality trimmed and mapped by Günter Raddatz to the 697 genes that showed variable methylation in the whole-genome bisulfite sequencing datasets (Gatzmann, 2019) using BSMAP (Xi and Li, 2009).

For the sake of identifying mechanisms for epigenetic adaptation in marbled crayfish, samples collected from the four previously described habitats were used for the analysis with the SureSelect Methyl-Seq assay. DNA was prepared from two different tissues: hepatopancreas, which represent the main metabolic organ of crayfish and abdominal muscle, the larger muscle tissue forming the abdominal tail. In total, 73 samples were analyzed. Hepatopancreas DNA was prepared from 47 animals (11-12 per location), while abdominal muscle DNA was prepared from a subset of the same animals (N=26, 12-4 per location) (Table 2).

Table 2: List of samples for the SureSelect Methyl-Seq assay, including samples from Singlis, Reilingen, Andragnaroa, and Ihosy and two different tissues (hepatopancreas and abdominal muscle).

	Singlis	**Reilingen**	**Andragnaroa**	**Ihosy**
Hepatopancreas (N)	12	12	13	11
Abdomial muscle (N)	12	7	4	4

A set of five abdominal muscle samples from two locations (2 x Singlis, 3 x Reilingen) were prepared and sequenced in the first batch. Subgenome capture was found to be both efficient and specific, providing a minimum of 10 million mapped reads per sam-

ple under stringent conditions and enrichment of reads in target genes (Figure 7). The capture efficiency, displayed by the percentage of captured genes, was 99.86 %.

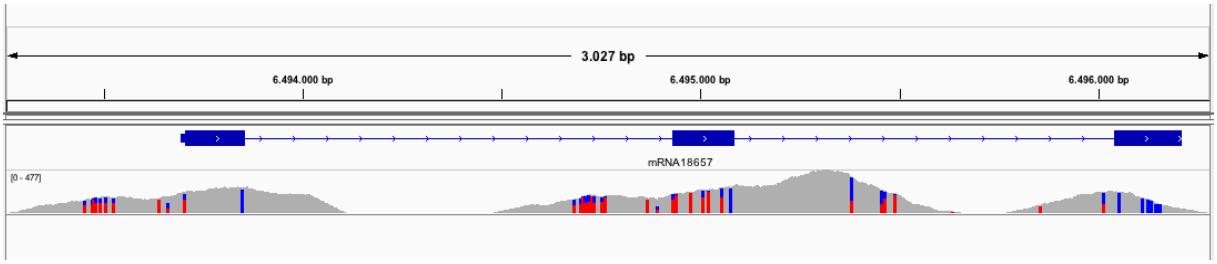

Figure 7: Enrichment of sequencing reads in a target gene. Example gene (3.027 bp) used for the capture-based assay. Capture-design was based on the first genome assembly and covered mainly exons (blue bars in the mRNA) and the spanning regions. Introns, which contained still a relatively high number of unknown bases, are therefore not covered. The bars in grey indicate the enrichment of reads in the exon regions. Blue and red bars within the grey area show CpGs and their methylation level. The enrichment of the target regions provides proof for the specificity of the capture-design.

A draft analysis of the samples using a principal component analysis (PCA) and considering all captured genes, showed a separation of the samples from the two locations by the first principal component with 45.49 % total variance (Figure 8).

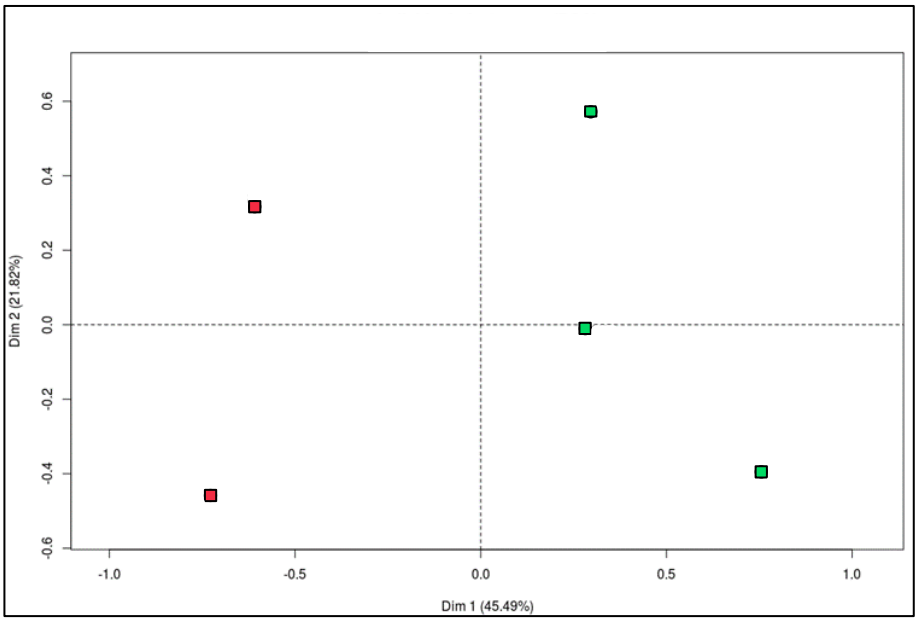

Figure 8: Location separation of Singliser See and Reilinger See. Principal component analysis showing the separation of abdominal muscle samples from Singliser See (red) and Reilinger See (green) by principal component 1. All 696 captured genes were taken into consideration.

Samples were prepared in different batches to establish and optimize the protocol for the custom-designed baits and the selected tissues. According to the manual's instructions, a minimum amount of 350 ng of the pre-prepared library was required for hybridization. For some samples, the required amount could not be reached but was still hy-

bridized. This revealed that the minimum amount of pre-prepared library needed is 165 ng. Samples hybridized and sequenced with less than 165 ng showed low coverage, resulting in a separation of the samples from all other samples in a PCA (Figure 9). Principal component 1 in this PCA reflects the coverage of the samples. Based on this, the minimum amount of 165 ng was set for further library preparation. Regardless of the three samples which needed to be excluded from the analysis (Sing3_hep, Reil16_hep, Sing3_abdM), a separation of the tissues by principal component 2 (13.92 % variance) is visible.

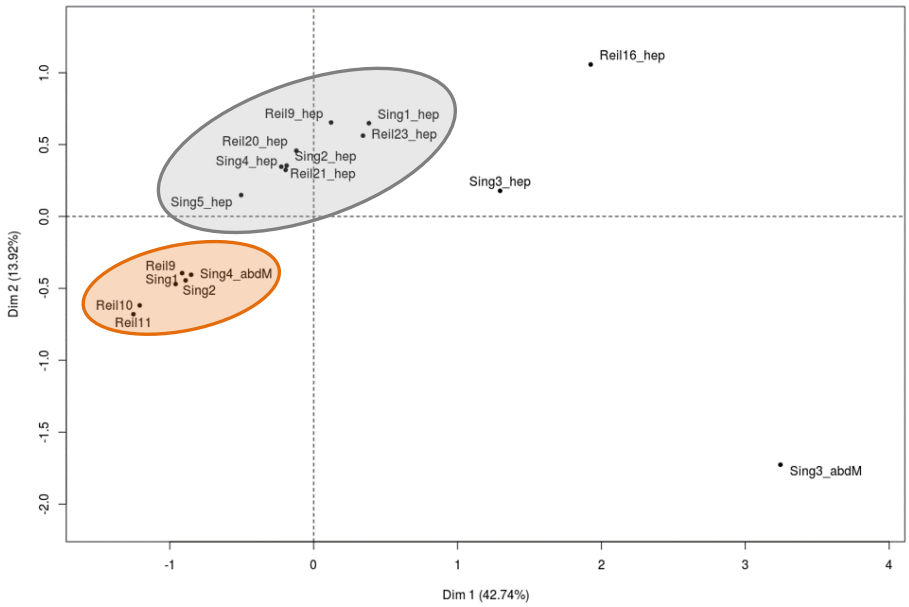

Figure 9: Establishment of library requirements. The PCA is showing three samples (Sing3_hep, Reil16_hep, Sing3_abdM) separated from the other samples (Dim 1 (42,74%)). These samples had a low amount of input DNA for the hybridization step during the library preparation. Furthermore, hepatopancreas samples (grey circle) show a separation from abdominal muscle samples (orange circle) by principal component 2 (Dim 2 (13.92%)

2.2.5.6 Identification of differentially methylated regions

Subsequent analysis was performed by Dr. Geetha Venkatesh. The methylation ratio for each CpG site was calculated using Python provided with BSMAP. Only those CpG sites present in all the samples with coverage of ≥5x were considered for further analysis. Additionally, the average methylation ratio was calculated only in genes with at least 5 CpG sites. Furthermore, genes in the bottom 10% in terms of methylation variance, genes with an average methylation level of < 0.1 or > 0.9, and genes with more than 50% Ns in their sequence have been excluded from the analysis, resulting in a gene set of 361 genes. This gene set was used to analyze samples for context-dependent methylation variances. In order to identify tissue-specific methylation differ-

ences, a Wilcoxon rank-sum test was applied (hepatopancreas vs. abdominal muscle samples from Singliser See and Reilinger See), and the p-values were corrected for multiple testing using the Benjamini-Hochberg method. Likewise, to identify location-specific methylation differences, a Kruskal-Wallis test was used, and the p-values were corrected for multiple testing using the Benjamini-Hochberg method. Samples were plotted in a principal component analysis (PCA) to see tissue-specific or location-specific clusters after filtering with the described criteria. Additionally, dmrseq (Korthauer et al., 2019) was used to identify tissue-specific and location-specific differentially methylated regions within our gene set. These regions were used to design markers for the validation of the Agilent Sure Select Methyl-Seq Assay results.

2.2.5.7 Gene ontology analysis

Gene ontology analysis was performed by Dr. Geetha Venkatesh and Dr. Vitor Coutinho Carneiro using the R tool topGO (Alexa and Rahnenführer, 2007).

2.2.5.8 Validation of differentially methylated regions

In order to validate the results from the Agilent assay, new samples were collected from the same 4 locations, and the same tissues were dissected, as used for the Agilent Sure Select Methyl-Seq Assay. As mentioned in section 2.2.5.3, samples were collected one to two years after the first sampling period. Genomic DNA from hepatopancreas and abdominal muscle was extracted, as mentioned in section 2.2.4.1, and bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research) following the manufacturer's instructions. Target regions were PCR amplified using region-specific primers with MiSeq specific adapters (see Appendix for primer list) using the Pyromark PCR Kit with the following protocol. One PCR reaction contains 12.5 µl Pyromark MasterMix, 2.5 µl Coral, 1 µl Mg^{2+}, 1 µl Primer Mix (forward and reverse, 10 nM), 7 µl H20, and 1 µl of target bisulfite converted DNA. The samples were amplified on a Bio-Rad Peltier Thermal Cycler with the following program: Initial denaturation with 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 56°C for 30 s and elongation at 72 °C for 30 s. A final elongation step at 72 °C for 10 min was performed. Samples were then cooled down to 4 °C until the PCR run was stopped.

PCR products were run on an agarose gel electrophoresis with a 1% agarose gel. The gel was put on a UV-table, and bands were cut out with a scalpel and put in a 1.5 ml

Eppendorf tube. DNA amplicons were gel-purified using the QIAquick Gel Extraction Kit (Qiagen). Subsequently, samples were indexed using the Nextera XT Index Kit v2 Set A (Illumina) using 25 µL of KAPA HiFi HotStart ReadyMix, 5 µL of Nextera XT Index Primer N7, 5 µL of Nextera XT Index Primer N5, 10 µL of water, and 5 µL of DNA amplicon to a final volume of 50 µL. An indexing PCR was performed as followed: Initial denaturation at 95°C for 3min, eight cycles of denaturation a 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, final elongation step at 72 °C for 5 min and cooling down to 4 °C until further use. The samples were purified with AMPure beads, and the final concentration of the samples was measured on the Qubit device with the DNA broad range protocol. Finally, the indexed DNA samples were pooled in equimolar amounts for a final volume of 130 µL at 10 nM final DNA concentration. The pooled library was submitted to the High Throughput Sequencing Core Facility (DKFZ, Heidelberg) and sequenced on a MiSeqV2 system using a paired-end 150 bp nano protocol.

Sequencing data were analyzed using BisAMP (Bormann et al., 2019). The tool generated heatmaps showing the methylation status per read and the methylation ratios per CpG. Samples with less than 140 reads were filtered out from the analysis. Subsequently, the results provided by the BisAMP platform were further analyzed using R studio (version 3.6.1) in order to compare the results to the methylation ratios from the initial analysis with the capture-based method.

In total, 17 samples (5 x abdominal muscle, 12 x hepatopancreas) for marker T595, and eight samples (4 x abdominal muscle, 4 x hepatopancreas) for marker T173 were analyzed. Mean methylation ratios per tissue for the two locations (Reilingen and Singlis) were calculated. Heatmaps were generated using R Studio (version 3.6.1).

2.2.5.9 Methylation analysis of marbled crayfish kept in controlled laboratory conditions and application of single-parameter-changes

The comparison of different wild populations revealed the possibility of distinguishing populations depending on their methylation pattern. To answer the question if the laboratory population shows a distinct methylation pattern and if the pattern can be changed by changing a single parameter, a laboratory experiment was designed to answer those questions. In total, 15 animals of the same age (hatched in March 2019) and similar size of ~ 3 cm, reared under the same conditions (tap water, similar tanks,

room temperature, and natural day-night rhythm, tailored feed), were kept in individual tanks (size: 25.6 cm x 18.1 cm x 13.6 cm). Three individuals were grouped randomly into five different trial conditions. The following conditions were applied (Table 3).

Table 3: Trial conditions for single-parameter-changes. Table showing the standard lab conditions used as control (group 1) and the parameter changed for groups 2 to 5.

Group	Name	Conditions
1	Standard (control)	• 20 °C room temperature • normal day-night rhythm • daily feeding (tailored feed) • tap water
2	Cold	Standard, but 10 °C room temperature
3	Warm	Standard, but 35 °C room temperature
4	Low manganese	Standard + 1 mg/L manganese
5	High manganese	Standard + 3 mg/L manganese

Individuals were kept under these conditions for six months. Animals were fed and checked upon daily, and water was changed bimonthly. If remaining feed could be observed, it was removed with a Pasteur pipet on the next day.

After the trial, animals were rinsed with ethanol, dried, and decapitated. Abdominal muscle and hepatopancreas tissues were extracted and immediately put in a 1.5 ml Eppendorf tube each. Samples were stored at -80 °C until further use.

To analyze the samples, the SureSelect Methyl-Seq Assay was used as described in section 2.2.5.4. As a further optimized gene set of 361 genes was available from the analysis of the wild populations, the custom-made baits were designed to capture just these genes. This allowed the sequencing of 16 samples on one sequencing lane of a High-Seq 4000 from Illumina.

Samples were compared to the wild populations in a PCA. Additionally, manganese treated and control animals were compared to Singliser See animals in a PCA.

2.2.6 Establishment of an aquaculture framework for marbled crayfish

2.2.6.1 Morphometric data analysis.

The size and weight structure of the population from the German lake Murner See (see Appendix for coordinates) was explored. All animals were caught by Frank Lenich while diving. In total, 768 marbled crayfish were collected, measured using a 15 cm

caliper and weight using an SJS 60007 scale (BASETech). Data were grouped and plotted in a histogram.

2.2.6.2 Isolation of chitin

Crayfish shells are a source for chitin, an often-used biopolymer, but the chitin content in different species varies a lot. Therefore, the chitin concentration in marbled crayfish shells was compared to shrimp shells (*Litopenaeus vannamei*). The following protocol is based on shellfish literature and was slightly modified to suit to marbled crayfish shells. The chemical extraction of the chitin was made by Jasmin Lohbeck and Dr. Aubry K. Miller from the Department of Cancer Drug Development (DKFZ) following the protocol described in Tönges et al. (Tönges et al., 2020a).

2.2.6.3 Feed development

In aquaculture, especially in closed-system aquaculture and for the hatchery and nursery of the juveniles, tailored feeds are needed to meet the crayfish´s requirements. Therefore, the analysis of the amino acid profile of marbled crayfish was carried out to get a basic idea of the amino acid requirements of the crayfish and to design a suited feed for them. Karthik Masagounder from Evonik Industries designed a basic feed formulation. Additionally, feeds with different methionine contents were produced and tested in a feeding trial with marbled crayfish.

First, one randomly selected laboratory-reared marbled crayfish (14.77 g) was decapitated and snap-frozen in liquid nitrogen and freeze-dried using a freeze dryer (Christ) (final mass: 4.35 g). This crayfish was fed with a common aquarium fish feed (NovoPleco, JBL, Neuhofen, Germany). The sample was sent to Evonik Industries to analyze the amino acid profile of this crayfish and to design a suitable feed. The analysis was performed using ion-exchange chromatography (AMINOLab®) except for tryptophan, which was estimated using high-performance liquid chromatography (HPLC) at Evonik Nutrition & Care, Germany. Additionally, the amino acid content of the aquarium fish feed (NovoPleco, JBL, Neuhofen, Germany) was also analyzed. This feed was used as a control feed in the feeding trial.

Dr. Karthik Masagounder from Evonik Nutrition & Care designed the feed using the crayfish's amino acid profile and knowledge from previous feed designs for shrimps. Designing method and feed composition of the feeds are described in Tönges et al.

(Tönges et al., 2020a). Five feeds with the same basic feed formulation, but different methionine levels were handed to DKFZ for the feeding trial.

2.2.6.4 Feed trial

In order to test the acceptance of the feed and the effects on the growth rate of the animals depending on the different methionine levels, each feed was tested for three months with 100 adolescent marbled crayfish (mean total length: 1.75 cm, SD: 0.25 cm; mean weight: 0.11 g, SD: 0.05 g) from our laboratory stock. Four tanks (25.6 x 18.1 x 13.6 cm) with five animals each were kept with a natural day-night rhythm and 20 °C room temperature for each feed. The animals were fed daily at 5 pm with 0.08 g of feed. Crayfish have a specific feeding behavior of prolonged feeding time and preferences to stay hidden during the daytime. Thus, it was not possible to determine accurate feed intake. A fixed amount of feed was provided in each tank, and no feed was left the next day. Occasionally more feed was provided but refused by the animals. All animals were measured and weighed once per week, and water parameters were checked for NH_4, NO_3, NO_2, and O_2 (JBL Test sets, Neuhofen, Germany) in the same rhythm. The room temperature was checked daily. The results confirmed good water quality for the entire trial and stable room temperatures (Table 4).

Table 4: Water parameters. Results of tested water parameters during the feeding trial to check the water quality. All parameters were measured once per week and indicated good water quality during trial runtime.

Parameter	Results
Temperature [°C]	20 ±1
O_2 [mg/l]	always 8
NH_4 [mg/l]	always <0.2
NO_3 [mg/l]	always <1
NO_2 [mg/l]	always <0.1

2.2.6.5 Feed trial data analysis

The mean weight gain per tank was determined by calculating the mean final body weight per tank minus the mean initial body weight per tank. Subsequently, the feed conversion ratio (FCR) was calculated by dividing the total feed intake per animal by the mean weight gain. Daily mean feed intake per animal was calculated by dividing

the amount of feed fed to a tank per day by the number of animals survived on that day. The mean daily feed intake value was summed for all days to calculate the total mean feed intake per animal. Mean FCR was calculated for each trial group, and to assess differences in the means of FCRs among the five feed groups, a one-way analysis of variances (ANOVA) was performed. As the total variances between groups were statistically significant (p-value = 0.00239), a more detailed pairwise comparison between all groups was performed using Tukey's HSD test. Julian Gutekunst performed the statistical analysis.

2.2.7 Establishment of environmental DNA (eDNA) detection method for marbled crayfish

2.2.7.1 Assay development for eDNA detection of marbled crayfish

Assay development for eDNA detection of marbled crayfish was carried out by Quentin Mauvisseau (Mauvisseau et al., 2019a) using a reference marbled crayfish DNA and genome information from Gutekunst *et al.* (2018). To accomplish the assay development, a field trial was designed and carried out in cooperation with Quentin Mauvisseau and Ranja Andriantsoa.

2.2.7.2 eDNA sampling

To prove the designed assay's sensitivity for marbled crayfish, 15 spots at 9 locations, including rivers, lakes, and a pond, were sampled in May 2018 in Baden-Württemberg and June 2018 in Hessen. Eight of the sampled spots were known for the presence of marbled crayfish (Dümpelmann and Bonacker, 2012; Lyko, 2017). To test DNA traces of a species from water samples, a sufficient amount of water needs to be filtered to concentrate DNA traces (of all species) from the water. Thus, two independent 1 l water samples were collected at each spot using a sterile polypropylene ladle and placed into a sterile plastic bag (Whirl-Pak® 1242 ml Stand-Up Bag Merck®, Darmstadt, Germany). If samples were collected from rivers, water was collected across the river's width, moving upstream to avoid disturbing the sediment, as described in Mauvisseau et al. (2019b). For sampling lentic water bodies, surface water subsamples were sampled across a ten-meter-wide strip, approximately 1 meter away from the bank. Samples were collected on different spots around the lentic water body, depending on the lake's accessibility.

