Review and Perspectives

Investigating the genetic and epigenetic basis of big biological questions with the new crayfish model *Procambarus virginalis*

GÜNTER VOGT

Centre of Organismal Studies (COS), University of Heidelberg, Im Neuenheimer Feld 230, 69120 Heidelberg, Germany, gunter.vogt@web.de

Abstract

In the last 15 years considerable attempts have been undertaken to develop the obligately parthenogenetic marbled crayfish *Procambarus virginalis* as a new model in biology. Its main advantage is the production of large numbers of offspring that are genetically identical to the mother, making this crustacean particularly suitable for research in epigenetics. Now, a draft genome, transcriptome and genome-wide methylome are available opening new windows for research. In this article, I summarize the biological advantages and genomic and epigenetic features of marbled crayfish and, based on first promising data, discuss what this new model could contribute to answering of "big" biological questions. Genome mining is expected to reveal new insights into the genetic specificities of decapod crustaceans, the genetic basis of arthropod reproduction, moulting and immunity, and more general topics such as the genetic underpinning of adaptation to fresh water, omnivory, biomineralization, sexual system change, behavioural variation, clonal genome evolution, and resistance to cancer. Epigenetic investigations with the marbled crayfish can help clarifying the role of epigenetic mechanisms in gene regulation, tissue specification, adult stem cell regulation, cell ageing, organ regeneration and disease susceptibility. Marbled crayfish is further suitable to elucidate the relationship between genetic and epigenetic variation, the transgenerational inheritance of epigenetic signatures and the contribution of epigenetic phenotype variation to the establishment of social hierarchies, environmental adaptation and speciation. These issues can be tackled by experiments with highly standardized laboratory lineages, comparison of differently adapted wild populations and the generation of genetically and epigenetically edited strains.

Keywords. Marbled crayfish; genomics; epigenetics; DNA methylation; immunity; regeneration; disease susceptibility; cancer resistance; environmental adaptation; speciation

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1. Introduction

The parthenogenetic all-female marbled crayfish or Marmorkrebs was introduced by us to science in the year 2003 (Scholtz *et al.* 2003). Its high potential for research was recognized from the very beginning, mainly because of the advantage of producing high numbers of genetically identical offspring. Meanwhile, more than 150 scientific papers, book chapters and theses are published on this animal, covering topics like morphology, physiology, neurobiology, behaviour, stem cell biology, ageing, toxicology, ecology and evolution (ref. in Faulkes 2016; Scholtz 2016; Vogt 2016, 2017a; Marmorkrebs web page: http://faculty.utrgv.edu/zen.faulkes/marmorkrebs/). Recently, a draft genome, transcriptome and genome-wide methylome have been completed (Falckenhayn 2016; Gutekunst 2017;

Gutekunst et al. 2018) setting the stage for numerous new research directions, particularly in genomics and epigenetics. The Division of Epigenetics at the German Cancer Research Center (DKFZ, Heidelberg, Germany) has established a Marmorkrebs Genome Portal, which provides a blast server and a manual curation tool for colleagues interested to search the marbled crayfish genome and to improve annotation (http://marmorkrebs.dkfz.de/).

Presently, some 20 research groups work with marbled crayfish coming mainly from zoology and ecology. Geneticists and epigeneticists have shown interest in this emerging model but mostly avoided stepping in because of unfamiliarity with its biology and missing genetic and epigenetic baseline data. The present review article attempts to close these gaps as far as possible and provides perspectives on the use of marbled crayfish for research on topical issues of biology and medicine. The article starts with a detailed account of the biological features and advantages of marbled crayfish, which one must know to optimally exploit this animal for research. Then, the genome, transcriptome and methylome are characterized. Thereafter, I will discuss major issues of biology to which marbled crayfish could contribute. These examples concern molecular biology, cell biology, development, physiology, reproduction, behaviour, pathology, ecology and evolution. Some examples are primarily of interest for carcinologists and arthropodologists but most examples are of more general interest. First marbled crayfish data are already available for several of the discussed topics indicating that it is worth to intensify these research directions. The article ends with suggestions on the generation of genetic and epigenetic mutants that help to identify the function of genes and their epigenetic modifications.

2. Biology of marbled crayfish

The marbled crayfish *Procambarus virginalis* (Crustacea: Decapoda: Astacidea: Cambaridae) (Figure 1A) is the only one of ~15.000 decapod crustaceans that reproduces by obligately apomictic parthenogenesis. All individuals are triploid females. The species was detected in the mid-1990s in the German pet trade (Werner 1998; Scholtz *et al.* 2003) and has since established wild populations in several countries on three continents as the result of releases (Chucholl 2016). Indigenous populations in the wild are unknown. Aside of these curiosities marbled crayfish has normal crayfish features as revealed by investigations on morphology, development, physiology, biochemistry, behaviour and ecology (ref. in Vogt 2008a, 2011, 2016; Faulkes 2016; Scholtz 2016).

2.1 Taxonomy, evolution and biogeography

Morphological criteria and the analysis of the mitochondrial *COI* and *12S rRNA* genes revealed a particularly close relationship of marbled crayfish to slough crayfish *Procambarus fallax* (Hagen 1870), which is native to Florida and southern Georgia (Scholtz *et al.* 2003; Martin *et al.* 2010). Therefore, marbled crayfish was considered as a parthenogenetic form of slough crayfish and provisionally named *Procambarus fallax* forma *virginalis* (Martin *et al.* 2010). We have established reproductive isolation and substantial genetic and life history differences between marbled crayfish and *P. fallax* and suggested to consider marbled crayfish as a species on its own named *Procambarus virginalis* (Vogt *et al.* 2015). A formal species description was recently published by Lyko (2017).

Genetic and morphological evidences indicate that triploidy in marbled crayfish has arisen by autopolyploidization (Vogt *et al.* 2015; Martin *et al.* 2016a) and not by hybridization between two closely related species, which is more common in the animal kingdom. Spontaneous triploidization of the whole genome is not very rare in animals but usually results in sterile offspring and remains undetected because it has no population or evolutionary effect (Glover *et al.* 2015). It can originate by an abrupt cold or heat shock in a

sensitive phase of egg development. Such a temperature shock, which prevents polar body extrusion in fertilised eggs, was repeatedly used in shrimp aquaculture to produce sterile autotriploids (Piferrer *et al.* 2009). In marbled crayfish, triploidization was accompanied by the transition from sexual reproduction to parthenogenesis making it one of the very rare cases of parthenogenetically reproducing triploid animals.