Samples from each spot were filtered with a 50 ml syringe (sterile Luer- Lock™ BD Plastipak™, Ireland) through a sterile 0.45 µm Sterivex™ HV filter (Sterivex™ filter unit, HV with Luer-lock outlet, Merck®, Millipore®, Germany). To fix the filtered material, Sterivex filters were then immediately filled with 2 ml of absolute ethanol and closed with Luer-lock caps (Uhs). Filters were stored at room temperature until the end of the field trip and at −80 °C in the laboratory until DNA extraction. Sterile equipment and disposable nitrile gloves were used during the sampling process and replaced at each location to avoid contamination.

2.2.7.3 DNA extraction

eDNA was extracted from Sterivex filters following the methods described in (Spens et al., 2017), using the DNeasy Blood and Tissue Kit (Qiagen) by Quentin Mauvisseau (Mauvisseau et al., 2019a).

A distribution map of the collected marbled crayfish was made with the open-source QGIS tool (version 3.6.0 with GRASS 7.6.0).

3 Results

3.1 Habitat diversity of marbled crayfish in Germany

It is known that Germany has existing wild populations of marbled crayfish in several different regions from Berlin in the north-east of Germany to Freiburg in the south-west (Chucholl and Pfeiffer, 2010; Dümpelmann and Bonacker, 2012; Lyko, 2017). Nevertheless, no systematic characterization of the habitats was conducted. In order to detect and collect marbled crayfish from wild populations and characterize the habitats, field trips were carried out in July and August 2017 at five different locations in Germany.

3.1.1 Habitat characterization and catch size of wild marbled crayfish populations in Germany

In total, 214 marbled crayfish were caught at all five locations, but the catch size was varying between the different locations. The following paragraphs will describe the catch size and habitats profiles of the five different locations. Water parameters were measured on-site with a multiparameter instrument (Hanna), and water samples were sent to Raiffeisen Laborservice (Ormot, Germany). Differences of parameters measured on-site and from the water samples sent to for chemical analysis could be caused by temperature shifts of the measurement directly at the lake, and the later analyzed water, later sampling, or the sample storage. Therefore, the pH and conductivity values of the direct measurements from the lakes are displayed in Figure 10C.

3.1.1.1 Krumme Lanke

At Krumme Lanke (Figure 5A) (Berlin) in two nights (collection time: five hours per night with two people), only five marbled crayfish were collected. The water parameters analysis revealed an overall good water quality, with only turbidity (8.21 NTU) reaching higher levels than for other lakes (Figure 10C). For fish, the chloride concentration of 63.1 mg/L is considered to be potentially harmful. With this being the only slightly increased parameter, the lake was still categorized as suited for fish.

3.1.1.2 Singliser See

In two nights at Singliser See (Figure 5A) (collection time: five hours in two nights with two people), 140 marbled crayfish were collected. Other crayfish species or fish were neither seen nor could be collected. A pH of 4.36 (calibrated to 25 °C) and a conductivity of 1205 µS/cm was detected with a multiparameter instrument (Hanna) on site. Singliser See was the only lake with acidic water, and the conductivity was almost twice as high as in the other lakes (Figure 10B, see Appendix for all parameters). A chemical analysis of the water (for a full report see Appendix) highlighted the unique water parameters compared to the other explored lakes. Because of former mining activities in this area, the water contained high amounts of aluminum (357.1 mg/L), manganese (2967 µg/L), and nickel (35.1 µg/L). These parameters are categorized as harmful for aquatic organisms, explaining the lack of fish and plants in the water. The marbled crayfish can still live under these conditions, and the high numbers of caught animals within a short time indicates a large population of marbled crayfish compared to the other explored lakes.

3.1.1.3 Reilinger See

At Reilinger See (Figure 5A), 15 marbled crayfish, and two spiny cheek crayfish were collected with two traps. By hand and hand nets, 37 marbled crayfish were collected by three persons within three hours. All parameters measured on-site suggested good water quality. The chemical analysis of the water confirmed this result. None of the parameters were critical for aquatic organisms (see Appendix for detailed report). Thus, Reilinger See is a lake with high-quality. As the lake is relatively small (9 ha) and has no connection to any flowing water, it freezes over in the winter, observed in February and March 2018 (Figure 5A). Temperatures had a difference of more than 20°C during the year (Figure 5B).

3.1.1.4 Baggersee Epple

During one night of catching (eight hours with two people) at Baggersee Epple (Figure 5A), only eight marbled crayfish could be collected. One of them was located at a connecting channel between the lake and a nearby stream. The water parameters showed, similar to Reilinger See, standard readings (Figure 10B). A detailed water analysis was not conducted as the report from the “Landesamt für Umwelt, Messung

und Naturschutz Baden-Württemberg" (2017) concludes excellent water quality (see Appendix for report).

3.1.1.5 Moosweiher

At Lake Moosweiher (Figure 5A), nine marbled crayfish were caught with two people within five hours. No *Faxonius limosus* was observed, even knowing that they are present in the lake. The water parameters indicated good water quality (Figure 10B), and the report from the Landesamt für Umwelt, Messung und Naturschutz Baden-Wüttemberg from 2017 also certified excellent water quality (see Appendix for report).

In conclusion, four of the five investigated lakes (Krumme Lanke, Reilinger See, Baggersee Epple, and Moosweiher) showed good to excellent water quality, with a variety of different fish. All five water bodies are lakes and therefore classified as lentic water bodies. As Germany has just one climatic zone, all lakes were located in a moderately continental climate zone and investigated during the summer. Therefore no significant differences regarding the water temperature were detected (Figure 10B). For Reilinger See, it is known by own observation and from the local fishing club that the lake freeze over during winter, with a water temperature of 4 °C on the lake's ground. An exceptional habitat among the five investigated lakes was Singliser See. This lake has harsh conditions like high amounts of aluminum, manganese, nickel, and an acidic pH of the water of 4.36. This was the only lake were no fish could be detected. Illustrating the ability of marbled crayfish is to adapt to different environments. Interestingly this lake showed the largest population of all the lakes, possibly related to the lack of predators.

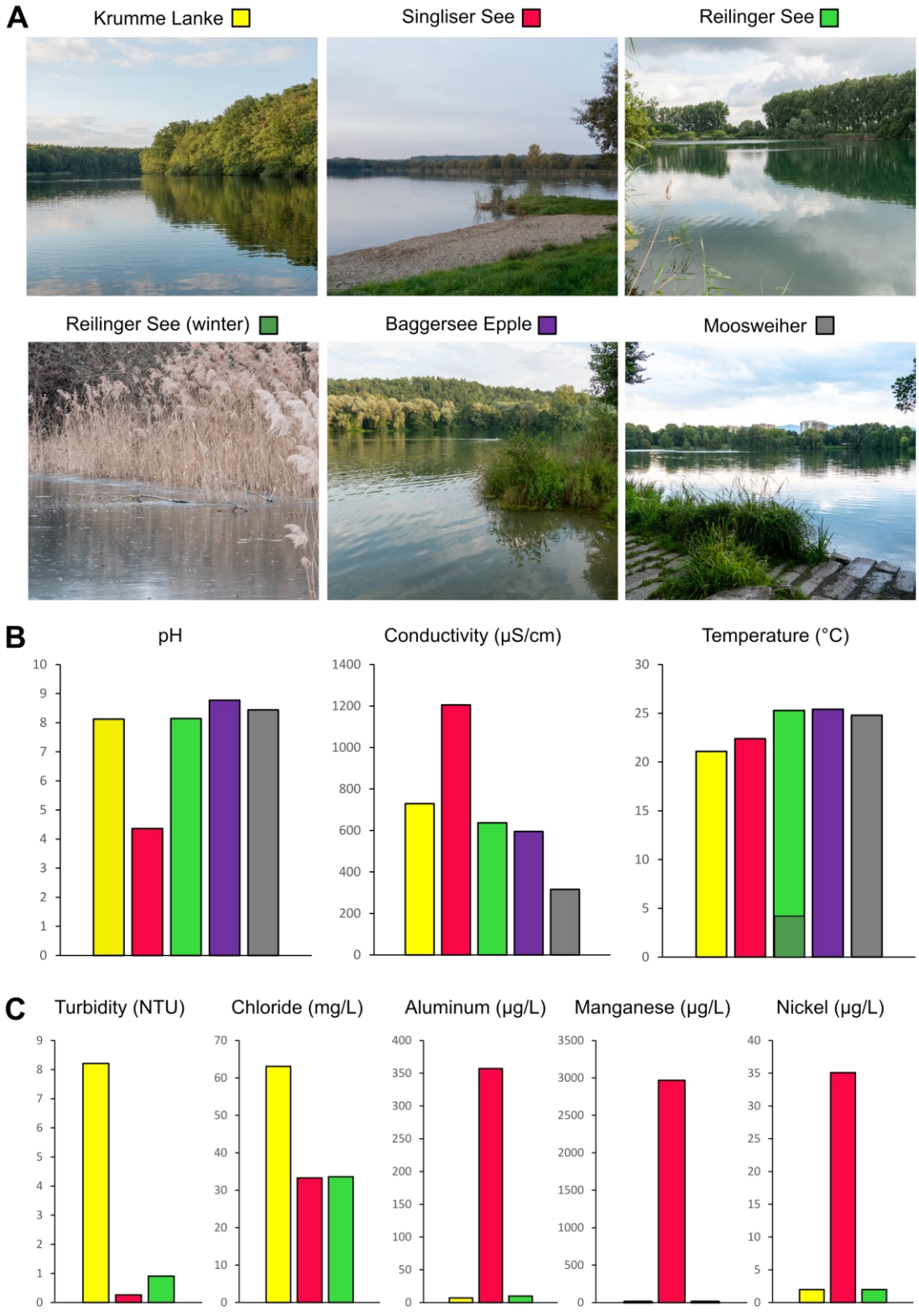

Figure 10: Habitat characteristics of German marbled crayfish populations. Colors display locations: Krumme Lanke, yellow; Singliser See, red; Reilinger See, light green; Reilinger See (winter), dark green; Baggersee Epple, purple; Moosweiher, grey (A) Pictures of the five locations. (B) Bar plots, showing the physical parameters pH, conductivity, and temperature measured on-site at the five lakes. The temperature for Reilinger See was measured during the sample collection in summer (light green) and in winter (dark green). (C) Bar plots, showing the physical and chemical parameters measured by Raiffeisen Laborservice (Ormot, Germany) for Krumme Lanke, Singliser See, and Reilinger See.

3.1.2 Morphological identification

The coloration of sampled marbled crayfish varied from dark brown to green, but all animals showed the characteristic marbled pattern of the carapace (Figure 11A-C). Animals from Singliser See showed a red to brown sediment attached to the carapace (Figure 11C). All crayfish, which were morphologically identified as marbled crayfish, were female. This was detected by the presence of the genital pore (anulus ventralis) on the base of the third walking legs (Figure 11D).

Additionally, at Reilinger See and at Krumme Lanke, two and 26 spiny cheek crayfish (*Faxonius limosus*) were detected, respectively (Figure 11E). One female *Faxonius limosus* from Reilinger See was dissected as a negative control for genetic authentication.

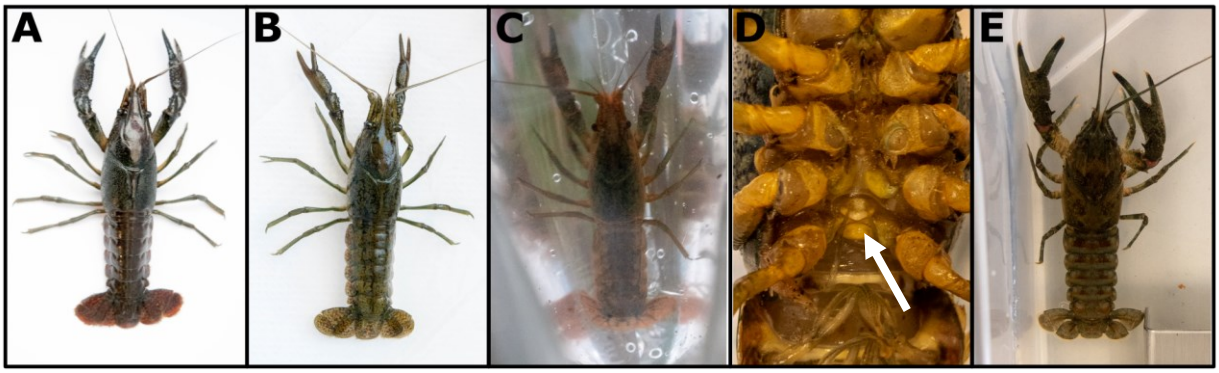

Figure 11: Morphometric characteristics of German marbled crayfish populations. (A) Dark-brown colored marbled crayfish from Reilinger See. (B) Green to brown colored marbled crayfish from Krumme Lanke. (C) Marbled crayfish from Singliser See with red to brown sediment on the carapace. (D) Ventral side of a marbled crayfish. White arc pointing to the annulus ventralis. (E) Spiny cheek crayfish (*Faxonius limosus*) from Reilinger See.

3.1.3 Genetic authentication

Additionally, to confirm the identity of collected crayfish, a genetic analysis of samples from each location was performed. For the analysis, abdominal muscular tissues from three individuals per location were extracted. A fragment of the mitochondrial Cytochrome b gene was analyzed by Sanger Sequencing and aligned to the reference genome of marbled crayfish. The workflow for the data analysis followed the description in Gutekunst et al., 2018. All 15 analyzed animals, morphologically identified as marbled crayfish, showed a 100% identity to the marbled crayfish reference, and not any

single nucleotide variance (SNP) was detected. In contrast, substantial sequence variations were detected for *Faxonius limosus* (Figure 12).

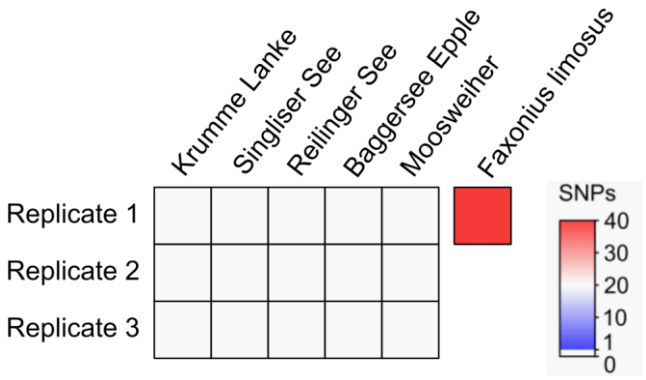

Figure 12: Genetic authentication of German marbled crayfish populations. All marbled crayfish samples show a 100% identity to the mitochondrial Cytochrome b fragment of the reference gene. Analysis of a sample from another crayfish species (*Faxonius limosus*) shows a distinct genetic sequence. SNPs: single nucleotide polymorphisms. Scale bar indicates the number of SNVs from 0 (white) to 40 (red).

In summary, morphological and genetic analysis of the collected crayfish at five different locations in Germany unambiguously identified them as *Procambarus virginalis*. Interestingly, they were found in various habitats ranging from small-sized eutrophic lakes with lots of fish and an additional crayfish species to a large oligotrophic lake with no fish at all and harsh conditions. Catch sizes varied a lot among the lakes indicating different population densities, with the highest density in the most challenging water condition.

3.1.4 Selection of samples for DNA methylation analysis

To explore context-dependent DNA methylation in marbled crayfish, animals were collected from four diverse populations (Figure 13, see Appendix for coordinates). Two populations were chosen from the previously investigated locations in Germany (Reilinger See and Singliser See, see section 2.2.2.2. for detailed descriptions).

Samples from two populations in Madagascar were chosen to complete the sample set to analyze methylation patterns of four distinct habitats. The marbled crayfish population in Andragnaroa was located in a river, flowing through a forest area at a relatively high altitude (1156 m) with soft mountain water (0.3 °dH) (for the detailed report see Appendix). Additionally, a lake in Ihosy was selected. This lake is characterized by highly turbid water, with high levels of pollution from nearby mining activities

(aluminum (2967 µg/l) and iron (2249 µg/l), report of water analysis in the Appendix). Detailed habitat descriptions can be found in Andriantsoa (2020). Altogether, the habitats differ considerably regarding climatic zones, water parameters, and flora and fauna.

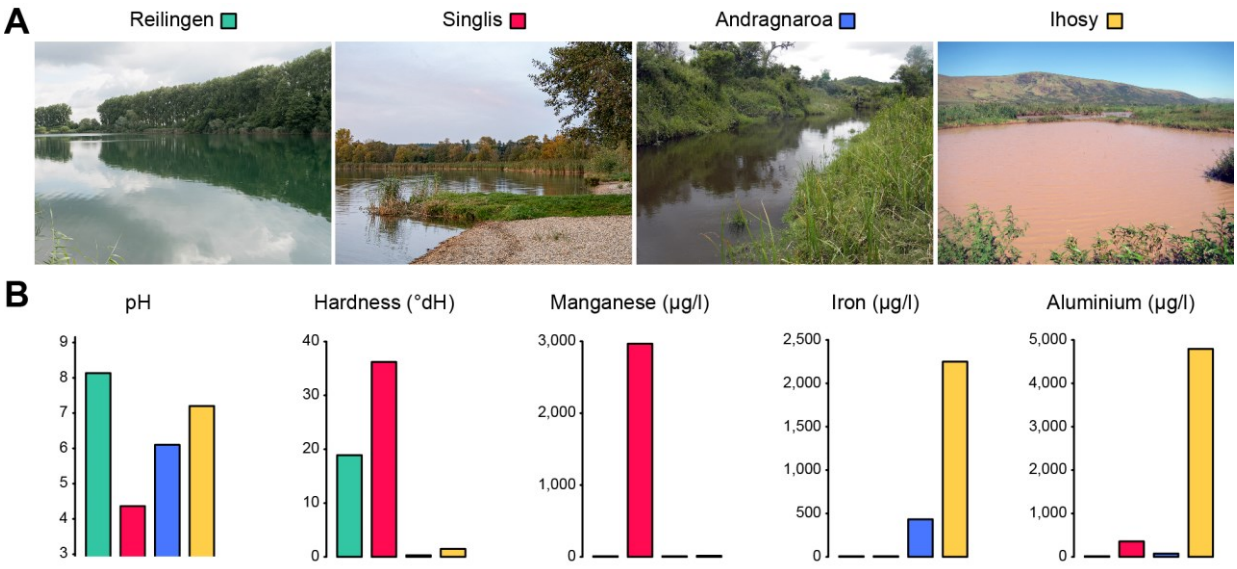

Figure 13: Marbled crayfish population habitats. (A) Habitat pictures of the four populations analyzed in with the capture-based assay (B) Bar plots showing specific water parameters of the four habitats analyzed. Pictures from Reilingen and Singlis and the parameters pH and manganese from these locations are also shown in figure 10 but included in this figure to compare the four habitats used for the capture-based analysis.

3.2 DNA methylation analysis in marbled crayfish

Overall, the marbled crayfish methylome is stably methylated between different tissues and animals from different sources (Gatzmann et al., 2018). Nevertheless, a subset of genes showed variable methylation among the eight samples analyzed with whole-genome bisulfite sequencing (see introduction, Figure 5).

To analyze methylation variations between tissues and within or between different marbled crayfish populations, a subset of 697 variable methylated genes was used to design a capture-based assay. This provides an opportunity to analyze a larger number of samples with high sequencing coverage in a cost- and time-effective manner. To capture the desired 697 genes from the whole genome, the Agilent SureSelect Methyl-Seq assay was used, and custom capture beads, called baits, were designed.

3.2.1 Characterization of the variably methylated gene set of 361 genes

The methylation analysis was based on a set of 361 variably methylated genes after applying several filtering parameters (see section 2.2.5.6 for more details).

Functional annotation of the core set of 361 genes identified 321 genes with predicted functions, while 40 genes remain to be annotated. For the 321 annotated genes, a gene ontology analysis was performed using topGO (Alexa and Rahnenführer, 2007) to get an insight into the functions of the variable methylated genes. This analysis revealed a significant enrichment of genes related to GTP-binding proteins. G proteins regulate various cellular activities, as they play a major role in transmitting signals from the extracellular matrix into the cell (Neves, 2002). Among others, genes found in the variably methylated gene set play a role in transcription and translation regulation, response to stress, RNA metabolism, and immune response to pathogens.

The characterization of the structural properties of the 361 genes showed that the average gene length of the subset of genes (~3900 bp) is shorter than the average gene length of all genes (~6900 bp) (Figure 14A). Furthermore, within the 361 genes, an enrichment of housekeeping genes could be observed compared to the percentage of housekeeping in the whole genome (8%, Figure 14B). The subset of 361 genes was mostly moderately methylated (Figure 14C). In comparison, most genes of the entire genome had either high or low methylation levels (Figure 14C). This pattern was already observed by Falckenhayn (Falckenhayn, 2016). The gene expression values (TPM: Transcripts per kilobase million) of the 361 genes were comparable to the TPMs of all genes and showed just a slightly decreased transcription level for the 361 genes (Figure 14D).

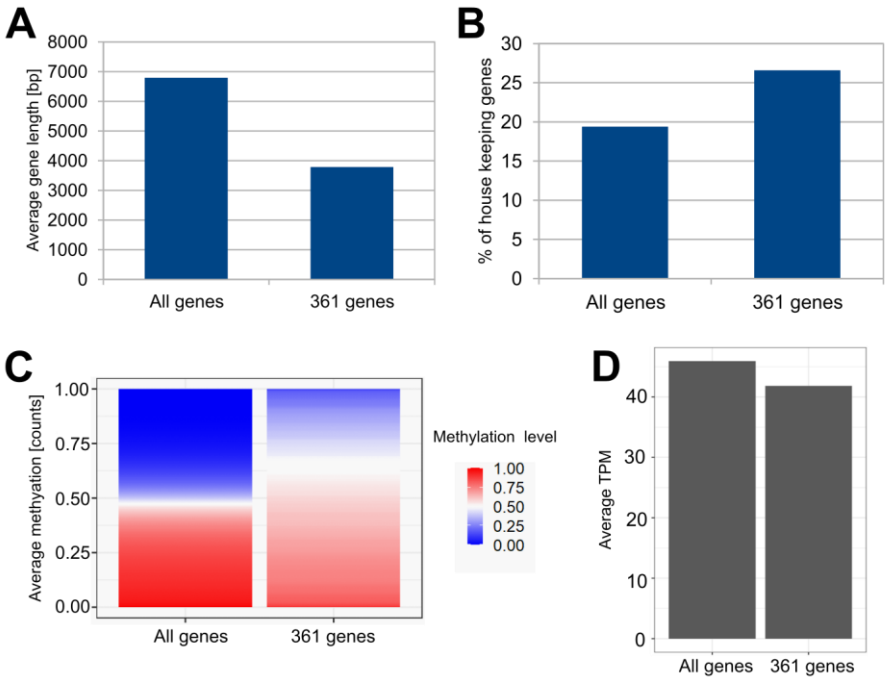

Figure 14: Characterization of the variably methylated gene set. (A) Bar plots showing the average gene length of all genes compared to the 361 variably methylated genes. (B) Bar plots indicating the percentage of housekeeping genes in all genes and the variably methylated gene set. (C) Stacked heatmap of the methylation level of all genes and the 361 variably methylated genes. (D) Bar plots showing the transcripts per kilobase million (TPM) for all genes and the variably methylated genes. Data provided by Dr. Geetha Venkatesh.

3.2.2 Capture-based methylation analysis of two tissues

For identification of tissue-specific methylation differences between hepatopancreas and abdominal muscle, a Wilcoxon rank-sum test ($p<0.05$ after Benjamini-Hochberg correction) was applied. With this test, 56 genes were found to generate a robust separation of the tissues in the largest dataset of a single origin (Singlis, N=24, 12 samples per tissue) in a principal component analysis (PCA) (Figure 15A). The first principal component (61.81%) is separating the tissue samples from each other. The same test was applied to the second-largest dataset from Reilingen (N=19, 12 samples hepatopancreas, 7 samples abdominal muscle), which detected 35 genes to separate the tissues in a PCA (Figure 15B) by the first principal component (66.52%). A total of 28 genes were overlapping both datasets. While tissue-specific methylation differences appeared relatively moderate for average gene methylation levels of the 28 overlapping genes from both datasets (Figure 15C), they appeared more pronounced at the CpG level (see Figure 15D for an example of a gene with a differentially methylated region (DMR) for tissue-specific methylation).

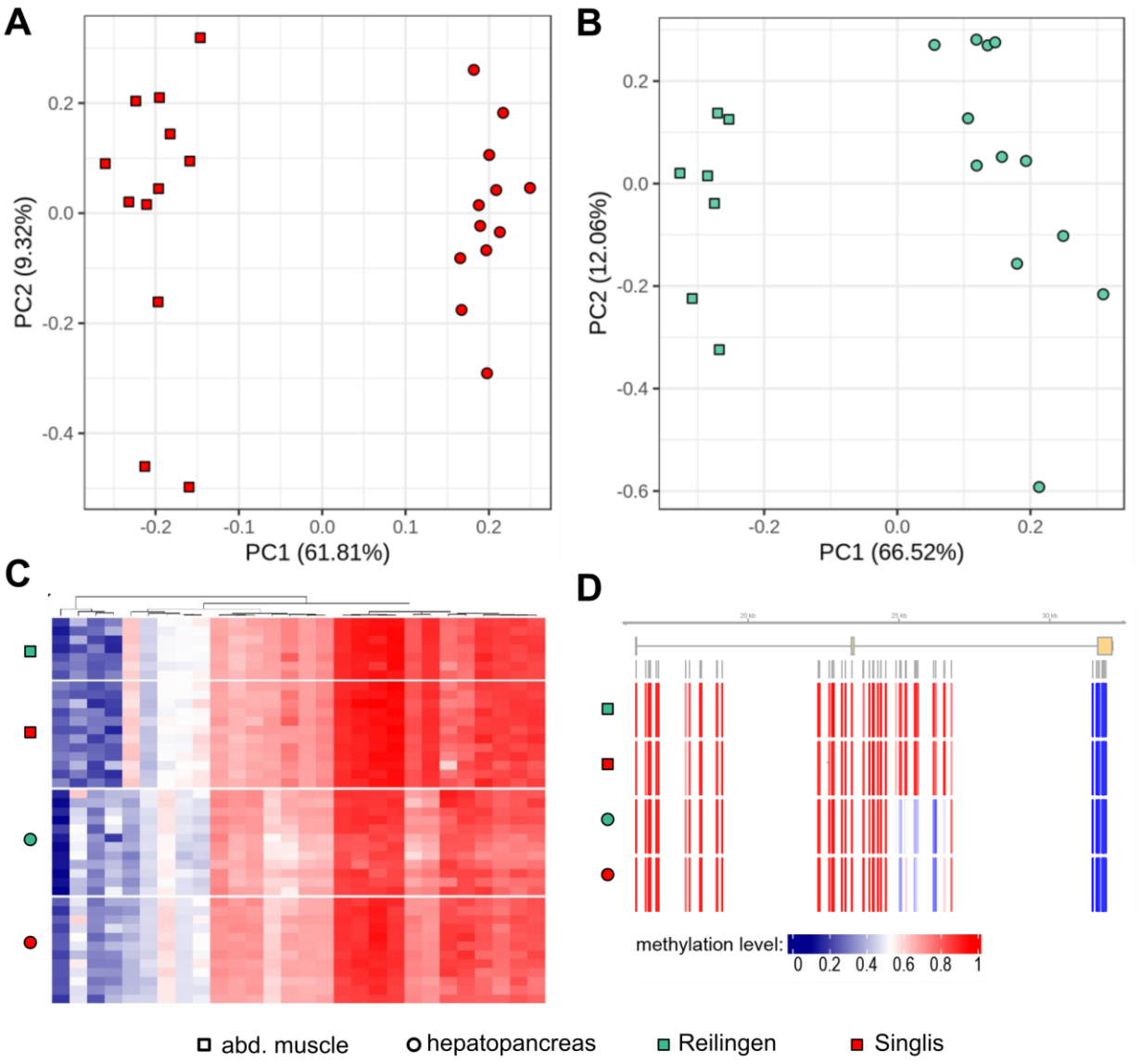

Figure 15: Tissue-specific differential methylation in marbled crayfish populations. (A) Principal component analysis of abdominal muscle (mus., square symbols) and hepatopancreas (hep., circular symbols) samples from Singlis, based on the methylation levels of 56 genes with tissue-specific methylation differences. (B) Principal component analysis of abdominal muscle (mus., square symbols) and hepatopancreas (hep., circular symbols) samples from Reilingen, based on the methylation levels of 35 genes with tissue-specific methylation differences. (C) Heatmap showing average methylation levels of 28 shared genes with tissue-specific methylation. Methylation levels are indicated on a scale from 0 (blue) to 1 (red) for Reilingen (green symbols) and Singlis (red symbols). (D) Example gene showing a differentially methylated region with tissue-specific methylation differences.

Comparative analysis of the mean methylation ratios per tissue from the tissue-specific methylated genes (N=28) and gene expression values (TPM) for the same genes from three abdominal muscle and three hepatopancreas samples analyzed in Gatzmann et al. (Gatzmann et al., 2018), revealed no correlation of methylation ratios to gene expression values (data not shown). Therefore, the expression levels do not explain the functional role of the methylation variation in these specific genes.

To conclude, methylation differences in different tissues detected with a small subset of genes suggests the existence of localized tissue-specific methylation patterns in marbled crayfish.

3.2.3 Capture-based methylation analysis of samples from four different wild populations

To analyze methylation patterns in different wild populations, a Kruskal-Wallis test ($p<0.05$ after Benjamini-Hochberg correction) for differential methylation between the four locations (Singlis, Reilingen, Andragnaroa, and Ihosy) was applied. This approach identified a set of 122 genes that allow a robust separation of the four locations in a PCA (Figure 16A) using the larger dataset of hepatopancreas samples (N=47). With principal component 1 (18.86%), German samples and Malagasy samples could be separated. Principal component 2 (17.77%) was separating the two locations of each country from each other. A more pronounced separation is visible between the German populations Singlis and Reilingen. The Malagasy populations are slightly mixed, but they can also be separated upon closer inspection of those two populations (Andriantsoa, 2020). Applying the same test to the smaller dataset of 26 abdominal muscle samples, 22 genes were identified to allow a robust separation of the German populations from each other and from the Malagasy samples (Figure 16B). Both datasets had 21 genes in common. Similar to the findings for tissue-specific methylation, location-specific methylation differences appeared moderate for average gene methylation levels (Figure 16C) of the 21 common genes, but more pronounced at the CpG level. Figure 16D shows two DMRs in one gene. Both DMRs together allow a clear separation of the four locations, using hepatopancreas tissue. These findings suggest the existence of defined location-specific methylation differences among marbled crayfish populations.

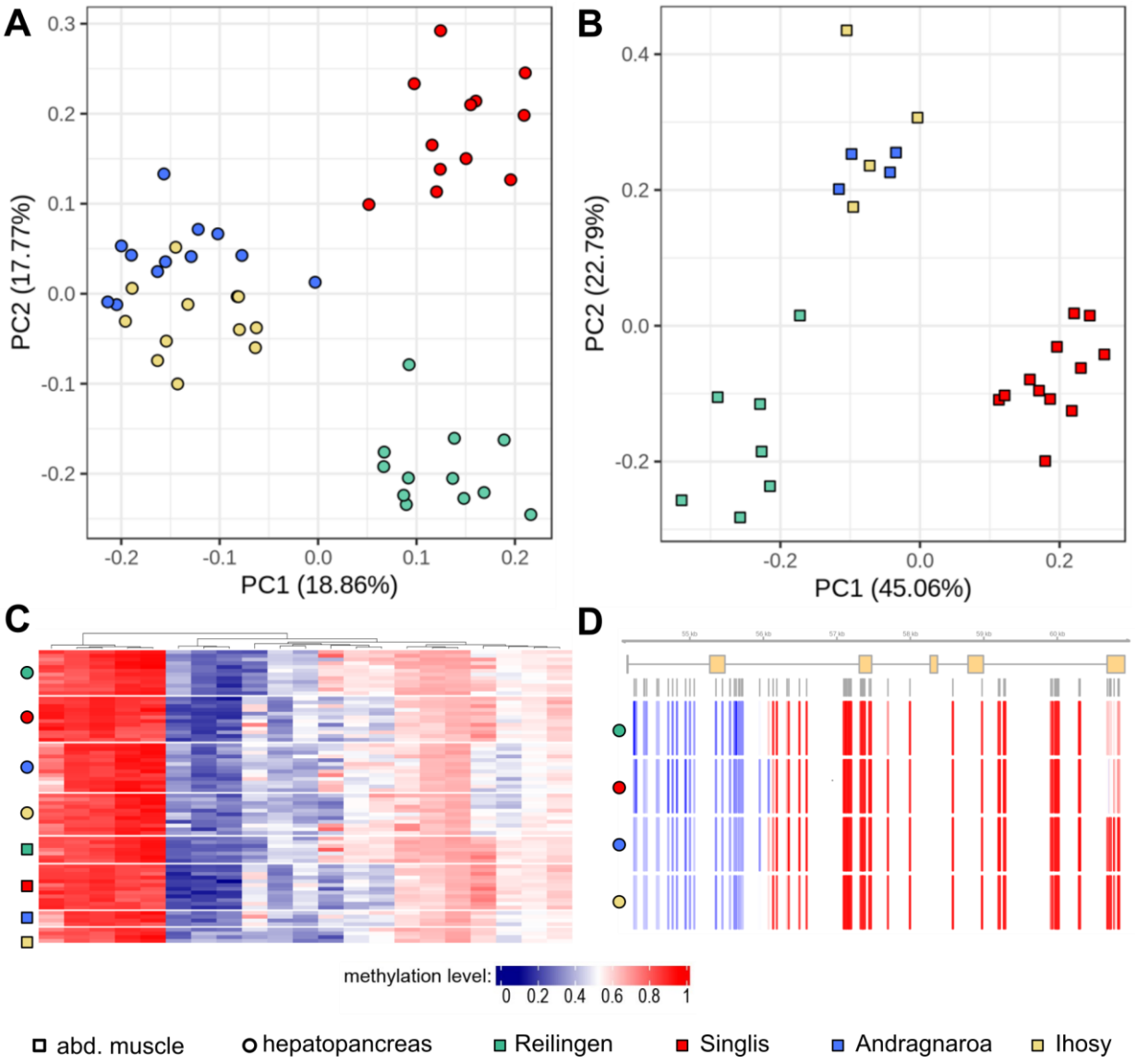

Figure 16: Location-specific differential methylation in marbled crayfish populations. (A) Principal component analysis of hepatopancreas samples from all locations, based on the methylation levels of 122 genes with location-specific methylation differences. (B) Principal component analysis of abdominal muscle samples from all locations, based on the methylation levels of 22 genes with location-specific methylation differences. (C) Heatmap showing average methylation levels of 21 shared genes with location-specific methylation. Methylation levels are indicated on a scale from 0 (blue) to 1 (red). (D) Example gene showing two differentially methylated regions with location-specific methylation differences.

In summary, with a set of 361 variable methylated genes, a robust separation between different tissues of marbled crayfish and different locations of marbled crayfish populations can be achieved.

3.2.4 Validation of context-dependent methylation patterns

In order to validate the results from the SureSelect Methyl-Seq assay, specific PCR assays for tissue-specificity (N=2) and location-specificity (N=3) were designed. Additionally, new samples were collected from the same four locations (Singlis, Reilingen, Andragnaroa, Ihosy), but one to two years after the first sampling, to explore the stability of the location-specific patterns in the population.

After identifying DMRs in genes responsible for either tissue or location separation of the samples, they were visually inspected for suitable markers to distinguish between the tissues or locations. In total, 15 primers were tested by PCR. Five primer pairs (2 tissue-specific and 3 location-specific) were selected as assays for the validation experiment (see Appendix for primer list).

The sequencing on the MiSeq platform allowed an in-depth analysis of the specific regions. The data analysis using BisAMP (Bormann et al., 2019) revealed an average coverage of 2431 aligned reads per region and sample. Average methylation ratios per CpG overall reads revealed the methylation ratio per CpG and sample used for further analysis and comparison to the capture-based methylation data.

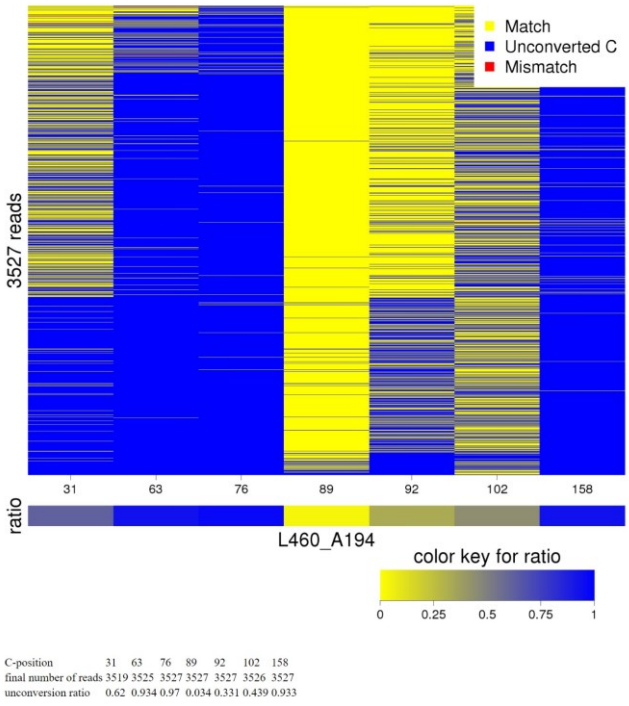

Figure 17: Deep amplicon sequencing of an example region used for the validation. The methylation status per CpG (column) and read (rows) are displayed for each sample. Yellow indicates an unmethylated Cytosine; blue indicates a methylated Cytosine mismatches are shown in red. Mean methyl-

ation ratio per CpG is shown as a bar under each column. Methylation ratios are indicated on a scale from 0 (yellow) to 1 (blue).

3.2.4.1 Tissue-specific patterns detectable with the chosen markers

Notably, the separation of the tissues was possible with the amplicon sequencing method and new samples (Figure 18, Amplicon Seq) and confirmed the results from the capture-based method. Even more important, the comparison between the methylation ratios of the two methods and the different samples were highly comparable to each other, suggesting a stable methylation pattern for tissue-specific methylated regions (Figure 18).

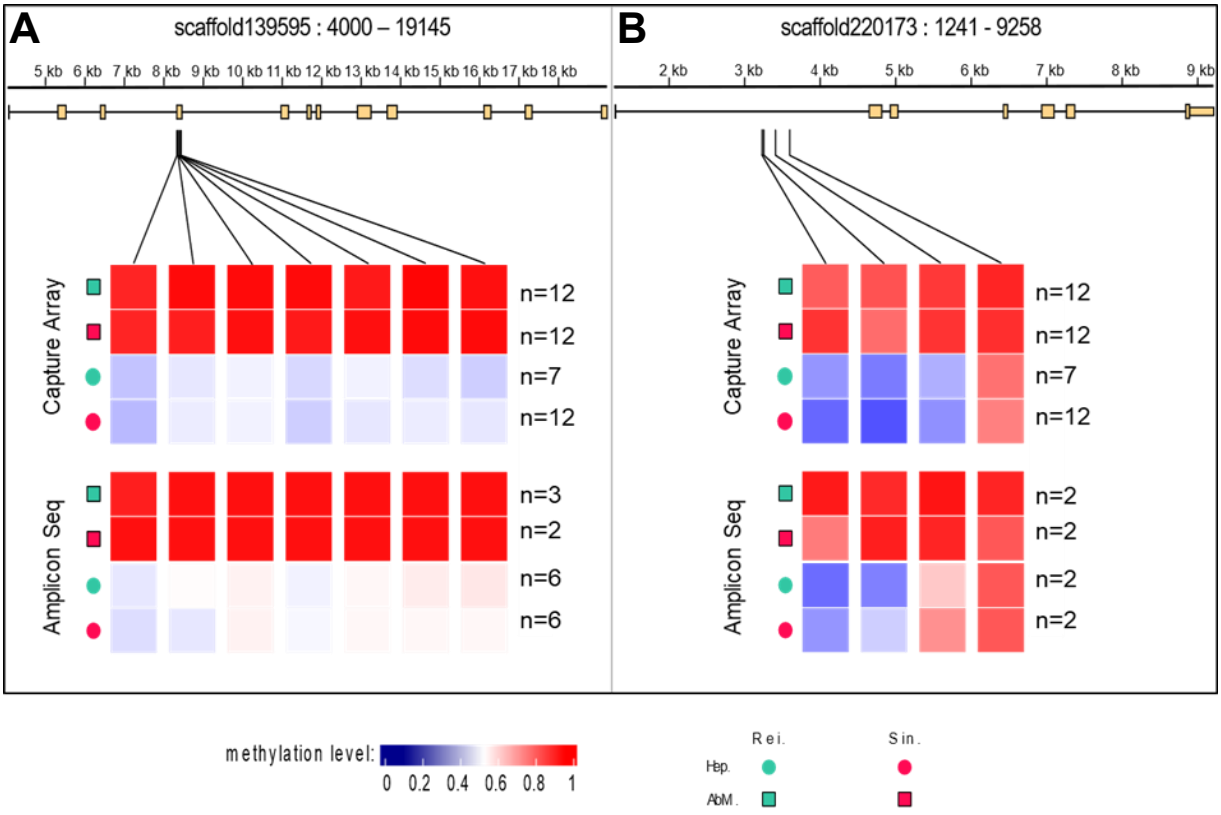

Figure 18: Validation of tissue-specific differential methylation in marbled crayfish. Results are shown for capture-based sequencing and the corresponding validation experiment with amplicon sequencing for two different genomic regions (A) Genomic region with one marker containing seven CpGs. (B) Genomic region with one marker containing 4 CpGs. Squares: abdominal muscle; circles: hepatopancreas; green: Reilingen; red: Singlis. Methylation levels are indicated on a scale from 0 (blue) to 1 (red). Differences in sample numbers for the amplicon sequencing are caused by differences in library preparation and/or sequencing.