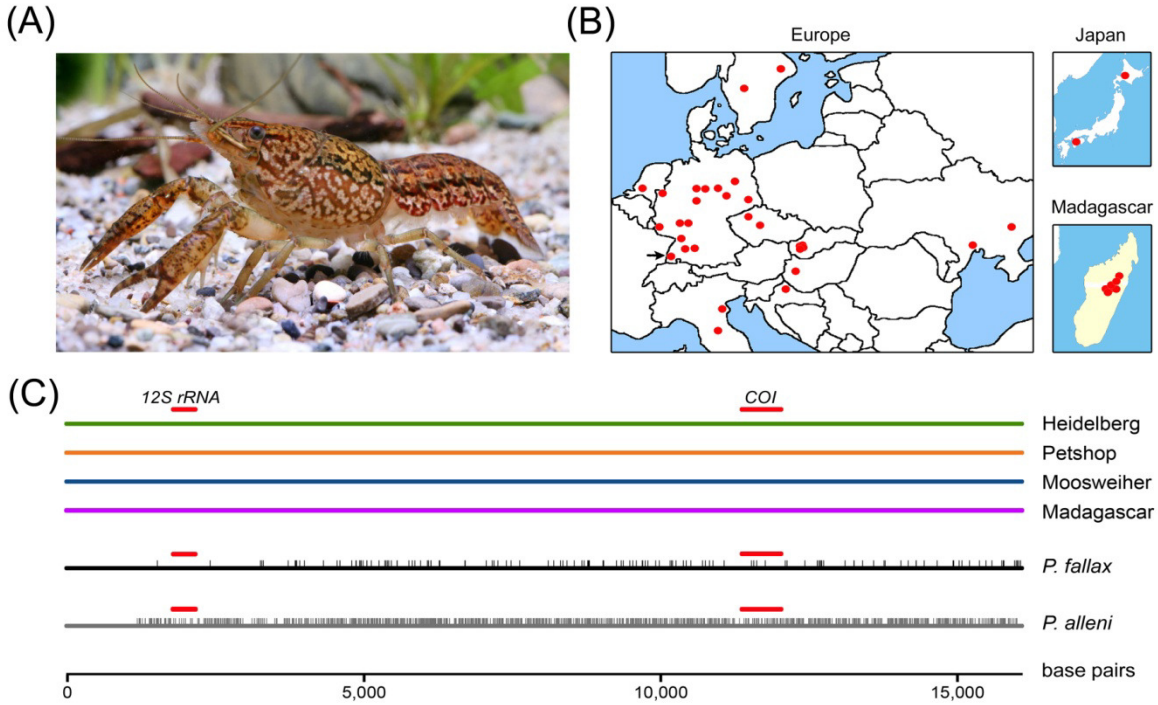

Figure 1. Morphology, biogeography and evolution of marbled crayfish *Procambarus virginalis*. (**A**) Habitus of marbled crayfish (photo by Chris Lukhaup). (**B**) Occurrence of populations in the wild. Arrow denotes Lake Moosweiher (data from Chucholl 2016, Lőkkös *et al.* 2016, Novitsky and Son 2016, Cvitanić 2017, Lipták *et al.* 2017 and Usio *et al.* 2017). (**C**) Comparison of complete mitochondrial genomes of *P. virginalis*, its parent species *P. fallax* and the congeneric *P. alleni*. The sequences of marbled crayfish from the Heidelberg and Petshop laboratory lineages and wild populations from Lake Moosweiher and Madagascar are completely identical suggesting single origin and monoclonality. The sequences of *P. fallax* and *P. alleni* differ in 144 and 1165 single nucleotide polymorphisms (vertical bars) from marbled crayfish, respectively. Horizontal red bars indicate positions of *12S rRNA* and *cytochrome oxidase subunit I* (*COI*) genes that are commonly used for phylogenetic analysis (redrawn and modified after Vogt *et al.* 2015).

Marbled crayfish populations were neither found in Florida nor southern Georgia, the distribution range of its parent species *Procambarus fallax* (Hobbs 1942, 1981; Hendrix and Loftus 2000; VanArman 2003; Van der Heiden and Dorn 2017), nor elsewhere in Northern and Central America, the distribution range of the Cambaridae (Hobbs 1989). This crayfish is so unusual that if it was out there, it should have been noticed. On the other hand, thriving populations have been found since the year 2003 in several European countries, Japan and Madagascar (Figure 1B) (Jones *et al.* 2009; Chucholl 2016). These populations are of single origin and monoclonal as shown by comparison of the *COI* sequences, complete mitochondrial genomes (Figure 1C) and nuclear microsatellite markers of specimens from laboratory lineages and wild populations (Martin *et al.* 2010; Vogt *et al.* 2015). Comparison of the *COI* sequences of specimens from the Heidelberg laboratory lineage (see chapter 3) and wild populations in Germany, Sweden, Italy, Madagascar and Japan suggest that they are all

descendants of the specimens that first appeared in 1995 in the German pet trade. They were apparently distributed by aquarists and the aquarium trade throughout the world and occasionally released into the wild. The biogeographical and genetic data prompted us to hypothesize that marbled crayfish has originated only once some 22+ years ago, perhaps in captivity by a temperature shock during transportation or storage (Vogt *et al.* 2015).

2.2 Characteristics and advantages

Marbled crayfish share some basic characteristics with other animal models such as easy culture, high fertility, breeding at any time of the year, easy accessibility of all life stages, adaptability to a wide spectrum of experimental conditions and high tolerance to physical manipulation. Special features are parthenogenetic reproduction, indeterminate growth, short-germ development, stereotyped cell lineage in early development, possession of numerous morphological traits easy to analyse, step-wise alteration of these phenotypic traits by moulting and broad behavioural repertoire (Vogt 2008a, 2011).

Marbled crayfish have an adult size (anterior tip of cephalothorax to posterior margin of pleon) of 4-11 cm (Figure 2A), which is suitable for both mass culture and biochemical and genomic analyses of individuals and single organs. The generation time is 6-7 months, which is longer than in some traditional animal models such as the nematode *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster*, mouse *Mus musculus* and rat *Rattus norvegicus*, within the range of zebrafish *Danio rerio* and chicken *Gallus gallus domesticus* and shorter than in clawed frog *Xenopus laevis*. Maximum longevity is 4.5 years but most adults live for 2-3 years (Vogt *et al.* 2004; Seitz *et al.* 2005; Vogt 2008a, b, 2010). The life cycle of marbled crayfish consists of embryonic, juvenile and adult periods (Figure 2A-E) (Vogt *et al.* 2004). The embryonic period starts with oviposition and ends with hatching of the first juvenile stage. The juvenile period includes approximately 15 stages with interspersed moults. The adult life period begins with first reproduction and is characterized by alternating reproduction and growth phases, which are separated by moults.

Obligately apomictic parthenogenesis results in all-female progeny genetically identical with their mother and among each other as shown by microsatellite analysis (Martin *et al.* 2007; Vogt *et al.* 2008, 2015). The high number of clutch-mates (>50) enables intense experimentation with isogenic specimens, which is particularly advantageous for epigenetics research. Despite genetic identity each individual is identified by a unique marmoration pattern (Figure 2A) resulting from stochastic developmental variation of pigmentation (Vogt 2015a). Marbled crayfish possess a rigid exoskeleton and numerous sensory setae easy to measure and count, principally facilitating correlations between phenotype, genes and epigenetic marks. They can regenerate damaged and lost appendages in all postembryonic life stages resulting in organs of different age in the same individual.

In the laboratory, marbled crayfish can reproduce all year round but peaks of egg-laying were observed around spring and autumn equinoxes (Vogt 2015b). Most females spawn twice a year but some spawn once or three times. The maximum number of clutches per female and lifetime recorded so far is seven. All eggs of a clutch are laid within less than an hour and are attached to the pleopods for brooding (Figure 2A). Clutch sizes vary between some 50 and 730 eggs depending on female size (Vogt 2010). The hatching rate can be up to 80% (Seitz *et al.* 2005; Vogt 2008b). The maximum number of hatchlings per clutch was 427 and the maximum number per female and lifetime was >800 (Vogt 2010). The duration of embryonic development (Figure 2B, C) depends on water temperature and varies from about 17 to 28 days. The embryos and first two lecithotrophic juvenile stages are permanently carried under the pleon (Figure 2D) allowing time- and stage-specific sampling. Stage-3 juveniles adhere most of the time to the maternal pleopods for shelter but leave them regularly for feeding. The

postembryonic brooding period lasts for 14-25 days, depending on environmental conditions (Vogt and Tolley 2004; Vogt 2008a, b).

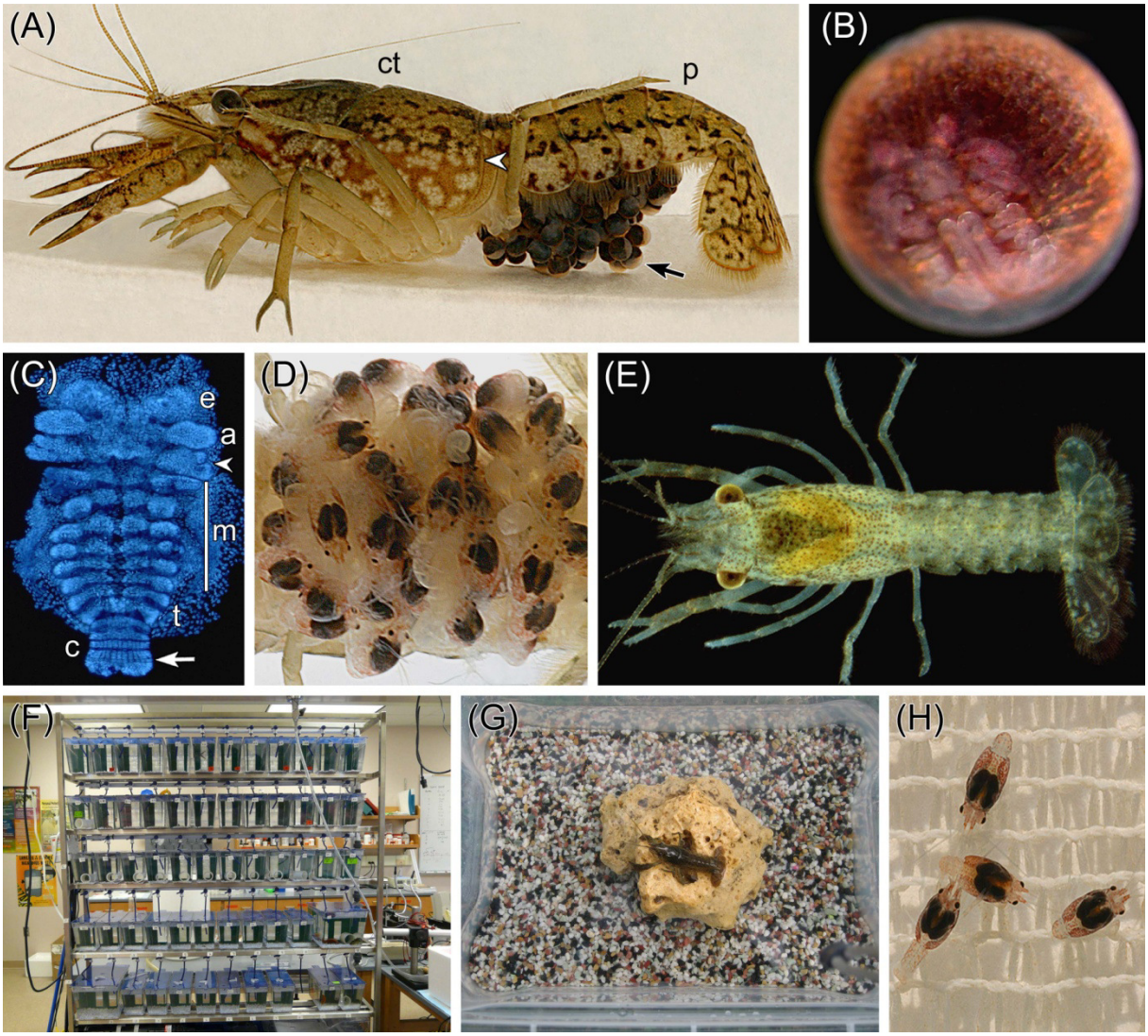

Figure 2. Biological features and rearing of marbled crayfish. (**A**) Female with embryos on pleopods (arrow). Arrowhead denotes marmoration pattern of posterio-lateral cephalothorax (ct) that enables individual recognition of specimens. p, pleon (from Vogt and Tolley 2004). (**B**) Developing embryo in the egg. (**C**) Isolated and stained embryo at 50% development illustrating short germ development and stereotyped cell lineage. The embryo consists of the segments of eyestalk (e), antenna (a), antennule (arrowhead), mouthparts (m) and first and second thoracopods (t) and the caudal papilla (c). The caudal papilla will later produce the missing three segments of the cephalothorax, five segments of the pleon and the tail fan. It shows distinct rings and rows of cells including the ectoteloblasts (arrow), which are embryonic stem cells (from Alwes and Scholtz 2006). (**D**) Stage-2 juveniles brooded on the maternal pleopods (from Vogt and Tolley 2004). (**E**) Stage 5 juvenile (from Vogt *et al.* 2008). (**F**) Stand-alone aquarium system for mass culture (from Jimenez and Faulkes 2010). (**G**) Simple setting for rearing of adults under highly controlled conditions (from Vogt *et al.* 2013). (**H**) Rearing of stage-2 juveniles in net culture system (from Vogt *et al.* 2008).

The embryos develop directly without larval stages (Vogt *et al.* 2004). At any time of development they are well visible through the egg membrane (Figure 2C) and can thus

exactly be staged and sampled at desired time points (Seitz *et al.* 2005; Alwes and Scholtz 2006). Embryogenesis is characterized by short-germ development and stereotyped cell lineage (Figure 2C). Short-germ development is the sequential addition of body segments to the cephalon anlage from a posterior growth zone over many days. This mode of morphogenesis is in contrast to long-germ development, the simultaneous appearance of all body segments, which is typical of the well established animal models. Short germ development and stereotyped cell lineage allows precise experimental interventions during morphogenesis.

Growth in marbled crayfish is indeterminate. This growth format is typical of many invertebrates and basal vertebrates and is considered phylogenetically ancestral (Hariharan *et al.* 2016). It stands in contrast to determinate growth, which is typical of fruit fly and the mammalian research models. Determinate growers reach their final size mostly at the beginning of adulthood. Indeterminate growers are particularly useful to investigate tissue homeostasis and stem cell activity until old age. Marbled crayfish show a broad behavioural repertoire including agonistic behaviours. They establish social hierarchies despite unisexuality and genetic identity of group members (Farca Luna *et al.* 2009; Vogt 2015a). These features facilitate investigations on the contribution of epigenetic mechanisms in establishing behavioural variation and social structures.

Marbled crayfish can be mass cultured in aerated tanks with shelters (Figure 2F) (Jimenez and Faulkes 2010). Such aquarium systems are commercially available for rearing of zebra fish. However, due to the ability to breathe atmospheric air they can also be kept in very simple containers without aeration (Figure 2G), if maximum standardization is necessary (Vogt 2008a; Vogt *et al.* 2015). They are best cultured at temperatures of 18–25°C. Maximum growth is obtained at 25°C and maximum survival at 20°C (Seitz *et al.* 2005). Marbled crayfish survive temperatures as low as 3°C and above 30°C for many weeks but mortality increases under such conditions and reproduction stops (Seitz *et al.* 2005; Kaldre *et al.* 2016). They are omnivorous and can be fed throughout life with commercial pellet food. Tap water is suitable as a water source (Vogt 2008a). Feeding of all life stages with pellet food and the use of tap water allows maximum standardization of the rearing conditions and prevents the introduction of diseases into the culture. Omnivory and the robustness against environmental challenges enable intense experimentation on environmental epigenetics.

The embryos and early juvenile stages can be raised either naturally on the maternal pleopods or in net culture systems (Figure 2H) and 12-well micro-plates (Vogt 2007; Vogt *et al.* 2008). For net culture, small containers should be filled to a water level of about 2 cm and be equipped with a fine-meshed net to enable attachment of the juveniles. Micro-plates are suitable for individual culture of embryos. In vitro culture facilitates research with embryonic and early juvenile stages.

A further advantage of marbled crayfish is its low protection status in animal welfare acts when compared to the vertebrate models, making experimentation much easier. At least in Germany, research with vertebrates requires formal permission and a lot of bureaucracy, whereas experiments with decapods must only be announced to the animal welfare officer. Furthermore, as an unwelcome invasive species marbled crayfish can be sampled in the wild without legal restrictions. Disadvantages of the marbled crayfish are the comparatively long generation time, the unfamiliarity of most experimental biologists with freshwater crayfish, and the scarcity of molecular and genetic tools. This article may help to overcome the latter limitation in the near future.