3.2.4.2 Validation of location-specific patterns

Re-sampled hepatopancreas samples from animals of the four locations were investigated by amplicon bisulfite sequencing to validate the capture-assay results for the

location-specific methylation patterns. Similar to the tissue-specific markers, it was possible to separate the samples from the four locations with the three location-specific markers. For the calculation of the mean methylation ratios, ten samples for marker L88_R1(three for German populations each, two for Madagascar populations each), 12 samples for marker L88_R2 (three samples per location), and 37 for marker L460 (8 x Reilingen, 9 x Singlis, 10 x Andragnaroa, 10 x Ihosy) were analyzed. With the markers, L88_R1 and L88_R2, targeting the same gene, a separation between populations was possible (Figure 19A). With the third marker, each location showed an individual methylation pattern (Figure 19B). Importantly, these patterns remained stable over time, as the methylation ratios were highly similar between the capture-based method and the amplicon sequencing (Figure 19).

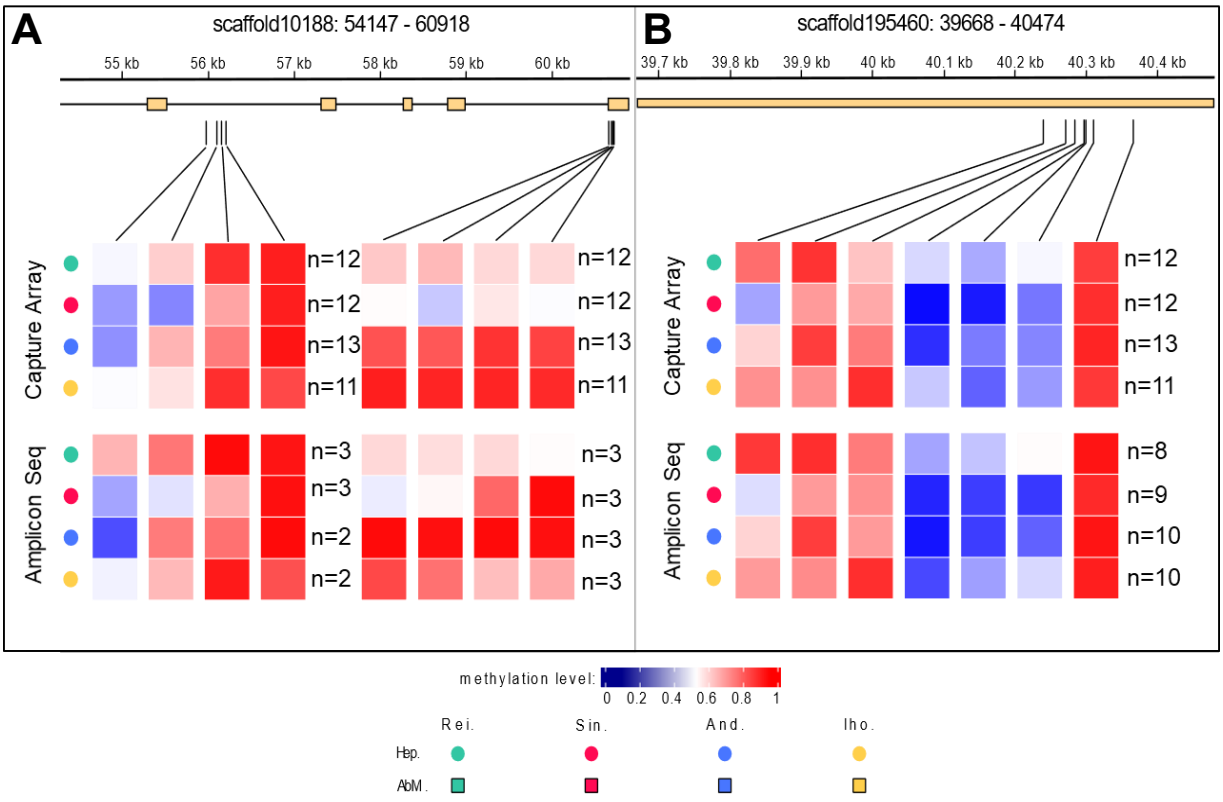

Figure 19: Validation of location-specific differential methylation in marbled crayfish. Results are shown for capture-based sequencing and the corresponding validation experiment with amplicon sequencing, for two different genomic regions by three markers. (A) Genomic region with two markers containing four CpGs each. (B) Genomic region with one marker containing seven CpGs. Circles: hepatopancreas; green: Reilingen; red: Singlis; blue: Andragnaroa; yellow: Ihosy. Methylation levels are indicated on a scale from 0 (blue) to 1 (red). Differences in sample numbers for the amplicon sequencing are caused by differences in library preparation and/or sequencing.

3.2.5 Genetic alterations do not influence methylation variations of selected genes

A recent study of single nucleotide variants (SNVs) in marbled crayfish from 10 different populations in eight countries revealed a low but significant number (16,564) of SNVs (Maiakovska et al., in revision). A comprehensive analysis of the SNVs found by Maiakovska et al. (in revision) with the gene set of 361 genes was conducted to identify a possible influence on the methylation data, driven by genetic variants. The analysis revealed 13 SNVs in a CpG context in the 361 genes. Only five SNVs were overlapping with genes identified for the location or tissue separation. No more than one SNV was found per gene. Therefore, the mean methylation ratio per gene was not influenced. None of the five SNVs were located in one of the validation genes. Therefore, all methylation differences detected in the capture-based analysis and the validation experiment can be considered purely epigenetic variations.

3.2.6 Experimental modeling of context-dependent methylation signatures

To model specific environments, it is essential to set up controlled conditions and change single parameters. Therefore, 15 animals were kept under the same primary conditions (see section 2.2.5.9). For 12 of them, a single parameter was changed (temperature, manganese concentration, see 2.2.5.9). All animals kept at 35°C room temperature died after four months, probably by bacterial or fungal contamination in the water. Additionally, one animal kept at 10°C room temperature died within the first month. Six animals were kept in water with two different concentrations of manganese (1 mg/L for Mn1, 3 mg/L for Mn3, 3 animals each) similar to two lakes in Germany with manganese contaminated water (Singliser See, ~3 mg/L, and Murner See, ~0,65 mg/L; see Appendix for coordinated and detailed water analysis).

In total, 11 hepatopancreas samples were analyzed with the capture-based assay. The comparison of samples from Singliser See (Figure 20A, red dots) and the manganese incubated animals (Figure 20A, yellow and blue dots) showed a clear separation of the Singliser animals and the laboratory animals, but no effect on manganese to the control group nor a shift toward the Singliser animals. Furthermore, the three animals kept at 10°C showed separation from the other laboratory animals (Figure 20B, dark red dots).

In the same PCA, the laboratory population (control, 10°C, Mn1, Mn3) showed a robust separation from the wild populations (Singlis, Reilingen, Andragnaroa, Ihosy; all in gray, Figure 20B), indicating that laboratory populations show a different epigenetic signature. These results are not related to an overall change of the methylation ratio (Figure 21), as there was no indication for a hyper- or hypomethylation of the laboratory animals compared to the wild animals.

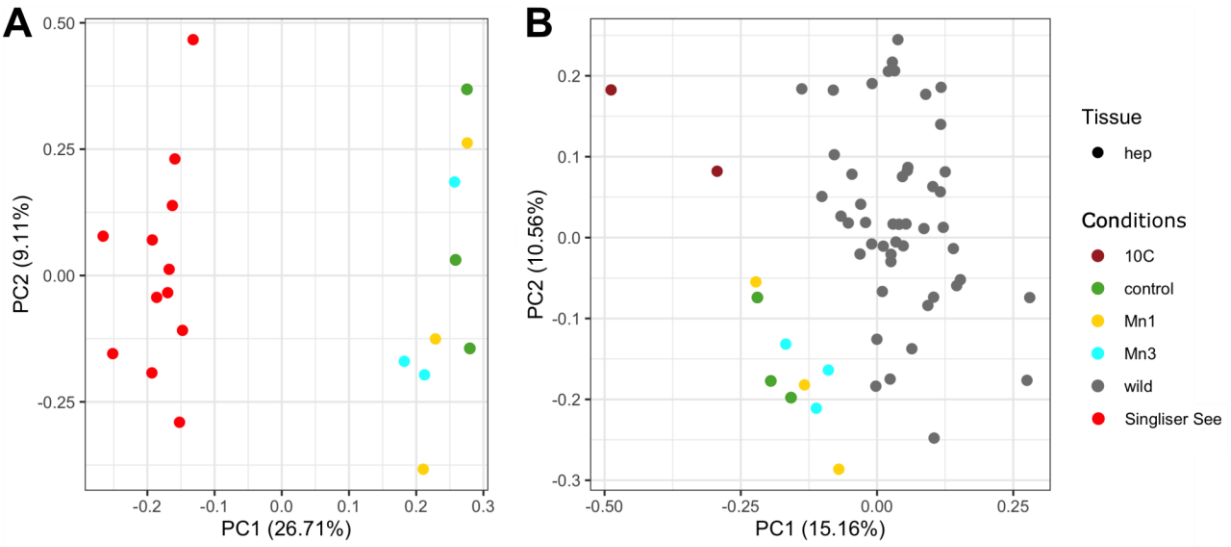

Figure 20: Experimental modeling of context-dependent methylation signatures. All samples of this analysis were taken from the hepatopancreas. Conditions are displayed in colors. Control, green; 10°C, dark red; manganese 1 mg/L (Mn1), yellow; manganese 3 mg/L (Mn3), blue. Animals from wild populations are shown in (A) red for Singliser See and (B) grey for all samples from wild populations. (A) PCA showing the separation of samples from Singliser See (red) and laboratory animals (green, yellow, and blue) by principal component 1 (26.71%). (B) PCA showing the separation of wild animals (gray) from laboratory animals (dark red, green, yellow, blue) by principal component 1 (15.16%) and the separation of low temperature kept animals (10 °C, dark red) from other laboratory animals (green, yellow, blue) by principal component 2 (10.56%).

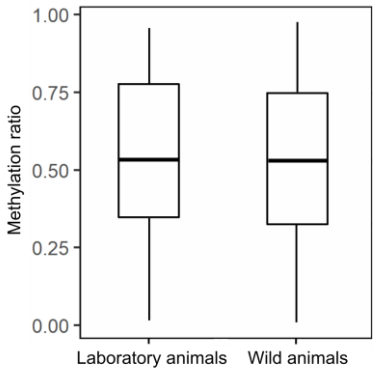

Figure 21: Methylation ratios of laboratory animals compared to wild animals. Average methylation ratios of the 361 genes from the wild animals compared to the laboratory animals show no difference in methylation ratios between the two groups.

3.3 The commercial potential of marbled crayfish

In order to explore marbled crayfish as aquaculture livestock and establish guidelines for safe aquaculture production, key characteristics of marbled crayfish were investigated, and a monitoring method was established.

3.3.1 Evaluation of key characteristics for commercial aquaculture

To evaluate the potential of marbled crayfish for commercial aquaculture, key characteristics of the species compared to the reference species *P. clarkii* were elaborated. *P. clarkii* is usually harvested at a weight of 20-25 grams (Wang et al., 2018). For the comparison, morphometric data of a population in Germany (Murner See) was analyzed. The size and weight of 768 marbled crayfish were measured and revealed the highest proportion of animals being 8 to 10 cm (47.8 %, Figure 22A) and 10 to 15 grams (24.48 %, Figure 22B). In this study, out of the 768 caught marbled crayfish, 24.74 % reached the targeted size of 20 grams or more (Figure 22B). The morphometric data used in this analysis were from wild populations with usually challenging environmental conditions compared to aquaculture habitats. Wild animals have to cope with cold climate, nutrient-poor habitats, and the presence of competitors and predators. Thus, the growth of a population in an aquaculture environment can likely be accelerated and enlarged. A good indication for this are wild-caught animals that were grown in aquarium cultures. They can reach a weight of more than 50 grams (Figure 22C).

Besides the commercial use of the tail meat as a protein source, shells of crayfish and other crustaceans can be salvaged as a source for chitin. Shells waste is produced as byproducts during meat processing and can further be used for chemical or biotechnological extraction of chitin. The chitin extraction from pre-cooked marbled crayfish shells revealed a mean chitin content per animal of 2.60% (Figure 22D). By processing shells from shrimps (*Litopenaeus vannamei*) in the same way, only 0.85% mean chitin content per animal could be determined (Figure 22D). Taken together, the key characteristics of marbled crayfish indicate high suitability for commercial aquaculture.

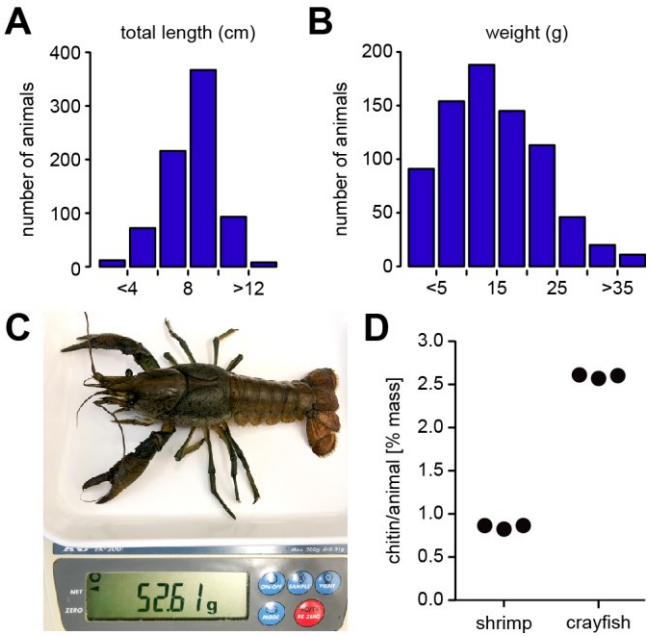

Figure 22: Physiological properties of marbled crayfish. (A) Size structure of 768 marbled crayfish from Murner See showing the highest proportion of animals being 8 to 10 cm (47.8 %). (B) Weight structure of 768 marbled crayfish from Murner See showing 24.74% of the animals having the target weight of 20 grams or more. (C) Example of a wild-caught marbled crayfish kept in aquarium culture showing a weight of 52.61 grams. (D) Chitin content per animals from marbled crayfish (2.60%) and shrimps (0.85%).

3.3.2 Formulation of tailored feeds for closed-system aquaculture

To minimize ecological concerns regarding marbled crayfish aquaculture, it is important to develop closed-system solutions, especially for the hatchery and nursery. An important prerequisite for closed-system aquaculture is the availability of tailored feeds to allow efficient utilization of the feed, to meet all nutritional requirements of the animals, and to promote good water quality. Therefore, Evonik Nutrition and Care developed tailored feeds for marbled crayfish (Evonik, Germany).

3.3.3 Effects of methionine supplements on growth

Methionine is an essential amino acid and often limiting (Bulbul et al., 2015). Different methionine concentrations were added to the basic feed formulation to determine the optimal methionine level for marbled crayfish, resulting in four different tailored feeds (D2-D5) for a feeding trial. Methionine levels were varied from 0.45% to 0.7%, and the common aquarium pet feed (NovoPleco, JBL) was used as a control feed (D1). D2 was designed with a methionine concentration of 0.45%, which is methionine deficient

compared to the marbled crayfish's amino acid profile and the analyzed pet shop feed (0.52%). The methionine concentration of D3 was matched to the pet shop feed. D4 is matched to the aa profile of the crayfish with a methionine concentration of 0.6%. D5 was designed to have a methionine surplus compared to the aa profile and the competitor feed, with a methionine concentration of 0.7%.

A feeding trial was designed to determine the effects of different methionine levels on the growth of adolescent marbled crayfish. Multiple independent groups of adolescent marbled crayfish (20 animals per group kept in 4 tanks with 5 animals each) were fed for three months. The animals were counted, measured, and weighed once per week, and no other food source was provided to the animals. The tailored feeds showed a noticeable effect on the growth of the animals. For example, tailored feed D3 (methionine matched to control) resulted in an increased weight gain when compared to the control feed (D1) (Figure 23A). This result shows that even with the same methionine concentration, the performance of the tailored feed is better than the control feed. By comparing the four tailored feeds, the influence of methionine was clearly visible. D2 (methionine deficient) and D4 (methionine matched to amino acid profile) showed a slower weight gain compared to D5 (surplus methionine) and D3 (methionine matched to control) (Figure 23B). Interestingly, D5 showed the fastest weight gain within the first eight weeks, while D3 performed the best during the later stages from week eight onwards (Figure 23B). These findings raise the possibility that D5 is more suited for juveniles and adolescents, while D3 is better suited for adults.

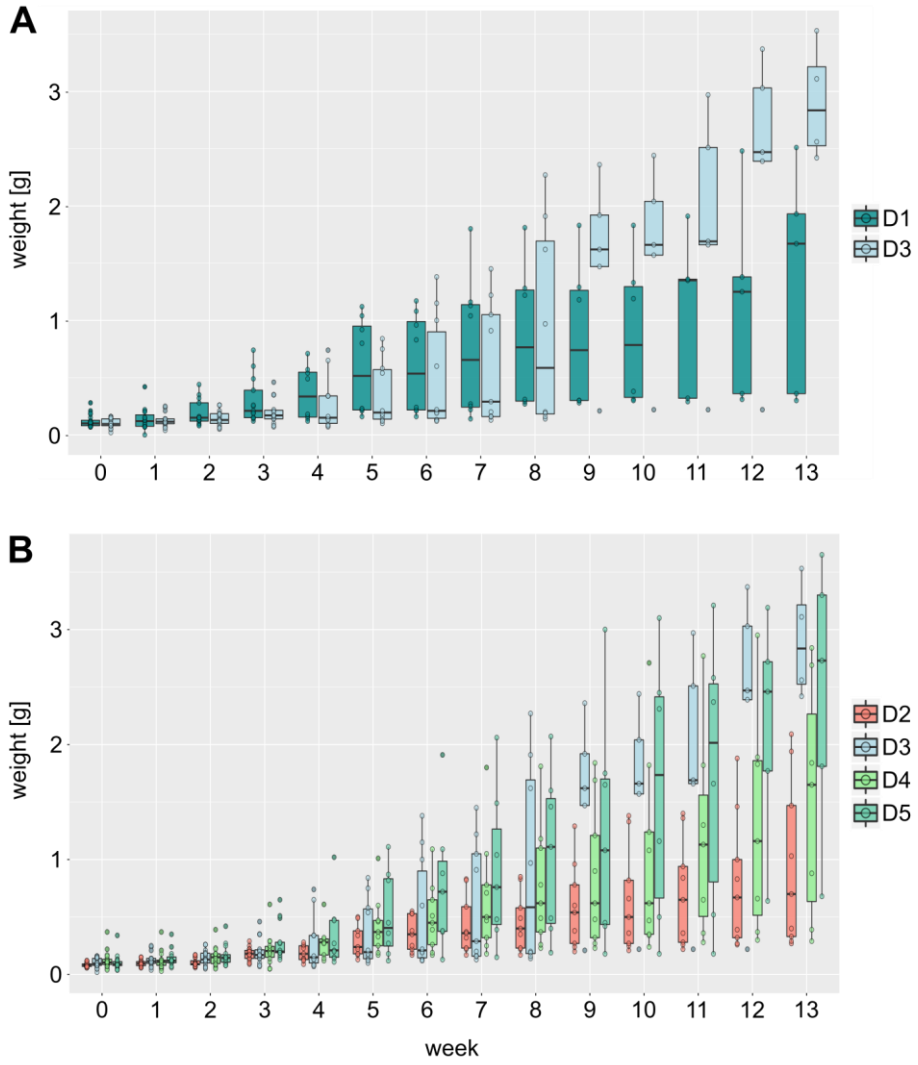

Figure 23: Effect of methionine feed supplements on the growth of marbled crayfish. Box plots showing the weight per group and week. D1: established standard (NovoPleco. 0.52% Met), D2: tailored. Met deficient (0.45% Met), D3: tailored. Met equivalent to standard (0.52% Met), D4: tailored. Met according to aa profile (0.6% Met), D5: tailored. Met surplus (0.7% Met) (A) Comparison of the control feed (D1) with the matched tailored feed (D3). The tailored feed D3 shows a higher weight gain compared to D1. (B) Comparison of all tailored feeds (D2-D5). D2 shows the lowest weight gain over time. D3 and D5 have the highest weight gain, but for D5 the weight gain is stronger at the beginning of the trial until week nine. From week nine till the end of the trial D3 shows the highest weight gain. Both groups have similar weight gains after 13 weeks with 2.81 g/animal for D3 and 2.63 g/animal for D5.