3. Properties of the genome, transcriptome and methylome

In the years 2003 and 2004, I have founded two laboratory lineages of marbled crayfish from single individuals at the Zoological Institute of the University of Heidelberg, Germany. These

lineages were first named A and B (Vogt *et al.* 2008) and later Heidelberg and Petshop, respectively (Vogt *et al.* 2015). The founder individual of the Heidelberg lineage originated from the oldest known marbled crayfish colony kept by Frank Steuerwald since 1995, the year of discovery of this animal. The founder specimen of the Petshop lineage was purchased from Kölle-Zoo in Ludwigshafen, Germany. Both lineages have been raised until today in the above described highly standardized settings. All animals were fed throughout life and over generations with Tetra Wafer Mix pellets only. Specimens of these disease-free lineages, which are now also cultured at the Epigenetics Department of the DKFZ in Heidelberg, were used for establishing the genome, transcriptome and methylome. These lineages may serve as reference for future genomics and epigenetics research.

3.1 Genome and transcriptome

In 2013, we have launched a project at the DKFZ to establish the complete genome sequence and genome-wide methylome of marbled crayfish (Vogt *et al.* 2013, 2015; Falckenhayn 2016; Gutekunst 2017; Lyko 2017; Gutekunst et al. 2018). I have raised and dissected most specimens mentioned in the following so the information on their life history features and functional phases is first-hand. The hepatopancreas and abdominal musculature of an approximately 3-year-old individual in reproduction phase from my Petshop lineage was used for sequencing, assembly and annotation of the reference genome (Gutekunst 2017; Gutekunst et al. 2018). The genomic data are accessible at the Marmorkrebs Genome Server of the DKFZ (http://marmorkrebs.dkfz.de). After registration the sequences can be blasted and manually curated. Meanwhile, the genomes of several further marbled crayfish from the laboratory and the wild have been fully sequenced (Gutekunst 2017; Gutekunst et al. 2018). For comparison, the genomes of males and females of *Procambarus fallax*, the parent species of marbled crayfish and the congeneric *Procambarus alleni* have been sequenced as well (Gutekunst 2017). The *P. fallax* and *P. alleni* were purchased from German aquarists and pet shops.

The statistical data of the reference genome of marbled crayfish are summarized in Table 1. Marbled crayfish were earlier shown to be triploid (Vogt *et al.* 2015; Martin *et al.* 2016a) having a haploid chromosome number of 92 (Figure 3A). Such a high chromosome value is not unusual in the Decapoda. The genome size was estimated to 3.5 Gb by k-mer analysis of the genome assembly and to 3.7 Gb by flow cytometry of haemocytes (Falckenhayn 2016; Gutekunst 2017). Comparison of nuclear genome sequences of a total length of 1.5 Mb in marbled crayfish from different populations revealed very low numbers of homozygous polymorphisms (Figure 10A). These data and the identity of the complete mitochondrial sequences of specimens from different populations (Vogt *et al.* 2015) indicate that all marbled crayfish are monoclonal and of single origin.

The genome of marbled crayfish comprises 21,772 predicted genes (Gutekunst 2017). The average gene length is 6.7 kb, with average exon and intron sizes of 253 bp and1960 bp, respectively. 1,356 genes code for the tRNAs of the 20 proteinogenic amino acids. The analysis of heterozygous sequence variants in marbled crayfish revealed a global rate of 0.53% (Gutekunst 2017), which is high compared to other sequenced genomes. Allelic frequency of sequence variants peaked at 0.33, which is in agreement with heterozygous loci in a triploid genome. For comparison, the allelic frequencies of the diploid *P. fallax* and *P. alleni* peaked at 0.5 and 1.0. Furthermore, the *P. virginalis* genome showed many more biallelic than triallelic sequence polymorphisms suggesting an AA'B genotype (Gutekunst 2017).

Repeat annotation detected 481,851 repeats covering 8.8% of the genome assembly (Gutekunst 2017). They were mainly composed of simple repeats, SINEs, LINEs and DNA transposons (Figure 3B). Repeat coverage is likely to increase in future versions of the

genome. Benchmarking with universal single-copy orthologs (BUSCO) indicated that the quality of the present version of the marbled crayfish genome assembly (version 0.4) is comparable to other, recently published arthropod genomes (Gutekunst 2017).

Table 1. Characteristics of the genome, transcriptome and methylome of marbled crayfish.

Genome and transcriptome	Methylome
genome triploid, 92 haploid chromosomes	global DNA methylation: 2.5%
whole genome seq. on Ilumina HiSeq2500	Illumina whole genome bisulfite sequencing
shotgun and long jumping distance libraries	33 Gb of sequence, 8.4x coverage
350.452 Mb of sequence, ~100x coverage	25% ubiquitous, 70% mosaic, 5% sporadic methylation
full genome assembly: 3,511 Mbp	methylation is CpG specific
genome size estimates: 3.5-3.7 Gbp	methylation is bimodal and symmetric
median scaffold size (N50): 28,228 bp	41% of genes highly methylated, 26% unmethylated
GC content: 43.31%	old, long and moderately expressed genes highly meth.
21,772 genes, 86,771 exons	housekeeping genes (HG) intensely methylated
gene length: average 6.7 kbp, max. 100 kbp	methylation of HG varies slightly between tissues
average exon: 253 bp; average intron: 1960 bp	gene bodies, exons and introns methylated
heterozygosity rate: 0.53%	repeats undermethylated
481,851 repeats, 8.8% of genome	DNA transposons highest methylation of repeat classes
transcriptome: Illumina RNAseq	evol. old repeats higher methylated than younger ones
48.6 Gb of sequence	single copies of Dnmt1, Dnmt3 and Tet enzymes
22,338 transcripts, 12,855 annotated	expression patterns of enzymes vary among tissues
4,306 predicted proteins unique	5-hydroxymethylcytosine level: 5.4-9.3 ppm

Data from Vogt *et al.* (2015), Falckenhayn (2016), Martin *et al.* (2016) and Gutekunst (2017).

The transcriptome of four pooled tissues (hepatopancreas, abdominal musculature, antennal gland and haematopoietic tissue) of a 2-year-old individual in growth phase from my Heidelberg lineage revealed 22,338 transcripts, which corresponds roughly to the numbers of predicted genes (Falckenhayn 2016; Gutekunst 2017). Benchmarking confirmed that the quality of the transcriptome was comparable to other, recently published arthropod transcriptomes. 12,855 transcripts (67.5%) could be automatically annotated.

The genome of marbled crayfish is an order of magnitude larger than the genomes of the invertebrate models *Caenorhabditis elegans* (100.2 Mb) and *Drosophila melanogaster* (122.6 Mb), in the same order of magnitude as the genomes of the vertebrate models *Danio rerio* (1.46 Gb), *Mus musculus* (2.8 Gb), *Rattus norvegicus* (3.04 Gb) and *Xenopus laevis* (3.1 Gb) and of similar size as the human genome (3.32 Gb). It is close to the means of crustaceans (3.1 Gb) and mammals (3.5 Gb), larger than the means of other species-rich animal groups like insects (1.6 Gb), molluscs (1.8 Gb) and fishes (1.9 Gb) but smaller than the means of amphibians (16.7 Gb) (Gregory *et al.* 2007; Vogt 2017b). The estimated number of genes is close to the published values of humans, mouse, *Caenorhabditis elegans* and *Daphnia pulex* but higher than in most sequenced insects (Hou and Lin 2009; Pertea and Salzberg 2010; Ye *et al.* 2017). Thus, the marbled crayfish seems to be a rather good animal representative with respect to genome size and gene number but is special with respect to chromosome number.

2.2 Genome-wide methylome

The features of the marbled crayfish methylome are summarized in Table 1. Global DNA methylation (5-methylcytosine per total cytosine) is about 2.5% as shown by mass spectrometry (Vogt *et al.* 2015). The reference methylome was obtained by whole genome bisufite sequencing on an Illumina platform of the hepatopancreas and abdominal musculature of a 2-year-old individual from my Heidelberg lineage (same specimen as used for transcriptome analysis) (Falckenhayn 2016; Gutekunst 2017). Analysis of the methylation pattern of the 20 longest scaffold sequences established that about 25% of the sequences were

ubiquitously methylated, 70% were mosaically methylated and 5% were sporadically methylated (Figure 3C) (Falckenhayn 2016). Ubiquitous DNA methylation is typical of vertebrates, whereas invertebrates usually show mosaic methylation or sporadic methylation (Albalat *et al.* 2012; Breiling and Lyko 2015). In the ubiquitously methylated genomes of vertebrates, more than 80% of the CpG dinucleotides are methylated and the unmethylated sites are typically associated with active regulatory elements. Mosaic-type methylation is characterized by the alternation of domains of methylated and unmethylated DNA.

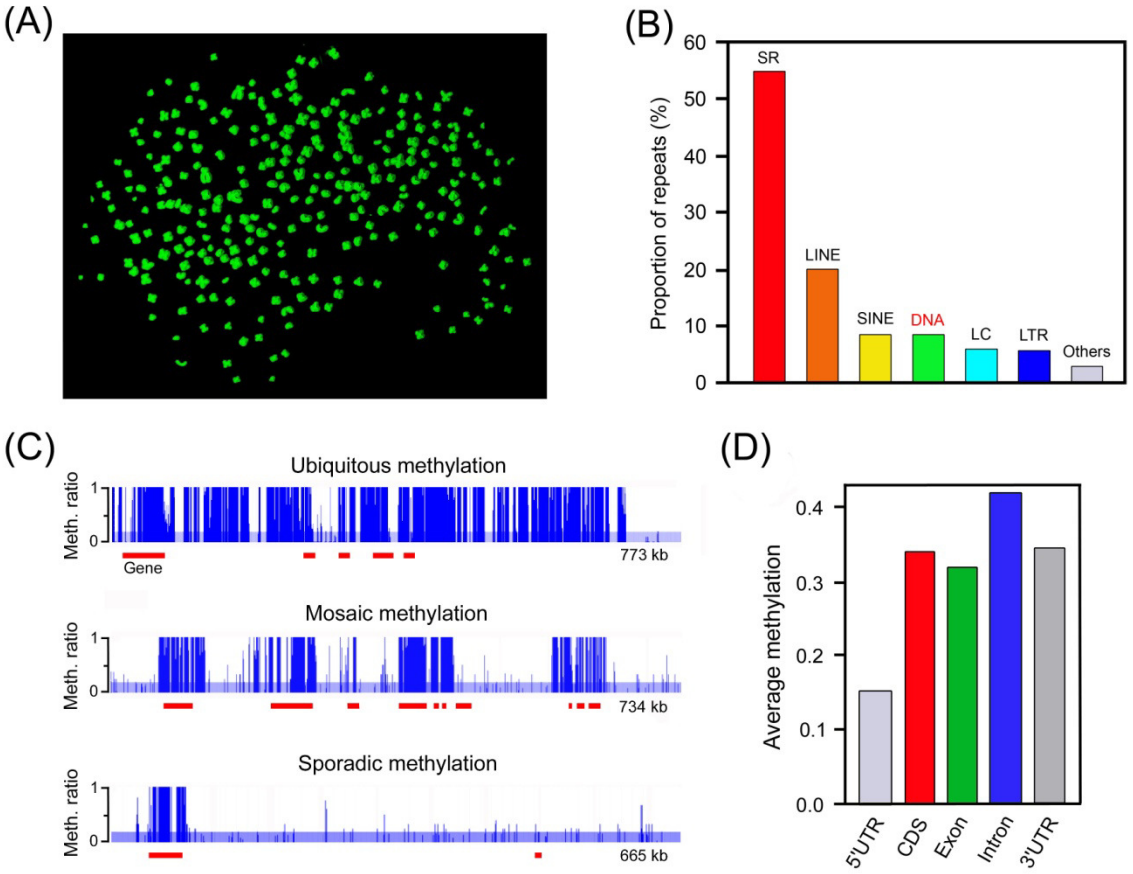

Figure 3. Genomic and epigenetic features of marbled crayfish. (**A**) Metaphase spread of embryonic body cell showing 276 chromosomes (from Martin *et al.* 2016). (**B**) Automatically annotated repetitive elements of genome distributed into major categories: DNA transposons (DNA), low complexity regions (LC), LINE repetitive DNA (LINE), LTR elements (LTR), SINE repetitive DNA (SINE) and simple repeats (SR). The DNA transposons show the highest methylation of all repeats (data from Falckenhayn 2016 and Gutekunst 2017). (**C**) Examples of ubiquitously, mosaically and sporadically methylated genomic scaffolds. Methylation ratios of CpGs are indicated by blue vertical bars. Methylation ratios below 0.2 are bisulfite conversion artefacts. Red horizontal bars indicate predicted genes (redrawn and modified after Falckenhayn 2016). (**D**) Methylation levels of untranslated regions (5'UTR and 3'UTR), protein-coding sequences (CDS) and exons and introns of predicted genes (redrawn and modified after Falckenhayn 2016).

Analysis of the methylome of marbled crayfish further revealed that 41% of the genes were heavily methylated and 26% were unmethylated (Falckenhayn 2016). DNA methylation is CpG-specific and present in both genes and repeats. Methylation was found in the 5'UTR, coding sequences, exons, introns and 3'UTR of genes (Figure 3D). Gene body methylation was highest in evolutionarily old genes predating the Arthropoda, genes with length of several

kb and moderately expressed genes (Falckenhayn 2016). Repeats are generally hypomethylated, and only repeats located within genes show higher methylation levels. DNA transposons show the highest methylation level of all repeat classes being close to the average gene body methylation level. Evolutionarily old repetitive elements display higher methylation levels than younger ones.

The DNA methylation toolkit of marbled crayfish consists of single homologues of the DNA methytransferases Dnmt1 and Dnmt3 and the ten-eleven translocation methylcytosine dioxygenase Tet (Falckenhayn 2016), providing a full complement of enzymes for de novo and maintenance methylation, demethylation and hydroxymethylation. The sequences are deposited in GenBank (Dnmt1: KM453737; Dnmt3: KM453738; Tet: KM453739). This pattern of single copies of the DNA methylation toolkit is supposed to be phylogenetically ancestral for arthropods and animals in general (Vogt 2017b).

Global DNA methylation in marbled crayfish is in the order of magnitude of zebrafish, the mammalian models and humans (Fraga *et al.* 2005; Xia *et al.* 2015; Liu *et al.* 2016a) but an order of magnitude higher than in most insects. The genomes of the invertebrate models *Caenorhabditis elegans* and *Drosophila melanogaster* are even unmethylated (Raddatz *et al.* 2013). The methylation and demethylation machinery of marbled crayfish is relatively simple when compared to mammals, which have one Dnmt1, three Dnmt3 and three Tet but obviously it is highly effective. Marbled crayfish shares crucial features of DNA methylation such as CpG methylation, gene body methylation and repeat methylation with many other animals. The majority of genes show mosaic methylation as is typical for many invertebrates but a considerable proportion of the genes is ubiquitously methylated like in vertebrates or sporadically methylated like in some higher insect orders. Thus, the marbled crayfish methylome seems to be a good representative for animals.