3.3.4 Determination of feed conversion ratios

The feed conversion ratio (FCR) is an important factor in determining the sustainable production of food proteins from an animal source. To determine the FCR of marbled crayfish, the mean weight gain per animal was calculated for the different feeding groups. A mean weight gain of 2.81 g (min: 2.3 g; max: 3.45 g; SD: 0.46) was observed for D3. In contrast, only a mean weight gain of 1.43 g (min:0.89 g; max: 2.38 g;

SD: 0.68) was observed for D1 (Table 5). This difference can also be detected by the FCRs of the two groups. While D3 has an FCR of 1.38, the FCR for D1 is 2.41, which is significantly poorer compared to D3 ($p<0.05$. ANOVA, RES=0.731). D2 showed the lowest FCR among all feeds (3.35), and the smallest mean weight gain with only 0.97 g (Table 5). As this is the group fed with the methionine deficient feed, the results highlight the importance of methionine for the growth of marbled crayfish.

Table 5: Weight gains and feed conversion ratios (FCR) of crayfish fed with different diets.

Feed	Description	Mean weight gain per animal	Mean feed intake per animal	FCR
D1	Control (0.52%)	1.43 g	3.62 g	2.41
D2	Tailored, Met-deficient (0.45%)	0.97 g	3.29 g	3.35
D3	Tailored, Met like control (0.52%)	2.81 g	3.81 g	1.38
D4	Tailored, Met-matched (0.60%)	1.56 g	3.11 g	2.22
D5	Tailored, Met surplus (0.70%)	2.63 g	4.29 g	1.72

FCR comparisons: D1:D3 p=0.041, D2:D3 p=0.003, D2:D5 p=0.012

3.3.5 Environmental safety (eDNA monitoring)

To minimize the risk of culturing a potentially invasive species like marbled crayfish, periodical monitoring of the surrounding environment can be implemented.

Detection of environmental DNA (eDNA) was found to be effective for identifying species in a particular water body (Baldigo et al., 2017; Cai et al., 2017; Mauvisseau et al., 2019b), including most European crayfish species (Agersnap et al., 2017; Baldigo et al., 2017; Cai et al., 2017; Harper et al., 2018; Mauvisseau et al., 2018). The method was established in collaboration with Quentin Mauvisseau (University of Derby, UK), who designed the primers and determined the quantification limits for the qPCR (Mauvisseau et al., 2019a).

A field study was conducted with samples from habitats with known marbled crayfish populations to evaluate the method. Additional samples were taken from regions with unknown status to obtain insights into the spread of marbled crayfish. Two lakes with known stable wild populations (Singliser See and Reilinger See; Figure 24A) were tested at four different spots each. In both lakes, three of the four spots were positive for eDNA of marbled crayfish (Figure 24B and C). These results show that the eDNA

assay can correctly detect marbled crayfish DNA. Several water bodies, including rivers, lakes, and a pond close to Reilinger See were also tested for the presence of marbled crayfish. For all spots, negative results were obtained (Figure 24D), which may indicate a more limited invasive potential of marbled crayfish.

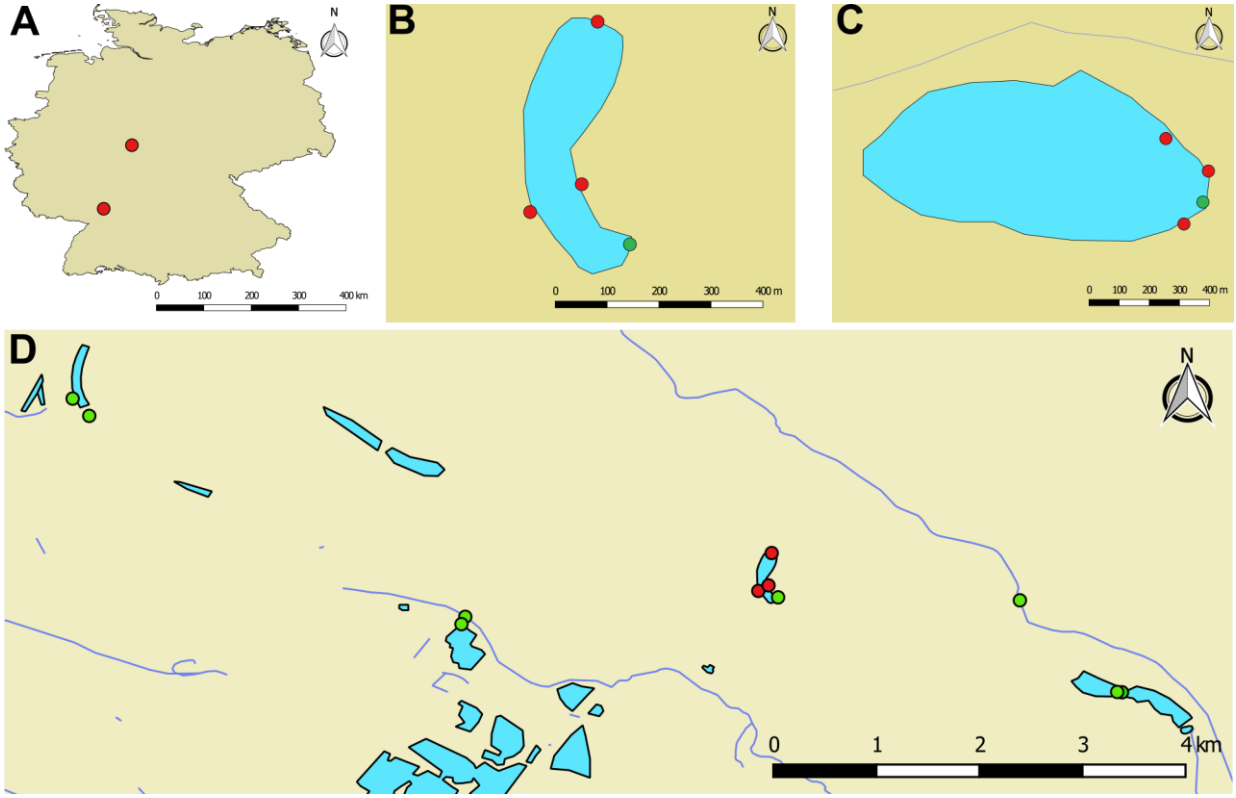

Figure 24: Detection of eDNA in established marbled crayfish populations in Germany. Red circles indicate sampling sites with marbled crayfish eDNA detection. Green circles indicate no detection of eDNA from marbled crayfish at this collection site. (A) Map of Germany showing eDNA sampling areas (Reilinger See (lower left red circle) and Singliser See (upper right red circle). (B) Map of Reilinger See with locations of the four sampling sites. (C) Map of Singliser See with locations of the four sampling sites. (D) Map of Reilinger See and surrounding water bodies with locations of eleven sampling sites.

4 Discussion

The marbled crayfish is a unique freshwater crayfish as it reproduces by parthenogenesis and emerged from a single origin. Thus, all known marbled crayfish are clonal. Furthermore, the species has a predicted age of 25-30 years and thus represents a remarkably young species (Scholtz et al., 2003). Despite their clonality, the animals can form stable wild populations in various countries in the world (Chucholl and Pfeiffer, 2010; Deidun et al., 2018; Gutekunst et al., 2018; Jones et al., 2009; Lyko, 2017). The Darwinian model of genetic adaptation (Özgür et al., 2006) is not applicable for a clonal species at this young age, raising the question of how this species adapts to different environments. Epigenetic mechanisms were often proposed to play a role in rapid adaptation (Carneiro and Lyko, 2020; Schübeler, 2015; Verhoeven et al., 2016), but insightful and statistically sound studies remain sparse (Carneiro and Lyko, 2020; Dubin et al., 2015; Lea et al., 2017).

In this thesis, the habitat diversity of German marbled crayfish populations was explored to show their adaptability. Furthermore, capture-based methylation analysis of the most variably methylated genes was performed on two different tissues and four independent populations to determine context-dependent methylation variants. Deep amplicon sequencing was used to validate the results from the capture-based analysis and confirmed the observed methylation patterns for tissues and locations in specific genes. Additionally, the physiological properties of marbled crayfish and their commercial potential were explored, and a method for marbled crayfish detection from water samples was developed to establish guidelines for sustainable aquaculture.

4.1 Clonal marbled crayfish show high adaptability to different environments

Different previous studies have already shown that marbled crayfish form stable populations in various climatic zones and habitats (Andriantsoa et al., 2019; Chucholl and Pfeiffer, 2010; Deidun et al., 2018; Gutekunst et al., 2018). For Germany, no systematic analysis of the stable wild populations has been conducted so far. The results of this study confirm the high adaptability of marbled crayfish. They could be found in a large variety of habitats, including a lake with pH 4.36 and high amounts of manganese,

aluminum, and nickel. Also, seasonal temperature changes of more than 20 °C within a year were observed. While wild populations of the sexually reproducing mother species *Procambarus fallax* are exclusively located in freshwater systems of Florida and southern Georgia (van der Heiden and Dorn, 2017), the marbled crayfish is observed in many countries (Andriantsoa et al., 2019; Chucholl and Pfeiffer, 2010; Deidun et al., 2018). This seems to distinguish the marbled crayfish from its mother species and may indicate a higher adaptive potential of marbled crayfish (Vogt et al., 2019). Notably, marbled crayfish show a global hypomethylation compared to its mother species *Procambarus fallax* (Gatzmann et al., 2018). A study on the mussel *Xenostrobus secures* showed a global hypomethylation of a recently evolved population compared to long-established populations. The hypomethylation was interpreted to promote higher phenotypic plasticity (Ardura et al., 2017). These findings are in line with our results and would support the assumption that marbled crayfish have a higher adaptability than *Procambarus fallax*. However, to confirm this assumption, studies on the adaptive potential of *P. fallax* would need to be carried out. Another closely related freshwater crayfish, *Procambarus clarkii,* is one of the most wide spread freshwater crayfish globally (Gherardi, 2006; Oficialdegui et al., 2020), but this species reproduces sexually and is, therefore, able to adapt genetically. However, epigenetic mechanisms may play a role in the rapid adaptation of this and other species. The number of publications on this topic is increasing (Carneiro and Lyko, 2020; Verhoeven et al., 2016) but they are often limited by confounding effects, low statistical power, or poor methodology (Dubin et al., 2015; Kucharski and Maleszka, 2020; Lea et al., 2017). The marbled crayfish has unique advantages for studying purely epigenetic adaptation mechanisms, as it combines obligatory apomictic parthenogenesis and a very small number of confounding genetic mutations caused by the young age of the species (Carneiro and Lyko, 2020; Gutekunst et al., 2018).

4.2 Marbled crayfish show highly localized methylation differences

Comparing samples from different animals, tissues, and an embryonic stage revealed a relatively stable and largely tissue invariant methylation pattern (Gatzmann, 2019). Nonetheless, a small subset of genes (697) showed more variable methylation levels, allowing a separation of hepatopancreas from abdominal muscle samples (Gatzmann,

2019). This subset of genes was used for a capture-based bisulfite sequencing assay to analyze 73 samples from two tissues and four locations on a single-base resolution. A small subset of genes was identified to allow a robust separation of the two tissues and the four locations. This was confirmed by deep sequencing of specific differentially methylated regions. These results indicate localized methylation differences in the marbled crayfish. Similar observations were seen in the honey bee (*Apis mellifera*), where Kucharski and Maleszka found condition-specific methylation patterns in the brain (Kucharski and Maleszka, 2020). These patterns were highly localized and position-dependent, similar to the patterns observed for marbled crayfish.

The tissue-specific patterns were independent of the location where the animals originated from, indicating a clear tissue specificity rather than an influence from the environment. This suggests a tissue-specific function of those genes. A comparison with gene expression patterns revealed no correlation between methylation and expression levels. Therefore, the functional relevance of these tissue-specific methylation patterns remains unclear and needs further investigation. These localized methylation changes could influence several functional mechanisms, like interactions with chromatin, microRNAs, or the modulation of transcription factor binding (Ashby et al., 2016; Neri et al., 2017; Wojciechowski et al., 2018).

4.3 Stable methylation patterns in marbled crayfish suggest the existence of epigenetic ecotypes

Interestingly, samples from the four different locations, representing four stable marbled crayfish populations, can be separated from each other, showing different methylation patterns. These findings suggest the influence of the specific environment on the population and a localized methylation change to cope with the need for adaptation. The role of epigenetic mechanisms in rapid adaptation has been discussed in the literature (Carneiro and Lyko, 2020; Schübeler, 2015; Verhoeven et al., 2016), and the existence of epigenetic ecotypes was proposed. Probably the most prominent example is *Arabidopsis thaliana*. Becker et al. identified methylation variations between distinct populations (Becker et al., 2011), but later studies linked those variations to genetic alterations (Alonso-Blanco et al., 2016; Dubin et al., 2015). Therefore, genetic ecotypes are an established concept in ecological adaptation (Alonso-Blanco et al., 2016),

but the existence of true epigenetic ecotypes has remained unclear. In the past years, some studies found evidence for differential methylation depending on the environment. Le Luyer et al. were able to identify methylation differences caused by hatchery rearing of salmon compared to their wild counterparts (Le Luyer et al., 2017). The hatchery environment led to a hypermethylation of genes in two distinct populations, which was suggested to play a role in the lower fitness of these groups.

Interestingly, in this study, a hypermethylation of laboratory animals compared to wild populations could not be observed. There were no investigations on the fitness of these two groups, as laboratory animals will not be released to the wild. However, altered physiological properties could not be observed. The analysis of the four wild populations used in this study revealed methylation variations among the populations. In a principal component analysis, animals from the same population were grouped together, while a robust separation to the other populations was achieved. These findings were consistent with the separation seen by Le Luyer et al. (Le Luyer et al., 2017).

The sequencing at a single-base resolution of the same genes in all samples allowed the identification of differentially methylated regions. A comparison revealed specific methylation patterns for each location, which were consistent within the population but different from other populations. The validation of those results by deep amplicon sequencing confirmed these findings. Additionally, taken both experiments together, it could be shown that the patterns remain stable over time, meaning populations from a certain environment maintain the methylation pattern. These results strongly suggest stable methylation patterns depending on the environment and, therefore, the presence of epigenetic ecotypes in marbled crayfish. A confounding effect, as seen in *Arabidopsis thaliana* (Dubin et al., 2015), is not likely in the clonal marbled crayfish. However, the used dataset of variably methylated genes was investigated for the few known population-related single-nucleotide variances (SNVs) (Maiakovska et al., in revision), and none of the validated location-specific genes were found to be affected by SNVs. Therefore, the detected methylation variants are not affected by genetic variants.

4.4 Methylation fingerprints allow origin tracing in marbled crayfish

The robust separation of populations and the stability of the pattern over time provides an opportunity to trace the environmental origin of a given population by identifying its methylation fingerprint. Each population forms its specific signature, depending on the specific habitat conditions. Therefore, origin tracing describes "environmental origin" and can be distinguished from the "geographical origin". As seen in the wild populations, the patterns also remain after sampling at a later timepoint and using another method. Also, the laboratory colony showed a different signature. The patterns seem to be stable and unique, but also plastic to some degree, which was indicated by the shift of the pattern in the animals kept at low temperatures for six months. Reasons for a visible shift in the methylation pattern for the low-temperature group, but not for the manganese treated groups, could be technical and biological. The analysis was based on a small subset of genes. These genes may not display the methylation change caused by manganese. Furthermore, the epigenetic response to different environmental stressors could potentially differ in time and magnitude. However, these results provide the opportunity to trace the environmental origin of a species by its methylation fingerprint.

The assessment of rearing conditions could potentially be made if genes can be identified that are affected by specific changes. Experiments in corals (*Stylophora pistillata*) showed methylation differences depending on different pH values of the water. These methylation differences were found in genes linked to growth and stress response (Liew et al., 2018). Also, methylation changes were reproducible in a second experiment with the same experimental setup (Liew et al., 2018), showing a correlation of environmental factor and affected gene. Interestingly, the results in corals are consistent with the findings in this study, where the methylation pattern remained stable under the same conditions. A gene ontology analysis on the 361 variably methylated genes revealed an enrichment of G-protein related genes with functions in transcription and translation regulation, response to stress, RNA metabolism, and immune response to pathogens. These genes could potentially confer plasticity in marbled crayfish. Nevertheless, further investigations could identify modulators for methylation changes in specific genes.

While further experiments need to be carried out, this thesis provides proof of concept for origin tracing of marbled crayfish by location-specific epigenetic fingerprints.

4.5 A concept for the sustainable aquaculture of marbled crayfish

The previously described findings improve the understanding of rapid epigenetic adaptation and potentially also have relevance for sustainable aquaculture. While marbled crayfish are not commercially used in aquaculture so far, there are indications that they are being considered for commercial production (Jurmalietis et al., 2019). How fast the species can be spread is exemplified in Madagascar, where the distribution area increased 100-fold within the last ten years (Gutekunst et al., 2018). The spread was mainly fueled by anthropogenic releases through the local population, as they quickly recognized that the marbled crayfish is a valuable protein source (Andriantsoa et al., 2020).

Aquaculture of crayfish is extensively performed in south-east Asia, China, southern Europe, and parts of the United States (Wang et al., 2018) and has a total market volume of more than 10 billion US dollars (FAO Fisheries and Aquaculture Department, 2018). The most prominent farmed crayfish species is *Procambarus clarkii,* with a market share of more than 90% (Wang et al., 2018). Marbled crayfish is a new freshwater crayfish species, which has been popular in aquaristics for 25 years (Scholtz et al., 2003). Moreover, the species is also considered as aquaculture livestock (Jurmalietis et al., 2019). However, the ecological concerns regarding marbled crayfish remain, and doubts about repeating mistakes made for *P. clarkii* aquaculture are high (Gherardi, 2006). Therefore, sustainable and safe production of (marbled) crayfish should be implemented. The results from this study could contribute to such a concept.

The marbled crayfish shows a comparable size and weight to *P. clarkii*. Furthermore, the feed conversion ratio (FCR) of marbled crayfish is comparatively low with 1.36. The FCR of livestock plays an essential role in the classification of an animal food source. Swine (FCR of 4.0) and cattle (FCR of 8.8) (Oonincx et al., 2015) are considered unsustainable and environmentally damaging because of high FCRs and methane emission. Insects are considered sustainable as they have the lowest known FCR (0.9; Van Huis, 2013) and do not emit greenhouse gases. However, insects are not generally accepted as a nutritional protein source, especially in non-Asian cultures.

Crayfish meat is more accepted, and the FCR for marbled crayfish calculated in this study compares favorably with meat from other livestock.

An additional commercial value of freshwater crayfish and crustaceans is provided by the shells of the animals, which are produced as waste during the meat processing, but can be used for chitin extraction. Marbled crayfish shells contain 2.6% chitin per animal, three times more than shrimp shells. Shrimps and crabs are currently the main sources for chitin (Younes and Rinaudo, 2015). In conclusion, marbled crayfish showed a comparable size and weight to *P. clarkii* and had a higher chitin content than shrimps, which suggests them as suitable candidates for aquaculture.

4.5.1 Establishment of an environmentally safe aquaculture of marbled crayfish

Commercial crayfish aquaculture is usually done in open systems. In China, the commonly used farming method is a rice field rotation system. In some cases, the co-culture with fish or crabs is pursued (Wang et al., 2018). Although crayfish maintain ecosystems, introducing invasive species can create significant ecological problems, such as competition with other species and the transmission of pathogens (Gherardi, 2006). One approach that limits a potential ecological damage by the aquaculture of an invasive species is the use of closed systems. An option for closed systems is a recirculating aquaculture system (RAS), but their operation is still too expensive to compete with open systems, and the production value is too low (Losordo and Westerman, 1994; Luo et al., 2014). However, closed systems are often used for the hatchery and nursery before the crayfish are released to the rice fields or ponds for grow-out (Wang et al., 2018). A pond culture system with no direct connection to any natural water body is not as safe as a completely closed system like a RAS but could minimize the ecological risk.