4. Benefits of marbled crayfish for functional, comparative and applied genomics

Full genomes of animals become increasingly available due to rapid advances in sequencing technology and the development of bioinformatics tools for genome assembly and annotation, broadening the data base for functional and comparative genomics. Despite of the high species number and ecological and economic importance of crustaceans, high-coverage genomes are only available for two species, the cladoceran *Daphnia pulex* (Colbourne *et al.* 2011; Ye *et al.* 2017) and the amphipod *Parhyale hawaiensis* (Kao *et al.* 2016). The marbled crayfish genome is now the first such genome for the Decapoda, which include the economically valuable shrimps, crabs, lobsters and crayfish. Incomplete low coverage genomes were earlier published for the shrimps *Neocaridina denticulata* and *Exopalaemon carinicauda* and the crab *Eriocheir sinensis* (Kenny *et al.* 2014; Song *et al.* 2016; Yuan *et al.* 2017). The high coverage genome of marbled crayfish seems well suitable for investigating the genetic underpinning of some topical issues of biology, particularly when combined with transcriptomics and gene expression studies, as will be discussed in the following.

4.1 Genetic peculiarities of decapod crustaceans and freshwater crayfish

Crustaceans comprise about 66,900 species (Ahyong *et al.* 2011) and are particularly diverse with respect to morphology, physiology, longevity, life history and ecology (Martin and Davis 2001). Some groups have central positions in food webs, some are keystone species in their ecosystems and some are exploited for human consumption. The Crustacea are also evolutionarily interesting because the insects, the largest animal group on earth (~1 million described species), have evolved from an ancient crustacean group, forming the Pancrustacea together with the crustaceans (Regier *et al.* 2005). The Decapoda comprise some 14,800

species (Ahyong *et al.* 2011) and the freshwater crayfish comprise 699 species (Crandall and De Grave 2017).

Comparison of the transcriptome of marbled crayfish with those of other crustaceans, insects and vertebrates revealed that about 41% of the marbled crayfish transcripts belong to the bilaterian core, 4% to the Pancrustacea, 5% to the Crustacea, 15% to the Decapoda and 16% to the Astacidea (Figure 4A). Approximately 19% are unique to marbled crayfish (Falckenhayn 2016).

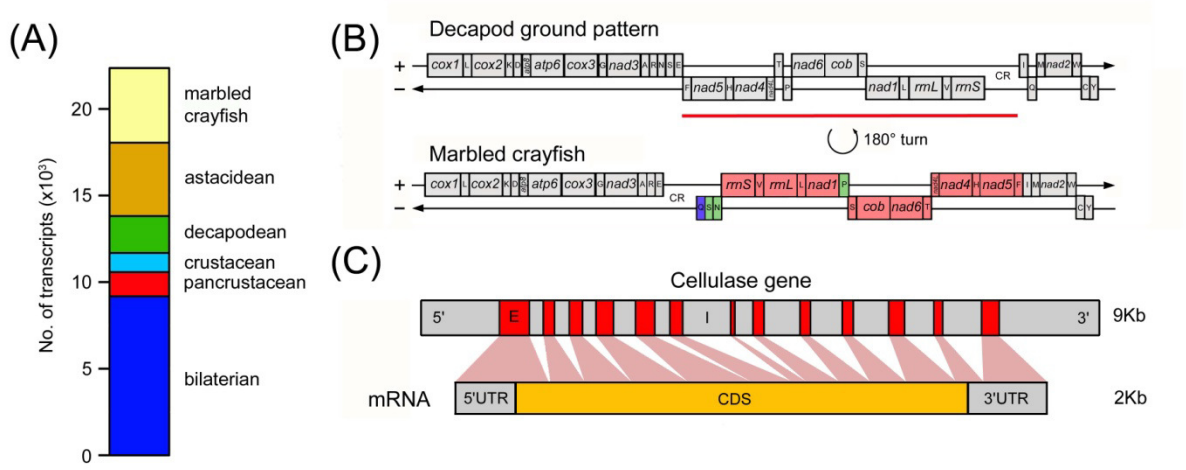

Figure 4. Exploitation of the marbled crayfish genome for evolutionary and ecological questions. (**A**) Transcripts of marbled crayfish classified by lineage. The graph shows bilaterian-specific, pancrustacean-specific, crustacean-specific, decapod-specific, crayfish-specific and species-specific proportions (redrawn and modified after Falckenhayn 2016). (**B**) Gene arrangement in the mitochondrial genome of marbled crayfish. Genes that kept their original position in comparison to the ground pattern of the Decapoda are in grey, genes inverted to the other strand are in red, genes translocated to other positions in the same strand are in blue and genes translocated to other positions in the other strand are in green. Red bar indicates inverted sequence stretch (redrawn and modified after Shen *et al.* 2013). (**C**) Gene structure and transcript of the GH9 cellulase gene of marbled crayfish, an important component of herbivory. CDS, coding sequence; E, exon; I, intron; UTR, untranslated region (redrawn and modified after Gutekunst 2017).

Analysis of the mitochondrial genome of marbled crayfish established a derived pattern when compared to the ground pattern of the Decapoda (Shen *et al.* 2013). The latter is almost identical to the ground pattern of the Ecdysozoa and Pancrustacea (Braband *et al.* 2010; Shen *et al.* 2013). In marbled crayfish, there are several genes inverted to the other strand by a 180° turn of a larger stretch of the mitochondrial genome (Figure 4B). Furthermore, there is one gene translocated to another position in the same strand and three genes to other positions in the other strand (Figure 4B) (Shen *et al.* 2013). Similar deviations from the decapod ground pattern have also been found in the European lobster *Hommarus gammarus*, another member of the Astacidea (Shen *et al.* 2013) but not in the parastacid crayfish *Cherax destructor* or other groups of the Decapoda (Shen 2012). These results suggest that the inversion has occurred in the stem group of the Astacidea, has been retained in the Nephropidae and Cambaridae but has been reverted in the Parastacidae (Shen *et al.* 2013).

Yuan *et al.* (2016) compared the genomes of four insects with the genome of *Daphnia pulex* and the transcriptomes of the shrimps *Penaeus monodon* and *Litopenaeus vannamei* and found losses and gains of genes within both the insect and crustacean lineages. They also detected significant gene family differences between water flea and shrimps. Detailed analysis

of the marbled crayfish genome and comparison with the available insect and crustacean genomes will certainly refine our picture on the genetic peculiarities and evolution of crustaceans, decapods and crayfish.

4.2 Genetic basis of crustacean reproduction

Understanding reproduction in crustaceans is vital for population ecology, fisheries and aquaculture. Here, I will focus on two topics, the regulation of female maturation and vitellogenesis, the provisioning of the eggs with yolk.

Endocrine research established that female maturation and vitellogenesis in decapods is controlled by the interplay of several inhibiting and stimulating neuropeptides, neuromodulators, and the hormone methyl farnesoate (Charniaux-Cotton and Payen 1985; Nagaraju 2011). The X-organ sinus gland complex is the major source of the neuropeptides including gonad inhibiting hormone, moult inhibiting hormone and crustacean hyperglycemic hormone, which regulate reproduction by acting on other hormone glands and the hepatopancreas and ovary. The gonad-stimulating factor and the gonadal development promoting methyl farnesoate are synthesized in the thoracic ganglia and mandibular organ, respectively (Vogt 2002). The hepatopancreas is the central organ of metabolism in decapods comparable to the liver of vertebrates. It synthesizes the metabolites required for growth of the oocytes, and in later stages of ovarian maturation, it synthesizes vitellogenins that are delivered to the oocytes and transformed into vitellins. These glycolipoproteins are accumulated and stored in yolk granules of the oocytes to be catabolized during embryonic development (Ferré *et al.* 2012).

Transcriptome and gene expression analyses in crayfish and shrimps identified additional molecules involved in the reproduction of decapods (Brady *et al.* 2012; Jiang *et al.* 2014; Peng *et al.* 2015; Saetan *et al.* 2016). For example, Jiang *et al.* (2014) identified several genes related to gonad development in crayfish *Procambarus clarkii* coding for cyclin B, cyclin-dependent kinases 2, Dmc1 and ubiquitin. Saetan *et al.* (2016) identified 1,025 genes in banana shrimp *Fenneropenaeus merguiensis*, which were differentially expressed between vitellogenic and non-vitellogenic stages.