Marbled crayfish tolerate small salt concentrations for a period of time but cannot reproduce in brackish water. Higher salt concentrations or prolonged exposure to saltwater are lethal for the animals (Veselý et al., 2017). Therefore, freshwater ponds could be surrounded by salt or brackish water ponds for fish and shrimp aquaculture (Figure 25). These ponds would function as an effective barrier that separates the marbled crayfish from the natural environment.

Figure 25: Concept for an environmentally safe marbled crayfish aquaculture. Freshwater grow-out ponds (turquoise) for marbled crayfish aquaculture, located in a salt- or brackish water (dark blue) aquaculture environment. Due to the low salinity tolerance of marbled crayfish (Veselý et al., 2017), the salt- or brackish water ponds would be an effective barrier to the surrounding environment.

Furthermore, continuous monitoring of the surrounding environment of the ponds could mitigate the risk and would allow a rapid reaction if the species is spreading. A monitoring method could be regular eDNA sampling and detection. Environmental DNA (eDNA) is a method to detect DNA traces of a particular species in the environment where the species can be found (Baldigo et al., 2017; Cai et al., 2017). Aquatic organisms are often difficult to detect visually, particularly if they are nocturnal, as the marbled crayfish. For fast and easy detection of this species from a water body, eDNA detection is a suitable method to determine the occurrence of a species and its distribution. The results in this study showed that the presence of marbled crayfish could successfully be detected from water samples using eDNA. This approach is available for many species and can, in theory, be established for any aquatic species (Baldigo et al., 2017; Cai et al., 2017; Mauvisseau et al., 2019b).

4.5.2 Importance of certification of aquaculture products

Aquaculture and agricultural meat production is strongly criticized by a growing part of the society, as sustainability and environmental protection are becoming increasingly important (Notarnicola et al., 2017; Tilman et al., 2011). Simultaneously, the demand for nutritional protein increases dramatically. Traditional meat production is often considered unsustainable and lacks transparency (Notarnicola et al., 2017; Tilman et al., 2011; Zaks and Kucharik, 2011), as the value chains of the agriculture and aquaculture industries are highly complex. Certificates have been established to

reinforce consumer relationships and trust. These certificates state animal welfare and sustainable production. However, certificates are based on audits at specific farms (MSC, 2020) and can be easily tampered by moving livestock from non-certified farms to certified farms (Papa, 2017; Schlag, 2010). This raises reasonable concerns about the certificates. Therefore, tempering resistant certification methods are needed.

Trace Element Fingerprinting (TEF) is currently being evaluated for certifying the origin of livestock (Ricardo et al., 2017). It is a method to detect different element/calcium ratios in mineralized samples like shells of bivalves. The combination of element/calcium ratios, measured by mass spectrometry, can identify the geographical origin of the sample. Ricardo et al. showed that the identification of the geographical origin of cockle (*Cerastoderma edule*) from different ecosystems was possible by detecting four element ratios (Mg/Ca, Mn/Ca, Sr/Ca, and Ba/Ca) (Ricardo et al., 2017). However, the method shows substantial limitations, such as the restriction to mineralized samples (shells and fish otoliths) or weather sensitivity. Therefore, frequent recalibrations (within months) would be needed (Ricardo et al., 2017).

Methylation fingerprinting could provide an alternative solution for a tampering resistant certificate. Marbled crayfish populations showed location-specific methylation patterns. These fingerprints can be detected from the meat and identify its origin. To protect this knowledge and the idea for using it commercially, a patent application was filed in July 2020 (Tönges et al., 2020b), and the trademark EpiTrace™ was submitted for registration in August 2020 (EUIPO, 2020). In addition, the plasticity of methylation patterns due to an external trigger indicated that rearing conditions could potentially be detected. This would be an additional benefit, as potentially (prohibited) treatments of the livestock, like antibiotics, could be detected.

4.6 Conclusions and outlook

In summary, marbled crayfish from different populations show differential methylation. These methylation differences are detectable by specific patterns in a small subset of genes but not on the average methylation level. The stability of these patterns in samples from a particular environment and over time suggests the existence of epigenetic ecotypes, which are not influenced by genetic variants. Furthermore, highly localized context-dependent methylation changes indicate context-dependent functions of these

methylation patterns. Genes involved in transcriptional and translational regulation, RNA metabolism, stress response, and immune response to pathogens may confer plasticity and explain the functional role of these genes in the adaptability of marbled crayfish. The suitability of marbled crayfish as aquaculture livestock was suggested by its physiological properties. Guidelines for an environmentally safe culture were established by implementing monitoring methods like eDNA detection and pond management with a combination of freshwater and saltwater. Furthermore, the unique methylation fingerprints of marbled crayfish could be used to certify the origin of the species and potentially also the rearing conditions. This could be used to establish a sustainable and transparent farming and value chain for marbled crayfish.

The results in this thesis also provide new insight into the methylation structure of marbled crayfish and the correlation to environmental adaptation, but further investigation is needed. The functional relevance of the differential methylation is still unclear. Combined analysis of methylation and expression data from the same samples would help to correlate expression values to methylation changes. The functional role of DNA methylation could also be investigated by knocking-down enzymes of the methylation machinery like DNMTs or TETs to observe phenotypic differences. Challenging those animals with environmental triggers would also help to understand the relevance of DNA methylation in the adaptation of those animals.

Furthermore, as shown in this thesis, the methylation pattern of the animals can be changed when they are exposed to an environmental trigger, but which genes are involved in these changes and which trigger causes which change could not be investigated yet. Further experiments with different environmental triggers could lead to an identification of specific changes depending on the trigger. This would provide a basis for an understanding of the role of methylation in these genes and the possibility to trace rearing conditions in marbled crayfish livestock. It would also be interesting to see how quickly a different signature will be established if an animal is placed from one to another environment and at which point this pattern remains stable. Studies on this topic would help to understand how fast methylation-related adaptation occurs.

Finally, additional locations should be included to generate methylation fingerprints for more populations and to obtain a better understanding of the specific features of the patterns. As similar results were obtained for marbled crayfish and salmon (Le Luyer et al., 2017), this indicates the possibility to apply the concept of traceability to other

aquaculture and agricultural livestock. The commercial potential of the respective market is huge, and this study provides an important proof of concept for origin tracing by DNA methylation fingerprinting.

5 Appendix

Table 6: Primer pair targeting a mitochondrial Cytochrome b region in the marbled crayfish genome used for genetic authentication of wild-caught marbled crayfish

Name	Sequence	Amplicon length [bp]
RA_009	CAGGACGTGCTCCGATTCATG	274
RA_010	GACCCAGATAACTTCATCCCAG	

Table 7: List of primers used for the validation experiment to identify tissue-specific (T) and location-specific (L) patterns in marbled crayfish. Sequences in red and blue are adapters required for the MiSeq platform.

Name	Sequence	Amplicon length [bp]
T173_fwd	tcgtcggcagcgtcagatgtgtataagagacagGAATTATTTTATTTGTGATATTTTTTTAAT	306
T173_rev	gtctcgtgggctcggagatgtgtataagagacagATTAATCCACATAATATTTCACCAC	
T595_fwd	tcgtcggcagcgtcagatgtgtataagagacagTGGAGATAAGTTAGTTTAATTAGGTTATAT	348
T595_rev	gtctcgtgggctcggagatgtgtataagagacagAATCATCTTAAAAATTCAAAAAAAA	
L460_fwd	tcgtcggcagcgtcagatgtgtataagagacagGGGTAGATAGAATTATTTTTTTT	184
L460_rev	gtctcgtgggctcggagatgtgtataagagacagTTTCCTAAAAACCACATTAAAACAC	
L88_R1_fwd	tcgtcggcagcgtcagatgtgtataagagacagTTATAATATATTAATGGTTTTGATGA	284
L88_R1_rev	gtctcgtgggctcggagatgtgtataagagacagCACAAAAAACAAAAACTACAAACTC	
L88_R2_fwd	tcgtcggcagcgtcagatgtgtataagagacagATTATATTTATATTGGATGGATTTAATTTA	126
L88_R2_rev	gtctcgtgggctcggagatgtgtataagagacagAAACAAACATCTTATACAATTCTTCTC	

Table 8: Coordinates of sampling sites in Germany and Madagascar (Andriantsoa et al., 2019).

Country	City/District	Water body's name/ Site	GPS Coordinates	
			Latitude	Longitude
Germany	Berlin	Krumme Lanke	N52°26,936'	E13°13,803'
Germany	Borken	Singliser See	N51°03,655'	E09°18,710'
Germany	Reilingen	Reilinger See	N49°17,649'	E08°32,672'
Germany	Freiburg	Moosweiher	N48°01,793'	E07°48,383'
Germany	Kirchentellinsfurt	Baggersee Epple	N48°32,537'	E09°08,989'
Germany	Wackersdorf	Murner See	N49°21,115'	E12°12,085'
Madagascar	Lalangina	Andragnaroa	S21°17,567'	E47°22,295'
Madagascar	Ihosy	Ihosy	S22°22,521'	E46°06,016'

Table 9: Water parameters measured during the field trips in Germany. All measurements were taken on-site using a Multiparameter instrument (Hanna).

City	Water body's name	Total catch size	Water parameters		
			pH	Conductivity (µS)	Temp. (°C)
Reilingen	Reilinger See	52	8,08	637	25,3
Berlin	Krumme Lanke	5	8,12	729	21,1
Borken	Singliser See	140	4,36	1205	22,1
Freiburg	Moosweiher	9	8,44	316	24,75
Kirchentellinsfurt	Baggersee Epple	8	8,77	595	25,4

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Ormont, den 29.06.2018
➔ **Analyse-Nr.: W 10564**

Untersuchungsbefund der Wasserprobe

Bezeichnung: Krumme Lanke (Bach, See)
Probenahme: 16.06.18; 17 Uhr 1563

	Meßwerte	Einheit	Grenzwert TrinkwV.	Richtwerte Binnengewässer	Beurteilung für Binnengewässer
Physikalisch-chemische Parameter					
Eingangstemperatur		°C	-		-
pH-Wert (bei 21°C)	7,71		6,5 - 9,5	6,5 - 8,0	schwach alkalisch
Redoxpotential (ORP)		mV	kein Grenzwert		
Leitfähigkeit bei 20 °C	726	µS/cm	2500	0	
Trübung	8,21	NTU	1,0	3,0	stark getrübt
Chemische Parameter (gelöste Stoffe)					
Gesamthärte	14,8	° dH	= 2,63 mmol Erdalkaliionen/Liter		hart
Säurekapazität bis pH 4,3		mmol/l			
Hygrogencarbonat		mg/l		6 - 8 °dKH	
Nitrat	2,76	mg/l	50 *	20	unbedenklich
Nitrit		mg/l	0,5	0,05	
Ammonium		mg/l	0,5	0,5	
Sulfat		mg/l	250	250	
Chlorid	63,1	mg/l	250		unbedenklich
PO_4 (Phosphat)	< 0,05	mg/l	6,7	1	unbedenklich
Ca (Calcium)	86,2	mg/l	kein Grenzwert		unbedenklich
K (Kalium)	8,7	mg/l	kein Grenzwert	175	unbedenklich
Mg (Magnesium)	11,8	mg/l	kein Grenzwert	250	unbedenklich
Na (Natrium)	38,9	mg/l	200 **	200	unbedenklich
Sr (Strontium)	0,41	mg/l	kein Grenzwert		unbedenklich
Al (Aluminium)	7,0	ug/l	200	500	unbedenklich
As (Arsen)	< 8	µg/l	10	10	unbedenklich
B (Bor)	67,9	µg/l	1000	1000	unbedenklich
Ba (Barium)	40,2	µg/l	1000	1000	unbedenklich
Cd (Cadmium)	< 1	µg/l	3	3	unbedenklich
Cr (Chrom)	< 2	µg/l	50	50	unbedenklich
Cu (Kupfer)	3,5	µg/l	2000 ***	2000	unbedenklich
Fe (Eisen, Fe^{2+})	< 1	µg/l	200	200	unbedenklich
Mn (Mangan)	< 1	µg/l	50	100	unbedenklich
Ni (Nickel)	< 2	µg/l	20	20	unbedenklich
Pb (Blei)	< 5	µg/l	10	10	unbedenklich
U (Uran)	< 3	µg/l	10	10	unbedenklich
Zn (Zink)	2,8	µg/l	3000 ****	3000	unbedenklich

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Untersuchungsbefund der Wasserprobe

Bezeichnung: Krumme Lanke (Bach, See)
Probenahme: 16.06.18; 17 Uhr 1563

	Meßwerte	Einheit	Grenzwert TrinkwV.	Richtwerte Binnengewässer	Beurteilung für Binnengewässer
Weitere chemische Parameter					
O_2 (Sauerstoff) gelöst		mg/l			
CO_2 (Kohlendioxid)		mg/l	-		
Gesamt-Eisen (Fe^{2+} + Fe^{3+})		µg/l	200	200	
Gesamt-Mangan		µg/l	50	100	
Mikrobiologische Parameter					
Koloniezahl bei 22 °C		KBE/ml	100	-	
Koloniezahl bei 36 °C		KBE/ml	100	-	
Coliforme Keime		KBE/100 ml	0	-	
Escherischia coli		KBE/100 ml	0	-	
Enterokokken		KBE/100 ml	0	-	
Sensorische Prüfung					
Aussehen/Farbe	zart gelblich, leicht trüb, Schwebepartikel, Bodenablagerungen				

Bewertung anhand der geprüften Parameter			
Vorgaben der TrwVO erfüllt:	nein	Eignung als Poolwasser:	nein
Eignung als Tränkewasser:	ja	Eignung als Aquarienwasser	nein
Eignung als Gießwasser:	ja		

Die Trübung ist erhöht. Wir empfehlen die Untersuchung auf Gesamt-Eisen und Bakterien.

* Die WHO hat für Kinder einen Grenzwert von 10 mg/Liter festgelegt. EU-Nitratrichtwert: 25 mg/l.

** Für Säuglinge ist natriumarmes Wasser mit weniger als 20 mg Na/Liter empfehlenswert.

*** Die Empfehlung der Europäischen Union liegt bei 100 µg Cu/Liter für Säuglinge.

**** Die Weltgesundheitsorganisation WHO hat für Zink einen Leitwert von 3000 µg/Liter herausgegeben.

Analysemethoden: pH-Wert und Leitfähigkeit elektrometrisch. Nitrat nach DIN 13395. Chlorid argentometrisch. As mit HG-AAS. Bestimmung der übrigen Elemente mit ICP-OES nach DIN 11885. Mikrobiol. Parameter nach TrinkWV Anl. 3. / DIN EN ISO 8199.

Zur Qualitätssicherung wurden in der Meßreihe mehrere zertifizierte Referenzmaterialen mitgeführt.

Der Befund bezieht sich ausschließlich auf den Prüfgegenstand.

Dieser Befund dient nur Ihrer persönlichen Information. Er kann nicht für das Gesundheitsamt, gutachterliche Nachweise sowie juristische Auseinandersetzungen genutzt werden. Reklamation werden nur innerhalb einer Frist von zwei Wochen anerkannt.

Bitte kontaktieren Sie uns bei Fragen zum Qualitätsmangel dieser Wasserprobe (gebührenfrei). 39,41 €

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Ormont, den 29.06.2018
Analyse-Nr.: **W 10564**

Parameter	Einheit	Ihr Meßwert	Eignung als… Trinkwasser	Tränkewasser	Gießwasser	Fischgewässer	Poolwasser	Wärmepumpe
pH-Wert	-	7,71	J	J	J	J	J	J
Leitfähigkeit	µS	726	J	J	J	J	J	N
Trübung	NTU	8,21	N	J	J	J	N	N
Gesamthärte	° dH	14,8						
Carbonathärte/Hydrogencabonat	° KH/mg/l							
Sauerstoff gelöst	mg/l							
Nitrat	mg/l	2,76	J	J	J	J	J	J
Nitrit	mg/l							
Ammonium	mg/l							
Sulfat	mg/l							
Chlorid	mg/l	63,1	J	J	J	N	J	J
PO4 (Phosphat)	mg/l	< 0,05	J	J	J	J	J	J
Ca (Calcium)	mg/l	86,2	J	J	J	J	J	J
K (Kalium)	mg/l	8,7	J	J	J	J	J	J
Mg (Magnesium)	mg/l	11,8	J	J	J	J	J	J
Na (Natrium)	mg/l	38,9	J	J	J	J	J	J
Sr (Strontium)	mg/l	0,41	J	J	J	J	J	J
Al (Aluminium)	µg/l	7,0	J	J	J	J	J	J
As (Arsen)	µg/l	< 8	J	J	J	J	J	J
B (Bor)	µg/l	67,9	J	J	J	J	J	J
Ba (Barium)	µg/l	40,2	J	J	J	J	J	J
Cd (Cadmium)	µg/l	< 1	J	J	J	J	J	J
Cr (Chrom)	µg/l	< 2	J	J	J	J	J	J
Cu (Kupfer)	µg/l	3,5	J	J	J	J	J	J
Fe (Eisen)	µg/l	0,0	J	J	J	J	J	J
Mn (Mangan)	µg/l	0,0	J	J	J	J	J	J
Ni (Nickel)	µg/l	< 2	J	J	J	J	J	J
Pb (Blei)	µg/l	< 5	J	J	J	J	J	J
U (Uran)	µg/l	< 3	J	J	J	J	J	J
Zn (Zink)	µg/l	2,8	J	J	J	J	J	J
Koloniezahl bei 22 °C	KBE/ml							
Koloniezahl bei 36 °C	KBE/ml							
Coliforme Keime	KBE/100ml							
Escherichia coli	KBE/100ml							
Enterokokken	KBE/100ml							

Beurteilung anhand der geprüften Parameter. Falls es sich bei Ihrer Probe um eine Stagnations-Leitungswasserprobe handelt, gelten die obigen Hinweise zu den Schwermetallen nur für Trink- und Tränkewasser.

N = NEIN, Qualitätsmangel

J = JA, Qualität entspricht den Richtwerten bzw. gesetzlichen Bestimmungen.

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Figure 26: Report of water analysis from Krumme Lanke (sampling 16.06.18). Analysis performed by Raiffeisen Laborservice (Ormont, Germany).