Comparison of the genomes of immature, mature and vitellogenic marbled crayfish may now help to unravel the genetic underpinning of female gonad maturation and vitellogenesis in more detail. The findings may be helpful in developing new ideas for the optimization of reproduction in crustacean aquaculture, an industry that produces 6.9 million tons of shrimps, crabs and crayfish per annum with a value of 36.17 billion US$ (FAO 2014).

4.3 Genetic components of the arthropod immune system

Knowledge of the immune system of arthropods is of great importance to prevent diseases in cultured species like bees and shrimps and to combat agricultural pests and human disease vectors. The immune system of arthropods is different from that of vertebrates in as far as it provides rapid defence against infections in a generic way but does not confer long lasting immunity to the host. However, it is obviously highly effective as well, particularly in decapods that can reach ages of several decades (Vogt 2012a). The immune system of crustaceans includes haemolymph clotting components, antimicrobial peptides, protease inhibitors, phagocytotic cells and the melanization and encapsulation of pathogens and infected tissue areas (Cerenius *et al.* 2008; Gherardi *et al.* 2010; Cerenius and Söderhäll 2013, 2017).

In the last years, new information on the immune system of crustaceans has been obtained from the analysis of transcriptomes of healthy and diseased specimens (Chen *et al.* 2013; Verbruggen *et al.* 2015; Theissinger *et al.* 2016; Lai and Aboobaker 2017). By

comparison of the transcriptomes of 55 malacostracan species, Lai and Aboobaker (2017) identified 7407 genes from 39 gene families involved in different aspects of immune defence, demonstrating the dynamic evolution of immunity components within this group. Chen *et al.* (2013) established that in shrimp *Litopenaeus vannamei* infected with white spot syndrome virus 767 host genes were significantly up-regulated and 729 genes were significantly down-regulated when compared to healthy specimens. Verbruggen *et al.* (2015) found transcripts for a series of pathogen recognition receptors and immune response proteins in crab *Carcinus maenas* representing the RNAi, Toll-like receptor signalling, IMD and JAK/STAT pathways.

The genome of marbled crayfish may now help to provide deeper insight into the immune system of decapods, and arthropods in general. The comparison of healthy specimens and specimens infected with known viral, bacterial, fungal and protozoan disease agents of crayfish (Longshaw 2011) are expected to reveal new components of the immune defence system of the Decapoda and help to identify potential differences in the defence strategies against the major classes of pathogens. The results may help in both the fight against arthropod diseases and eradication of arthropod pests.

4.4 Genetic underpinning of moulting and biomineralization

Malacostracan crustaceans, particularly the Decapoda, are exceptional among animals because they possess a mineralized chitinous exoskeleton that is regularly moulted (Luquet 2012; Roer *et al.* 2015). This feature makes them suitable models to study both moulting and biomineralization. They are particularly suitable to investigate decalcification and recalcification processes that may be of some interest for human biology.

Moulting is a key process in the life history of the Ecdysozoa (Ewer 2005), the largest animal clade that includes the Nematoda, Arthropoda and several smaller phyla. The ability to moult the exoskeleton is currently believed to have evolved only once during the early ecdysozoan evolution (Ewer 2005). Curiously, the most important model for the genomics of moulting in ecdysozoans is not a crustacean or an insect but the tiny nematode *Caenorhabditis elegans* (Frand *et al.* 2005; Turek and Bringmann 2014). Unlike arthropods that have a rather rigid chitinous cuticle, this worm has a flexible cuticle composed of intensely cross-linked collagen-like molecules.

In crustaceans, moulting can be subdivided into three phases, the synthesis of a new cuticle underneath the old cuticle (premoult), shedding of the old cuticle (ecdysis) and hardening of the new cuticle (postmoult). These processes require different metabolic pathways and sophisticated regulatory mechanisms (Chang and Mykles 2011). To better understand the molecular mechanisms of exoskeleton development and reconstruction in decapods, Gao *et al.* (2017) analysed transcriptomes of developmental and adult stages of the shrimp *Litopenaeus vannamei*. They identified more than 600 unigenes related to exoskeleton development and moulting. Lv *et al.* (2017) investigated the transcriptomes of the eyestalk of premoult, intermoult and postmoult specimens in the crab *Portunus trituberculatus* and identified 1,394 moult-related and differentially expressed genes.

Decalcification and recalcification of the cuticle during moulting requires additional metabolic pathways and regulatory genes. The Decapoda have evolved a dual carbonate and phosphate mineralization system that enables the deposition of amorphous calcium carbonate, amorphous calcium phosphate, calcite and apatite at various skeletal locations. This is in contrast to vertebrates that use only calcium phosphate for skeletal construction and most other invertebrates that use only calcium carbonate (Bentov *et al.* 2016). Decapods can also combine these minerals to form composites materials, for instance in the particularly hard mandibles that serve for food processing (Bentov *et al.* 2016). These features may inspire materials scientists to mimic such calcified structures and their properties for technological and biomedical purposes (Nudelman and Sommerdijk 2012).

The most intensely used invertebrate models for studying biomineralization are probably the bivalves, which possess a rigid calcified shell that increases in size and thickness throughout life. Since this shell is never shed or decalcified it is only suitable to study the calcification process. Sequencing of peptides from the shell of oyster *Crassostrea gigas* and mapping to the fully sequenced genome has identified 259 proteins involved in formation of the shell, 61 of them being apparently related to the mineralization process itself (Zhang *et al.* 2012).

In freshwater crayfish, the calcium is withdrawn from the cuticle during premoult and stored in gastroliths within the stomach wall. After ecdysis the stored calcium is remobilized and integrated into the chitinous network of the new cuticle (Shechter *et al.* 2008). Additionally, calcium salts are absorbed from the water. Abehsera *et al.* (2016) investigated gene expression in the gastrolith and the epithelium of the mandible and identified genes involved in the synthesis and breakdown of chitin that form the matrix for mineralization. However, they did not report on genes involved in the calcification and decalcification process.

The comparison of the genomes of premoult, intermoult and postmoult individuals of marbled crayfish should provide deeper insight into the genetic networks and pathways involved in moulting and decalcification and recalcification. The results are expected to contribute to better understanding of moulting in arthropods and biomineralization in general. Unravelling of the decalcification and recalcification pathways in marbled crayfish may provide new ideas for treatment of osteoporosis, a severe skeletal disease of elderly people (Ralston 2010).

4.5 Genetic underpinning of omnivory

Freshwater crayfish and many other decapod crustaceans are omnivorous, which means that they eat animals, plants and detritus. They can not only digest the soft intracellular constituents of their food items but also the relatively solid and voluminous extracellular matrix components collagen, cellulose and chitin. Cellulose is the most frequent macromolecule on earth making up 50% of the cell wall of plants (Watanabe and Tokuda 2001). Collagen is the most widespread protein in the animal kingdom and occurs in all multicellular animals. In mammals, it constitutes about 20% of the proteins (Harkness *et al.* 1978). Chitin is an important component of arthropod prey of crayfish and the fungal destruents of organic matter (Rathore and Gupta 2015). Moreover, chitin is the main constituent of the shed cuticle of crayfish, which is often used as first food after moulting.

Freshwater crayfish secrete a broad set of digestive enzymes into the lumen of the stomach including proteinases, carbohydrases, cellulases, chitinases and lipases (Vogt 2002; Crawford *et al.* 2004; Coccia *et al.* 2011). These enzymes are highly stable because they are stored for hours and days in active form in the stomach lumen to await the next meal. This is in sharp contrast to vertebrates, which store their digestive enzymes intracellularly as inactive proenzymes and discharge and activate them only when food enters the digestive tract (Vogt *et al.* 1989). Of particular interest is the crayfish endopeptidase astacin, which can cleave the native collagen triple-helix at neutral pH. In mammals, the collagen must first be denaturated by the acidic pH of the stomach before it is cleaved by pepsin and other proteinases. It would be interesting to see, how many digestive enzymes are encoded in the genome of marbled crayfish and if there are multiple copies of those enzymes that are produced in high quantities. Gutekunst (personal communication) has already identified the astacin gene and a cellulase gene (Figure 4C) in the marbled crayfish genome.