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Raiffeisen Rhein-Ahr-Eifel Handelsgesellschaft mbH

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Im Neuenheimer Feld 280
69120 Heidelberg

Ormont, den 29.06.2018
➔ **Analyse-Nr.: W 10565**

Untersuchungsbefund der Wasserprobe

Bezeichnung: Sinliser See (Bach, See)
Probenahme: 15.06.18; 18:10 Uhr 1564

	Meßwerte	Einheit	**Grenzwert** TrinkwV.	**Richtwerte** Binnengewässer	**Beurteilung für** Binnengewässer
Physikalisch-chemische Parameter					
Eingangstemperatur		°C	-		-
pH-Wert (bei 21°C)	**5,19**		6,5 - 9,5	6,5 - 8,0	korrosiv
Redoxpotential (ORP)		mV	kein Grenzwert		
Leitfähigkeit bei 20 °C	1261	µS/cm	2500	0	
Trübung	0,26	NTU	1,0	3,0	klar
Chemische Parameter (gelöste Stoffe)					
Gesamthärte	36,2	° dH	= 6,44 mmol Erdalkaliionen/Liter		hart
Säurekapazität bis pH 4,3		mmol/l			
Hygrogencarbonat		mg/l		6 - 8 °dKH	
Nitrat	1,13	mg/l	50 *	20	unbedenklich
Nitrit		mg/l	0,5	0,05	
Ammonium		mg/l	0,5	0,5	
Sulfat		mg/l	250	250	
Chlorid	33,3	mg/l	250		unbedenklich
PO_4 (Phosphat)	< 0,05	mg/l	6,7	1	unbedenklich
Ca (Calcium)	173,2	mg/l	kein Grenzwert		unbedenklich
K (Kalium)	7,1	mg/l	kein Grenzwert	175	unbedenklich
Mg (Magnesium)	51,6	mg/l	kein Grenzwert	250	unbedenklich
Na (Natrium)	20,8	mg/l	200 **	200	unbedenklich
Sr (Strontium)	2,03	mg/l	kein Grenzwert		unbedenklich
Al (Aluminium)	**357,1**	ug/l	200	500	leicht belastet
As (Arsen)	< 8	µg/l	10	10	unbedenklich
B (Bor)	250	µg/l	1000	1000	unbedenklich
Ba (Barium)	26,0	µg/l	1000	1000	unbedenklich
Cd (Cadmium)	< 1	µg/l	3	3	unbedenklich
Cr (Chrom)	< 2	µg/l	50	50	unbedenklich
Cu (Kupfer)	2,1	µg/l	2000 ***	2000	unbedenklich
Fe (Eisen, Fe^{2+})	< 1	µg/l	200	200	unbedenklich
Mn (Mangan)	**2967**	µg/l	50	100	unbrauchbar
Ni (Nickel)	**35,1**	µg/l	20	20	unbrauchbar
Pb (Blei)	< 5	µg/l	10	10	unbedenklich
U (Uran)	< 3	µg/l	10	10	unbedenklich
Zn (Zink)	72,3	µg/l	3000 ****	3000	unbedenklich

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Ormont, den 29.06.2018
➔ **Analyse-Nr.: W 10565**

Untersuchungsbefund der Wasserprobe

Bezeichnung: Sinliser See (Bach, See)
Probenahme: 15.06.18; 18:10 Uhr 1564

	Meßwerte	Einheit	Grenzwert TrinkwV.	Richtwerte Binnengewässer	Beurteilung für Binnengewässer
Weitere chemische Parameter					
O_2 (Sauerstoff) gelöst		mg/l			
CO_2 (Kohlendioxid)		mg/l	-		
Gesamt-Eisen (Fe^{2+} + Fe^{3+})		µg/l	200	200	
Gesamt-Mangan		µg/l	50	100	
Mikrobiologische Parameter					
Koloniezahl bei 22 °C		KBE/ml	100	-	
Koloniezahl bei 36 °C		KBE/ml	100	-	
Coliforme Keime		KBE/100 ml	0	-	
Escherischia coli		KBE/100 ml	0	-	
Enterokokken		KBE/100 ml	0	-	
Sensorische Prüfung					
Aussehen/Farbe	Schwebepartikel				

Bewertung anhand der geprüften Parameter			
Vorgaben der TrwVO erfüllt:	nein	Eignung als Poolwasser:	nein
Eignung als Tränkewasser:	nein	Eignung als Aquarienwasser	nein
Eignung als Gießwasser:	nein		

* Die WHO hat für Kinder einen Grenzwert von 10 mg/Liter festgelegt. EU-Nitratrichtwert: 25 mg/l.
** Für Säuglinge ist natriumarmes Wasser mit weniger als 20 mg Na/Liter empfehlenswert.
*** Die Empfehlung der Europäischen Union liegt bei 100 µg Cu/Liter für Säuglinge.
**** Die Weltgesundheitsorganisation WHO hat für Zink einen Leitwert von 3000 µg/Liter herausgegeben.
Analysemethoden: pH-Wert und Leitfähigkeit elektrometrisch. Nitrat nach DIN 13395. Chlorid argentometrisch. As mit HG-AAS. Bestimmung der übrigen Elemente mit ICP-OES nach DIN 11885. Mikrobiol. Parameter nach TrinkWV Anl. 3. / DIN EN ISO 8199.
Zur Qualitätssicherung wurden in der Meßreihe mehrere zertifizierte Referenzmaterialen mitgeführt.
Der Befund bezieht sich ausschließlich auf den Prüfgegenstand.
Dieser Befund dient nur Ihrer persönlichen Information. Er kann nicht für das Gesundheitsamt, gutachterliche Nachweise sowie juristische Auseinandersetzungen genutzt werden. Reklamation werden nur innerhalb einer Frist von zwei Wochen anerkannt.
Bitte kontaktieren Sie uns bei Fragen zum Qualitätsmangel dieser Wasserprobe (gebührenfrei). 39,41 €

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Ormont, den 29.06.2018

Analyse-Nr.: **W 10565**

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Parameter	Einheit	Ihr Meßwert	Eignung als… Trinkwasser	Tränkewasser	Gießwasser	Fischgewässer	Poolwasser	Wärmepumpe
pH-Wert	-	5,19	N	N	J	N	N	N
Leitfähigkeit	µS	1261	J	J	J	N	J	N
Trübung	NTU	0,26	J	J	J	J	J	J
Gesamthärte	° dH	36,2						
Carbonathärte/Hydrogencabonat	° KH/mg/l							
Sauerstoff gelöst	mg/l							
Nitrat	mg/l	1,13	J	J	J	J	J	J
Nitrit	mg/l							
Ammonium	mg/l							
Sulfat	mg/l							
Chlorid	mg/l	33,3	J	J	J	J	J	J
PO4 (Phosphat)	mg/l	< 0,05	J	J	J	J	J	J
Ca (Calcium)	mg/l	173,2	J	J	J	J	J	J
K (Kalium)	mg/l	7,1	J	J	J	J	J	J
Mg (Magnesium)	mg/l	51,6	J	J	J	J	J	J
Na (Natrium)	mg/l	20,8	J	J	J	J	J	J
Sr (Strontium)	mg/l	2,03	J	J	J	J	J	J
Al (Aluminium)	µg/l	357,1	N	N	J	N	N	N
As (Arsen)	µg/l	< 8	J	J	J	J	J	J
B (Bor)	µg/l	250	J	J	J	J	J	J
Ba (Barium)	µg/l	26,0	J	J	J	J	J	J
Cd (Cadmium)	µg/l	< 1	J	J	J	J	J	J
Cr (Chrom)	µg/l	< 2	J	J	J	J	J	J
Cu (Kupfer)	µg/l	2,1	J	J	J	J	J	J
Fe (Eisen)	µg/l	0,0	J	J	J	J	J	J
Mn (Mangan)	µg/l	2967	N	N	N	N	N	N
Ni (Nickel)	µg/l	35,1	N	J	J	J	N	J
Pb (Blei)	µg/l	< 5	J	J	J	J	J	J
U (Uran)	µg/l	< 3	J	J	J	J	J	J
Zn (Zink)	µg/l	72,3	J	J	J	J	J	J
Koloniezahl bei 22 °C	KBE/ml							
Koloniezahl bei 36 °C	KBE/ml							
Coliforme Keime	KBE/100ml							
Escherichia coli	KBE/100ml							
Enterokokken	KBE/100ml							

Beurteilung anhand der geprüften Parameter. Falls es sich bei Ihrer Probe um eine Stagnations-Leitungswasserprobe handelt, gelten die obigen Hinweise zu den Schwermetallen nur für Trink- und Tränkewasser.

N = NEIN, Qualitätsmangel

J = JA, Qualität entspricht den Richtwerten bzw. gesetzlichen Bestimmungen.

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Figure 27: Report of water analysis from Sinliser See (sampling 15.06.18). Analysis performed by Raiffeisen Laborservice (Ormont, Germany).

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Ormont, den 18.05.2018

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➔ **Analyse-Nr.: W 10072**

Untersuchungsbefund der Wasserprobe

Bezeichnung: Bach, See
Probenahme: 10.05.18; 10:20 Uhr 1077

	Meßwerte	Einheit	Grenzwert TrinkwV.	Richtwerte Binnengewässer	Beurteilung für Binnengewässer
Physikalisch-chemische Parameter					
Eingangstemperatur	13,2	°C	-		-
pH-Wert (bei 21,2°C)	8,41		6,5 - 9,5	6,5 - 8,0	schwach alkalisch
Redoxpotential (ORP)		mV	kein Grenzwert		
Leitfähigkeit bei 20 °C	731	µS/cm	2500	0	
Trübung	2,56	NTU	1,0	3,0	schwach getrübt
Chemische Parameter (gelöste Stoffe)					
Gesamthärte	18,9	° dH	= 3,36 mmol Erdalkaliionen/Liter		hart
Säurekapazität bis pH 4,3		mmol/l			
Hygrogencarbonat		mg/l		6 - 8 °dKH	
Nitrat	5,07	mg/l	50 *	20	unbedenklich
Nitrit		mg/l	0,5	0,05	
Ammonium		mg/l	0,5	0,5	
Sulfat		mg/l	250	250	
Chlorid	32,0	mg/l	250		unbedenklich
PO_4 (Phosphat)	< 0,05	mg/l	6,7	1	unbedenklich
Ca (Calcium)	108,7	mg/l	kein Grenzwert		unbedenklich
K (Kalium)	3,6	mg/l	kein Grenzwert	175	unbedenklich
Mg (Magnesium)	15,8	mg/l	kein Grenzwert	250	unbedenklich
Na (Natrium)	17,7	mg/l	200 **	200	unbedenklich
Sr (Strontium)	0,36	mg/l	kein Grenzwert		unbedenklich
Al (Aluminium)	< 10	ug/l	200	500	unbedenklich
As (Arsen)	< 8	µg/l	10	10	unbedenklich
B (Bor)	39,2	µg/l	1000	1000	unbedenklich
Ba (Barium)	92,8	µg/l	1000	1000	unbedenklich
Cd (Cadmium)	< 1	µg/l	3	3	unbedenklich
Cr (Chrom)	< 2	µg/l	50	50	unbedenklich
Cu (Kupfer)	0,8	µg/l	2000 ***	2000	unbedenklich
Fe (Eisen, Fe^{2+})	8,5	µg/l	200	200	unbedenklich
Mn (Mangan)	1,2	µg/l	50	100	unbedenklich
Ni (Nickel)	< 2	µg/l	20	20	unbedenklich
Pb (Blei)	< 5	µg/l	10	10	unbedenklich
U (Uran)	< 3	µg/l	10	10	unbedenklich
Zn (Zink)	< 2	µg/l	3000 ****	3000	unbedenklich

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➔ **Analyse-Nr.: W 10072**

Untersuchungsbefund der Wasserprobe

Bezeichnung: Bach, See
Probenahme: 10.05.18; 10:20 Uhr 1077

	Meßwerte	Einheit	Grenzwert TrinkwV.	Richtwerte Binnengewässer	Beurteilung für Binnengewässer
Weitere chemische Parameter					
O_2 (Sauerstoff) gelöst		mg/l			
CO_2 (Kohlendioxid)		mg/l	-		
Gesamt-Eisen (Fe^{2+} + Fe^{3+})		µg/l	200	200	
Gesamt-Mangan		µg/l	50	100	
Mikrobiologische Parameter					
Koloniezahl bei 22 °C		KBE/ml	100	-	
Koloniezahl bei 36 °C		KBE/ml	100	-	
Coliforme Keime		KBE/100 ml	0	-	
Escherischia coli		KBE/100 ml	0	-	
Enterokokken		KBE/100 ml	0	-	
Sensorische Prüfung					
Aussehen/Farbe	zart gelblich, Bodenablagerungen, Schwebepartikel				

Bewertung anhand der geprüften Parameter			
Vorgaben der TrwVO erfüllt:	nein	Eignung als Poolwasser:	nein
Eignung als Tränkewasser:	ja	Eignung als Aquarienwasser	ja
Eignung als Gießwasser:	ja		

Die Trübung ist erhöht. Wir empfehlen die Untersuchung auf Gesamt-Eisen und Bakterien.

* Die WHO hat für Kinder einen Grenzwert von 10 mg/Liter festgelegt. EU-Nitratrichtwert: 25 mg/l.
** Für Säuglinge ist natriumarmes Wasser mit weniger als 20 mg Na/Liter empfehlenswert.
*** Die Empfehlung der Europäischen Union liegt bei 100 µg Cu/Liter für Säuglinge.
**** Die Weltgesundheitsorganisation WHO hat für Zink einen Leitwert von 3000 µg/Liter herausgegeben.
Analysemethoden: pH-Wert und Leitfähigkeit elektrometrisch. Nitrat nach DIN 13395. Chlorid argentometrisch. As mit HG-AAS. Bestimmung der übrigen Elemente mit ICP-OES nach DIN 11885. Mikrobiol. Parameter nach TrinkWV Anl. 3. / DIN EN ISO 8199.
Zur Qualitätssicherung wurden in der Meßreihe mehrere zertifizierte Referenzmaterialen mitgeführt.
Der Befund bezieht sich ausschließlich auf den Prüfgegenstand.
Dieser Befund dient nur Ihrer persönlichen Information. Er kann nicht für das Gesundheitsamt, gutachterliche Nachweise sowie juristische Auseinandersetzungen genutzt werden. Reklamation werden nur innerhalb einer Frist von zwei Wochen anerkannt.
Bitte kontaktieren Sie uns bei Fragen zum Qualitätsmangel dieser Wasserprobe (gebührenfrei). 39,41 €

Seite 2 von 2

Figure 28: Report of water analysis from Reilinger See (sampling 10.05.18). Analysis performed by Raiffeisen Laborservice (Ormont, Germany).

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Raiffeisen Rhein-Ahr-Eifel Handelsgesellschaft mbH

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Ormont, den 18.05.2018

Analyse-Nr.: W 10073

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69120 Heidelberg

Untersuchungsbefund der Wasserprobe

Bezeichnung: Andracnaroa / Madagaskar (Bach, See)
Probenahme: 15.03.18; 9:45 Uhr 1078

	Meßwerte	Einheit	Grenzwert TrinkwV.	Richtwerte Binnengewässer	Beurteilung für Binnengewässer
Physikalisch-chemische Parameter					
Eingangstemperatur	13,2	°C	-		-
pH-Wert (bei 21,2°C)	7,50		6,5 - 9,5	6,5 - 8,0	schwach alkalisch
Redoxpotential (ORP)		mV	kein Grenzwert		
Leitfähigkeit bei 20 °C	29	µS/cm	2500	0	
Trübung	3,25	NTU	1,0	3,0	trüb
Chemische Parameter (gelöste Stoffe)					
Gesamthärte	0,3	° dH	= 0,05 mmol Erdalkaliionen/Liter		weich
Säurekapazität bis pH 4,3		mmol/l			
Hygrogencarbonat		mg/l		6 - 8 °dKH	
Nitrat	0,18	mg/l	50 *	20	unbedenklich
Nitrit		mg/l	0,5	0,05	
Ammonium		mg/l	0,5	0,5	
Sulfat		mg/l	250	250	
Chlorid	3,8	mg/l	250		unbedenklich
PO_4 (Phosphat)	< 0,05	mg/l	6,7	1	unbedenklich
Ca (Calcium)	1,3	mg/l	kein Grenzwert		unbedenklich
K (Kalium)	0,0	mg/l	kein Grenzwert	175	unbedenklich
Mg (Magnesium)	0,3	mg/l	kein Grenzwert	250	unbedenklich
Na (Natrium)	1,7	mg/l	200 **	200	unbedenklich
Sr (Strontium)	0,01	mg/l	kein Grenzwert		unbedenklich
Al (Aluminium)	76,7	ug/l	200	500	unbedenklich
As (Arsen)	< 8	µg/l	10	10	unbedenklich
B (Bor)	< 10	µg/l	1000	1000	unbedenklich
Ba (Barium)	4,4	µg/l	1000	1000	unbedenklich
Cd (Cadmium)	< 1	µg/l	3	3	unbedenklich
Cr (Chrom)	< 2	µg/l	50	50	unbedenklich
Cu (Kupfer)	2,3	µg/l	2000 ***	2000	unbedenklich
Fe (Eisen, Fe^{2+})	433	µg/l	200	200	unbrauchbar
Mn (Mangan)	10,9	µg/l	50	100	unbedenklich
Ni (Nickel)	< 2	µg/l	20	20	unbedenklich
Pb (Blei)	< 5	µg/l	10	10	unbedenklich
U (Uran)	< 3	µg/l	10	10	unbedenklich
Zn (Zink)	< 2	µg/l	3000 ****	3000	unbedenklich

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Ormont, den 18.05.2018

Analyse-Nr.: W 10073

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Untersuchungsbefund der Wasserprobe

Bezeichnung: Andracnaroa / Madagaskar (Bach, See)
Probenahme: 15.03.18; 9:45 Uhr 1078

	Meßwerte	Einheit	Grenzwert TrinkwV.	Richtwerte Binnengewässer	Beurteilung für Binnengewässer
Weitere chemische Parameter					
O_2 (Sauerstoff) gelöst		mg/l			
CO_2 (Kohlendioxid)		mg/l	-		
Gesamt-Eisen (Fe^{2+} + Fe^{3+})		µg/l	200	200	
Gesamt-Mangan		µg/l	50	100	
Mikrobiologische Parameter					
Koloniezahl bei 22 °C		KBE/ml	100	-	
Koloniezahl bei 36 °C		KBE/ml	100	-	
Coliforme Keime		KBE/100 ml	0	-	
Escherischia coli		KBE/100 ml	0	-	
Enterokokken		KBE/100 ml	0	-	
Sensorische Prüfung					
Aussehen/Farbe	gelblich, Bodenablagerungen, Schwebepartikel				

Bewertung anhand der geprüften Parameter			
Vorgaben der TrwVO erfüllt:	nein	Eignung als Poolwasser:	nein
Eignung als Tränkewasser:	ja	Eignung als Aquarienwasser	nein
Eignung als Gießwasser:	ja		

Die Trübung ist erhöht. Wir empfehlen die Untersuchung auf Gesamt-Eisen und Bakterien.

* Die WHO hat für Kinder einen Grenzwert von 10 mg/Liter festgelegt. EU-Nitratrichtwert: 25 mg/l.

** Für Säuglinge ist natriumarmes Wasser mit weniger als 20 mg Na/Liter empfehlenswert.

*** Die Empfehlung der Europäischen Union liegt bei 100 µg Cu/Liter für Säuglinge.

**** Die Weltgesundheitsorganisation WHO hat für Zink einen Leitwert von 3000 µg/Liter herausgegeben.

Analysemethoden: pH-Wert und Leitfähigkeit elektrometrisch. Nitrat nach DIN 13395. Chlorid argentometrisch. As mit HG-AAS. Bestimmung der übrigen Elemente mit ICP-OES nach DIN 11885. Mikrobiol. Parameter nach TrinkWV Anl. 3. / DIN EN ISO 8199.

Zur Qualitätssicherung wurden in der Meßreihe mehrere zertifizierte Referenzmaterialen mitgeführt.

Der Befund bezieht sich ausschließlich auf den Prüfgegenstand.

Dieser Befund dient nur Ihrer persönlichen Information. Er kann nicht für das Gesundheitsamt, gutachterliche Nachweise sowie juristische Auseinandersetzungen genutzt werden. Reklamation werden nur innerhalb einer Frist von zwei Wochen anerkannt.

Bitte kontaktieren Sie uns bei Fragen zum Qualitätsmangel dieser Wasserprobe (gebührenfrei). 39,41 €

Raiffeisen Laborservice
Raiffeisen Rhein-Ahr-Eifel Handelsgesellschaft mbH

Raiffeisen-Laborservice
Ulmenstr. 4
54597 Ormont
Ansprechpartner: Josef Lux
Tel. 06557/9203 30 (für Rückfragen)
eMail: Josef.Lux@raiffeisenservice.de
www.Raiffeisen-Laborservice.de

Raiffeisen-Laborservice, Ulmenstr. 4, 54597 Ormont

An
DKFZ Abt. Epigenetik (130)
Im Neuenheimer Feld 280
69120 Heidelberg

Ormont, den 18.05.2018

Analyse-Nr.: **W 10073**

Parameter	Einheit	Ihr Meßwert	Eignung als… Trinkwasser	Tränkewasser	Gießwasser	Fischgewässer	Poolwasser	Wärmepumpe
pH-Wert	-	7,50	J	J	J	J	J	J
Leitfähigkeit	µS	29	J	J	J	J	J	J
Trübung	NTU	**3,25**	N	J	J	J	N	N
Gesamthärte	° dH	0,3						
Carbonathärte/Hydrogencabonat	° KH/mg/l							
Sauerstoff gelöst	mg/l							
Nitrat	mg/l	0,18	J	J	J	J	J	J
Nitrit	mg/l							
Ammonium	mg/l							
Sulfat	mg/l							
Chlorid	mg/l	3,8	J	J	J	J	J	J
PO4 (Phosphat)	mg/l	< 0,05	J	J	J	J	J	J
Ca (Calcium)	mg/l	1,3	J	J	J	J	J	J
K (Kalium)	mg/l	0,0	J	J	J	J	J	J
Mg (Magnesium)	mg/l	0,3	J	J	J	J	J	J
Na (Natrium)	mg/l	1,7	J	J	J	J	J	J
Sr (Strontium)	mg/l	0,01	J	J	J	J	J	J
Al (Aluminium)	µg/l	76,7	J	J	J	J	J	J
As (Arsen)	µg/l	< 8	J	J	J	J	J	J
B (Bor)	µg/l	< 10	J	J	J	J	J	J
Ba (Barium)	µg/l	4,4	J	J	J	J	J	J
Cd (Cadmium)	µg/l	< 1	J	J	J	J	J	J
Cr (Chrom)	µg/l	< 2	J	J	J	J	J	J
Cu (Kupfer)	µg/l	2,3	J	J	J	J	J	J
Fe (Eisen)	µg/l	**433**	N	J	J	N	N	N
Mn (Mangan)	µg/l	10,9	J	J	J	J	J	J
Ni (Nickel)	µg/l	< 2	J	J	J	J	J	J
Pb (Blei)	µg/l	< 5	J	J	J	J	J	J
U (Uran)	µg/l	< 3	J	J	J	J	J	J
Zn (Zink)	µg/l	< 2	J	J	J	J	J	J
Koloniezahl bei 22 °C	KBE/ml							
Koloniezahl bei 36 °C	KBE/ml							
Coliforme Keime	KBE/100ml							
Escherichia coli	KBE/100ml							
Enterokokken	KBE/100ml							

Beurteilung anhand der geprüften Parameter. Falls es sich bei Ihrer Probe um eine Stagnations-Leitungswasserprobe handelt, gelten die obigen Hinweise zu den Schwermetallen nur für Trink- und Tränkewasser.

N = NEIN, Qualitätsmangel

J = JA, Qualität entspricht den Richtwerten bzw. gesetzlichen Bestimmungen.

Seite 3 von 3

Figure 29: Report of water analysis from Andragnaroa (sampling 15.03.18). Analysis performed by Raiffeisen Laborservice (Ormont, Germany).

Raiffeisen Laborservice
Raiffeisen Rhein-Ahr-Eifel Handelsgesellschaft mbH

Raiffeisen-Laborservice
Ulmenstr. 4
54597 Ormont
Ansprechpartner: Dr. Diana Finken
Tel. 06557/9203 38 (für Rückfragen)
eMail: Diana.Finken@Raiffeisen-Laborservice.de
www.Raiffeisen-Laborservice.de

Raiffeisen-Laborservice, Ulmenstr. 4, 54597 Ormont

An
Sina Tönges DKFZ Abt. Epigenetik (130)
Im Neuenheimer Feld 280
69120 Heidelberg

Ormont, den 06.09.2019

➔ **Analyse-Nr.: W 16846**

Untersuchungsbefund der Wasserprobe

Bezeichnung: Ihosy (Bach, See)
Probenahme: 02.04.19; 10 Uhr 4215

	Meßwerte	Einheit	**Grenzwert** TrinkwV. 2011	**Richtwerte** Binnengewässer	**Beurteilung für** Binnengewässer
Physikalisch-chemische Parameter					
Eingangstemperatur	14,7	°C	-		-
pH-Wert (bei 20,6°C)	6,94		6,5 - 9,5	6,5 - 8,0	schwach sauer
Redoxpotential (ORP)		mV	kein Grenzwert		
Leitfähigkeit bei 20 °C	87	µS/cm	2500	800	unbedenklich
Trübung	29	NTU	1,0	3,0	stark getrübt
Chemische Parameter (gelöste Stoffe)					
Gesamthärte	1,5	° dH	= 0,27 mmol Erdalkaliionen/Liter		weich
Säurekapazität bis pH 4,3		mmol/l			
Hygrogencarbonat		mg/l		6 - 8 °dKH	
Nitrat	0,08	mg/l	50 *	20	unbedenklich
Nitrit		mg/l	0,5	0,05	
Ammonium		mg/l	0,5	0,5	
Sulfat		mg/l	250	250	
Chlorid	3,5	mg/l	250		unbedenklich
PO_4 (Phosphat)	< 0,05	mg/l	6,7	1	unbedenklich
Ca (Calcium)	5,3	mg/l	kein Grenzwert		unbedenklich
K (Kalium)	2,9	mg/l	kein Grenzwert	175	unbedenklich
Mg (Magnesium)	3,5	mg/l	kein Grenzwert	250	unbedenklich
Na (Natrium)	6,5	mg/l	200 **	200	unbedenklich
Sr (Strontium)	0,04	mg/l	kein Grenzwert		unbedenklich
Al (Aluminium)	354	ug/l	200	500	leicht erhöht
As (Arsen)	< 8	µg/l	10	10	unbedenklich
B (Bor)	9,6	µg/l	1000	1000	unbedenklich
Ba (Barium)	29,4	µg/l	1000	1000	unbedenklich
Cd (Cadmium)	< 1	µg/l	3	3	unbedenklich
Cr (Chrom)	< 2	µg/l	50	50	unbedenklich
Cu (Kupfer)	2,7	µg/l	2000 ***	2000	unbedenklich
Fe (Eisen, Fe^{2+})	138	µg/l	200	200	leicht erhöht
Mn (Mangan)	1,5	µg/l	50	100	unbedenklich
Ni (Nickel)	< 2	µg/l	20	20	unbedenklich
Pb (Blei)	< 5	µg/l	10	10	unbedenklich
U (Uran)	< 3	µg/l	10	10	unbedenklich
Zn (Zink)	< 2	µg/l	3000 ****	3000	unbedenklich

Raiffeisen Laborservice
Raiffeisen Rhein-Ahr-Eifel Handelsgesellschaft mbH

Raiffeisen-Laborservice
Ulmenstr. 4
54597 Ormont
Ansprechpartner: Dr. Diana Finken
Tel. 06557/9203 38 (für Rückfragen)
eMail: Diana.Finken@Raiffeisen-Laborservice.de
www.Raiffeisen-Laborservice.de

Raiffeisen-Laborservice, Ulmenstr. 4, 54597 Ormont
An
Sina Tönges DKFZ Abt. Epigenetik (130)
Im Neuenheimer Feld 280
69120 Heidelberg

Ormont, den 06.09.2019
Analyse-Nr.: W 16846

Untersuchungsbefund der Wasserprobe

Bezeichnung: Ihosy (Bach, See)
Probenahme: 02.04.19; 10 Uhr 4215

	Meßwerte	Einheit	Grenzwert TrinkwV. 2011	Richtwerte Binnengewässer	Beurteilung für Binnengewässer
Weitere chemische Parameter					
DOC (gelöster org. Kohlenstoff)	3,63	mg/l	ohne anormale Veränderung		Ziel: < 7
O_2 (gelöster Sauerstoff)		mg/l			
CO_2 (Kohlendioxid)		mg/l	-		
Gesamt-Eisen (Fe^{2+} + Fe^{3+})		µg/l	200	200	
Gesamt-Mangan		µg/l	50	100	
Mikrobiologische Parameter					
Koloniezahl bei 22 °C		KBE/ml	100	-	
Koloniezahl bei 36 °C		KBE/ml	100	-	
Coliforme Keime		KBE/100 ml	0	-	
Escherischia coli		KBE/100 ml	0	-	
Enterokokken		KBE/100 ml	0	-	
Sensorische Prüfung / Bemerkungen					
Aussehen/Farbe	gelbbräunlich, Schwebepartikel, Bodenablagerungen				

Bewertung anhand der geprüften Parameter			
Vorgaben d. TrinkwV erfüllt:	nein	Eignung als Poolwasser:	nein
Eignung als Tränkewasser:	nein	Eignung als Aquarienwasser	nein
Eignung als Gießwasser:	ja		

Die Trübung ist erhöht. Wir empfehlen die Untersuchung auf Gesamt-Eisen und Bakterien.

* Die WHO hat für Kinder einen Grenzwert von 10 mg/Liter festgelegt. EU-Nitratrichtwert: 25 mg/l.

** Für Säuglinge ist natriumarmes Wasser mit weniger als 20 mg Na/Liter empfehlenswert.

*** Die Empfehlung der Europäischen Union liegt bei 100 µg Cu/Liter für Säuglinge.

**** Die Weltgesundheitsorganisation WHO hat für Zink einen Leitwert von 3000 µg/Liter herausgegeben.

Analysemethoden: pH-Wert und Leitfähigkeit elektrometrisch. Nitrat nach DIN 13395. Chlorid argentometrisch. As mit HG-AAS. Bestimmung der übrigen Elemente mit ICP-OES nach DIN 11885. Mikrobiol. Parameter nach TrinkWV Anl. 3. / DIN EN ISO 8199.

Zur Qualitätssicherung wurden in der Meßreihe mehrere zertifizierte Referenzmaterialen mitgeführt.

Der Befund bezieht sich ausschließlich auf den Prüfgegenstand.

Dieser Befund dient nur Ihrer persönlichen Information. Er kann nicht für das Gesundheitsamt, gutachterliche Nachweise sowie juristische Auseinandersetzungen genutzt werden. Reklamation werden nur innerhalb einer Frist von zwei Wochen anerkannt.

Bitte kontaktieren Sie uns bei Fragen zum Qualitätsmangel dieser Wasserprobe (gebührenfrei). 59,31 €

Raiffeisen Laborservice
Raiffeisen Rhein-Ahr-Eifel Handelsgesellschaft mbH

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An
Sina Tönges DKFZ Abt. Epigenetik (130)
Im Neuenheimer Feld 280
69120 Heidelberg

Ormont, den 06.09.2019
Analyse-Nr.: **W 16846**

Parameter	Einheit	Ihr Meßwert	Eignung als… Trinkwasser	Tränkewasser	Gießwasser	Fischgewässer	Poolwasser	Wärmepumpe
pH-Wert	-	6,94	J	J	J	J	J	N
Leitfähigkeit	µS	87	J	J	J	J	J	J
Trübung	NTU	29	N	N	J	N	N	N
Gesamthärte	° dH	1,5						
Carbonathärte/Hydrogencabonat	° KH/mg/l							
Sauerstoff gelöst	mg/l							
Nitrat	mg/l	0,08	J	J	J	J	J	J
Nitrit	mg/l							
Ammonium	mg/l							
Sulfat	mg/l							
Chlorid	mg/l	3,5	J	J	J	J	J	J
PO4 (Phosphat)	mg/l	< 0,05	J	J	J	J	J	J
Ca (Calcium)	mg/l	5,3	J	J	J	J	J	J
K (Kalium)	mg/l	2,9	J	J	J	J	J	J
Mg (Magnesium)	mg/l	3,5	J	J	J	J	J	J
Na (Natrium)	mg/l	6,5	J	J	J	J	J	J
Sr (Strontium)	mg/l	0,04	J	J	J	J	J	J
Al (Aluminium)	µg/l	353,9	N	N	J	N	N	N
As (Arsen)	µg/l	< 8	J	J	J	J	J	J
B (Bor)	µg/l	9,6	J	J	J	J	J	J
Ba (Barium)	µg/l	29,4	J	J	J	J	J	J
Cd (Cadmium)	µg/l	< 1	J	J	J	J	J	J
Cr (Chrom)	µg/l	< 2	J	J	J	J	J	J
Cu (Kupfer)	µg/l	2,7	J	J	J	J	J	J
Fe (Eisen)	µg/l	138	J	J	J	J	N	J
Mn (Mangan)	µg/l	1,5	J	J	J	J	J	J
Ni (Nickel)	µg/l	< 2	J	J	J	J	J	J
Pb (Blei)	µg/l	< 5	J	J	J	J	J	J
U (Uran)	µg/l	< 3	J	J	J	J	J	J
Zn (Zink)	µg/l	< 2	J	J	J	J	J	J
Koloniezahl bei 22 °C	KBE/ml							
Koloniezahl bei 36 °C	KBE/ml							
Coliforme Keime	KBE/100ml							
Escherichia coli	KBE/100ml							
Enterokokken	KBE/100ml							

Beurteilung anhand der geprüften Parameter. Falls es sich bei Ihrer Probe um eine Stagnations-Leitungswasserprobe handelt, gelten die obigen Hinweise zu den Schwermetallen nur für Trink- und Tränkewasser.

N = NEIN, Qualitätsmangel

J = JA, Qualität entspricht den Richtwerten bzw. gesetzlichen Bestimmungen.

Seite 3 von 3

Figure 30: Report of water analysis from Ihosy (sampling 02.04.19). Analysis performed by Raiffeisen Laborservice (Ormont, Germany).

Landesanstalt für Umwelt, Messung und Naturschutz
Baden-Württemberg

LUBW

Badegewässerprofil 26.07.2017

Moosweiher DEBW_PR_0294

Stammdaten

BW-Identifikationsnummer: *DEBW_PR_0294*

See-Identifikationsnummer: 10858

Nuts-Code:
R18310000108311001

Profil erstellt am:
12.05.2016

Name des Badegewässers:
Moosweiher

EU-Anmeldung (nach Stichjahr 2008):

Name des Gewässers:

EU-Abmeldung (endgültig):

Gewässerart:
See

EU-Badegewässer im Stichjahr 2008:
NEIN

Gemeinde:
Freiburg im Breisgau (8311000)

Bezeichnung in der Papierkarte:
FRS 1

Zuständigkeit:
Freiburg im Breisgau, Stadt (8311)

Kurzbezeichnung:
frs001

Sonstiges:

Spezielle Badegewässerdaten

Art des Badegewässers:
Baggersee

Geographische Länge in Grad:
7.80430

Wassererneuerung bei Seen:
Grundwasserzustrom bzw. -durchströmung

Geographische Breite in Grad:
48.03051

Größe des Einzugsgebietes bei Fliessgewässern:
keine Angaben

Höhenwert in Meter:

Trophiestufe:
keine Angaben

Maximale Wassertiefe in Meter:
8

Salzgehalt:
Süßwasser: < 0,5

Sichttiefe im Mittel:
> 2 - 5 m

Ökologische Zustandsklasse (Trophie) gem. WRRL:
keine Angaben

Beschaffenheit des Uferbereichs:
Kies, Wiese

Sonstiges:

Qualitätswerte der letzten 5 Jahre

2012	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)
2013	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)
2014	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)
2015	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)
2016	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)

Infrastruktur

Beschreibung:

Anfahrt:
für Fußgänger und Radfahrer über vorhandenes Wegenetz, keine Zufahrt für PKWs

Ergänzende Angaben:

Nichtfachliche Beschreibung:
Naherholungsgebiet für Stadtteil Mooswald

Piktogramme:
Angeln, Baden ohne Aufsicht, Rudern, Spielen

Mögliche Gefahren bzw. Verschmutzungen

Indirekte Verschmutzung

Name des Fliessgewässers:

Sonstiges:

Kläranlagen im Einzugsbereich:

Niederschlagswasser:
nicht relevant

Direkte Verschmutzung

Kläranlagen:
NEIN

Niederschlagswasser:
NEIN

Ablauf von Fischteichanlagen:
NEIN

Fischzucht:
NEIN

Versiegelte Flächen, Straßen:
NEIN

Hafen/Liegeplätze:
NEIN

Wohngebiete:
NEIN

Industriegebiet:
NEIN

Campingplätze:
NEIN

Abläufe von landwirtschaftlichen Nutzflächen:

Sonstiges:

Sonstige Verschmutzung

Gefahr durch Massenvermehrung durch Phytoplankton/Cyanobakterien/Blaualgen in den letzten Jahren:
Keine beobachtet

Gefahr der Massenvermehrung von Makroalgen (z.B. Schlingpflanzen, etc.):
Keine beobachtet

Massenauftreten von Vögeln:
keine beobachtet

Gefahr, an einer Badedermatitis zu erkranken, verursacht durch Zerkarien (hier: Enten-Bilharziose):
gering

Sonstiges:

Voraussichtliche Dauer:

Während der kurzzeitigen Verschmutzung ergriffene Bewirtschaftungsmaßnahmen:

Angabe der für diese Maßnahmen zuständigen Stelle und Einzelheiten der Kontaktaufnahme:

Zeitplan für die Beseitigung der Verschmutzungsursachen:

Figure 31: Report of the water analysis from Lake Moosweiher (12.05.2016). Report downloaded from the website of the "Landesamt für Umwelt, Messung und Naturschutz Baden-Württemberg" (https://rips-dienste.lubw.baden-wuerttemberg.de/rips/ripsservices/apps/badegewaesser/steckbrief/select.aspx, accessed 26.07.2017)

Landesanstalt für Umwelt, Messung und Naturschutz
Baden-Württemberg

LUBW

Badegewässerprofil 26.07.2017

Baggersee Kirchentellinsfurt DEBW_PR_0234

Stammdaten

BW-Identifikationsnummer: ***DEBW_PR_0234***

See-Identifikationsnummer: 6984

Nuts-Code:
R18420000608416001

Profil erstellt am:
17.02.2017

Name des Badegewässers:
Baggersee Kirchentellinsfurt

EU-Anmeldung (nach Stichjahr 2008):

Name des Gewässers:
Epplesee

EU-Abmeldung (endgültig):

Gewässerart:
See

EU-Badegewässer im Stichjahr 2008:
JA

Gemeinde:
Kirchentellinsfurt (8416022)

Bezeichnung in der Papierkarte:
TÜ 1

Zuständigkeit:
Tübingen (8416)

Kurzbezeichnung:
tue001

Sonstiges:

Spezielle Badegewässerdaten

Art des Badegewässers:
Baggersee

Geographische Länge in Grad:
9.15335

Wassererneuerung bei Seen:
Grundwasserzustrom bzw. -durchströmung

Geographische Breite in Grad:
48.54355

Größe des Einzugsgebietes bei Fliessgewässern:
keine Angaben

Höhenwert in Meter:
304

Trophiestufe:
keine Angaben

Maximale Wassertiefe in Meter:
5

Salzgehalt:
Süßwasser: < 0,5

Sichttiefe im Mittel:
1 - 2 m

Ökologische Zustandsklasse (Trophie) gem. WRRL:
keine Angaben

Beschaffenheit des Uferbereichs:
Wiese

Sonstiges:
Steilufer, kein Flachbereich

Qualitätswerte der letzten 5 Jahre

2012	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)
2013	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)
2014	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)
2015	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)
2016	Ausgezeichnete Qualität (Perzentilbeurteilung gemäß RL 2006/7/EG)

Infrastruktur

Beschreibung:

-nur ein sehr kleiner Flachbereichsabschnitt
-an der Uferböschung bis zu 2,00 m Tiefe
-WC geöffnet wenn Parkplatz bewirtschaftet wird
-keine Badeaufsicht verfügbar!
-Hunde sind am See nicht erlaubt
-Campen ist nicht gestattet
-am See sind keine Duschen verfügbar
-weitere Informationen über: www.kirchentellinsfurt.de

Anfahrt:

-B 27 Abfahrt Kirchentellinsfurt

Ergänzende Angaben:

- See wird auch von Seglern außerhalb des Badebereiches genutzt

Nichtfachliche Beschreibung:

-Grillen ist nur an den Grillstellen zulässig
-Wildparken wird geahndet
-direkt am Zugang zum Badestrand gebührenpflichtiger Parkplatz (keine gebührenfreie Parkplätze direkt am See)
-Höhe der Parkgebühren unter: http://www.kirchentellinsfurt.de/de/Freizeit-und-Kultur/Baggersee abrufbar
-in 1,5 km Entfernung vom Badestrand nächster gebührenfreier Parkplatz

Piktogramme:

Angeln, Baden ohne Aufsicht, Parken, Segeln, Surfen, WC

Mögliche Gefahren bzw. Verschmutzungen

Indirekte Verschmutzung

Name des Fliessgewässers:

Neckar

Sonstiges:

Neckarhochwasser oder Überschwemmungen der angrenzenden Wiesen und Einleitung in den See bei Starkregen möglich.

Kläranlagen im Einzugsbereich:

Niederschlagswasser:

relevant

Direkte Verschmutzung

Kläranlagen:
NEIN

Niederschlagswasser:
JA

Ablauf von Fischteichanlagen:
NEIN

Fischzucht:
NEIN

Versiegelte Flächen, Straßen:
NEIN

Hafen/Liegeplätze:
NEIN

Wohngebiete:
NEIN

Industiregebiet:
NEIN

Campingplätze:
NEIN

Abläufe von landwirtschaftlichen Nutzflächen:

Sonstiges:

Sonstige Verschmutzung

Gefahr durch Massenvermehrung durch Phytoplankton/Cyanobakterien/Blaualgen in den letzten Jahren:
Keine beobachtet

Gefahr der Massenvermehrung von Makroalgen (z.B. Schlingpflanzen, etc.):
Keine beobachtet

Massenauftreten von Vögeln:
keine beobachtet

Gefahr, an einer Badedermatitis zu erkranken, verursacht durch Zerkarien (hier: Enten-Bilharziose):
Keine beobachtet

Sonstiges:

Voraussichtliche Dauer:

Während der kurzzeitigen Verschmutzung ergriffene Bewirtschaftungsmaßnahmen:

Angabe der für diese Maßnahmen zuständigen Stelle und Einzelheiten der Kontaktaufnahme:

Zeitplan für die Beseitigung der Verschmutzungsursachen:

Figure 32: Report of the water analysis from Baggersee Epple (17.02.2017). Report downloaded from the website of the "Landesamt für Umwelt, Messung und Naturschutz Baden-Württemberg" (https://rips-dienste.lubw.baden-wuerttemberg.de/rips/ripsservices/apps/badegewaesser/steckbrief/select.aspx, accessed 26.07.2017)

List of publications with personal contributions

Andriantsoa, R., Tönges, S., Panteleit, J., Theissinger, K., Carneiro, V.C., Rasamy, J., and Lyko, F. (2019). Ecological plasticity and commercial impact of invasive marbled crayfish populations in Madagascar. BMC Ecol. *19*, 1–10.

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