Comparison of marbled crayfish fed for longer periods of time either with pure animal, pure plant or mixed diets should identify the genetic underpinning of carnivory, herbivory and omnivory, because enzyme production in decapods is apparently adapted to the diet (Le

Moullac *et al.* 1994). Similar experiments were successfully performed to identify omnivory-related genes in the beetle *Coleomegilla maculata* (Allen 2015). The genes specifically involved in the digestion of cellulose, collagen and chitin could then be identified by feeding artificial diets that include cellulose, collagen or chitin as the sole digestible components. The identification of genes and pathways involved in their catabolism may help to optimize feeding in crustacean aquaculture and to extract base materials for the food industry from these readily available natural macromolecules.

4.6 Genetic basis of adaptation to fresh water

The genetic and molecular basis of adaptive evolution is still poorly known. The transition from the sea to fresh water is a major step in the evolution of life and is therefore particularly suitable to investigate this topic in detail. Interesting models of the transition from the sea to fresh water are fishes and crustaceans. For example, the threespine stickleback *Gasterosteus aculeatus* colonized freshwater habitats repeatedly after the retreat of Pleistocene glaciers and evolved changes in salt handling, body shape, trophic specialization and life history. Jones *et al.* (2012) investigated the genomic alterations associated with these adaptations and identified a genome-wide set of loci that are consistently linked with the marine-fresh water transition. Both coding and regulatory changes occurred in these loci but regulatory changes were predominant.

The shrimp genus *Macrobrachium* is an interesting crustacean model system of the genetic underpinning of fresh water adaptation (Anger 2013; Moshtaghi *et al.* 2017). This genus has invaded fresh water at least nine times but most of the approximately 240 species still depend on brackish or sea water for larval development. However, some species like *M. koombooloomba* complete their entire life cycle in fresh water. Using a transcriptomics approach, Rahi *et al.* (2017) identified 43 potential genes that are likely involved in fresh water adaptation of this species, including genes for osmoregulation, cell volume regulation, haemolymph maintenance and epithelial permeability.

Freshwater crayfish deviate from the sticklebacks and shrimps as they invaded fresh water only once before the break-up of Pangaea some 200 million years ago (Rode and Babcock 2003). There is evidence that the fresh water invading stem group has already evolved a complete freshwater life cycle including direct development (Vogt 2013). Thus, freshwater crayfish are particularly suitable to investigate the genetic underpinning of an irreversible and phylogenetically old adaptation to freshwater conditions. The genome of marbled crayfish can now be searched for genes that promoted adaptation to fresh water, making use of the knowledge obtained with sticklebacks and *M. koombooloomba*. Further unknown loci involved in fresh water adaptation may be identified by comparing the crayfish genome with the available transciptomes of clawed lobsters (McGrath *et al.* 2016), the closest marine relatives of freshwater crayfish within the Astacidea (Toon *et al*. 2010).

4.7 Genetic causes of sexual system shifts

Sex in crayfish is determined by a WZ/ZZ chromosomal mechanism with the heterogametic sex being the female (Chandler *et al.* 2016; Vogt 2017c). The *Dsx*- and *mab-3*-related transcription factors are apparently key regulators of male sex determination. Females are the default sex. Endocrine research has shown that sexual differentiation of males is mediated by the insulin-like androgenic gland hormone, regulatory binding proteins, a tyrosine kinase insulin receptor and the upstream effects of the gonad inhibiting and moult inhibiting hormones (Ventura *et al.* 2015; Chandler *et al.* 2016; Sharabi *et al.* 2016). Comparison of the genomes of male and female *Procambarus fallax* is expected to reveal further genes and pathways involved in sex determination of crayfish.

In the course of speciation, the sexual system of marbled crayfish has changed from gonochorism to obligate parthenogenesis (Vogt *et al.* 2015), which was supposedly caused by mutation, loss or permanent silencing of meiosis-related genes. Infection with the feminizing bacterium *Wolbachia*, which can cause parthenogenesis in insects and induce all-female populations in isopod crustaceans, was already excluded (Vogt 2008a). The male-determining insulin-like androgenic gland hormone is present in marbled crayfish as was demonstrated by the group of Amir Sagi, Ben-Gurion University, Beer Sheva , Israel (GenBank: KX619618). Thus, the lack of males in marbled crayfish is not due to the loss of the insulin-like androgenic hormone gene. The Sagi group has started to study the parthenogenetic marbled crayfish model as an alternative to the common gonochoristic models of reproduction to investigate gender determination in decapods in more detail.

The genetic underpinning of the sexual system shift in marbled crayfish could be investigated by comparison of the genomes of marbled crayfish and *P. fallax* females. A first comparison via automatic genome annotation found evidence for potential loss of meiosis related genes. Most of the 93 meiosis related genes searched for were present but *Rec8* and *Msh5* genes were lacking (Gutekunst 2017). Rec8 protein is a meiosis-specific component of the cohesin complex that binds sister chromatids in preparation for the meiotic divisions. The *Msh5* gene encodes a member of the mutS proteins that are involved in meiotic recombination processes.

A further approach to identify the causes of sexual systems shifts is the comparison of the marbled crayfish genome with the genome of obligately parthenogenetic strains of water flea *Daphnia pulex*. *D. pulex* normally alternates between sexual and parthenogenetic reproduction but some strains have become obligate parthenogens like marbled crayfish. Using genome-wide association analysis, Xu *et al.* (2015) detected a set of 647 alleles with single nucleotide polymorphisms that were specific to these parthenogens. They classified 206 of these genes into orthologue groups but the specific factors underlying obligate parthenogenesis remained elusive. Eads *et al.* (2012) established that all obligate parthenogens of *D. pulex* carry a transposable element insertion and a frameshift mutation in the *Rec8* gene, suggesting that obligately parthenogenetic water fleas have lost the ability of chromosome segregation at meiosis I.

4.8 Relationship between genes, neurophysiology and behaviour

Neural development, brain function and behaviours are encoded by the genes (Bae *et al.* 2015). However, neural phenotypes are polygenic, extremely complex and can be greatly modulated by the environment (Roth 2013). Mental and physical activity can modify synapses and remodel the epigenetic signatures of the chromatin. Impoverished and enriched living conditions influence the proliferation and survival of the neurons in the brain even in invertebrates like crayfish (Sandeman and Sandeman 2000). Altering genetic programmes and epigenetic profiles of the neurons can have positive effects but can also impair brain function and behaviours leading to severe diseases (Yao and Wu 2014).

Due to genetic identity the marbled crayfish is particularly suitable to dissect the genetic, stochastic developmental and environmental proportions of development and functioning of the nervous system and resulting behaviours (Vogt 2015a). Vilpoux *et al*. (2006) and Sintoni (2010) described the development of the nervous system of marbled crayfish and adult neurogenesis in detail (Figure 5A), providing the structural basis for such research. The relationship between genes, neurophysiology and behaviours can be investigated by knock out of genes involved in brain functioning and recording of the resulting behavioural changes. Such work has been initiated by the groups of Wolfgang Stein and Andrés Vidal-Gadea at Illinois State University, Normal, IL, USA (Gahrs *et al.* 2016; Benson *et al.* 2017). For example, Benson *et al.* (2017) injected innexin-4 dsDNA into juvenile crayfish to suppress

expression of the innexin-4 gene. The innexins are structural components of the gap junctions and build transmembrane channels that facilitate cell-to-cell communication. The authors observed reduced walking behaviour and reduced tail flip escape responses after suppression of the innexin-4 gene.

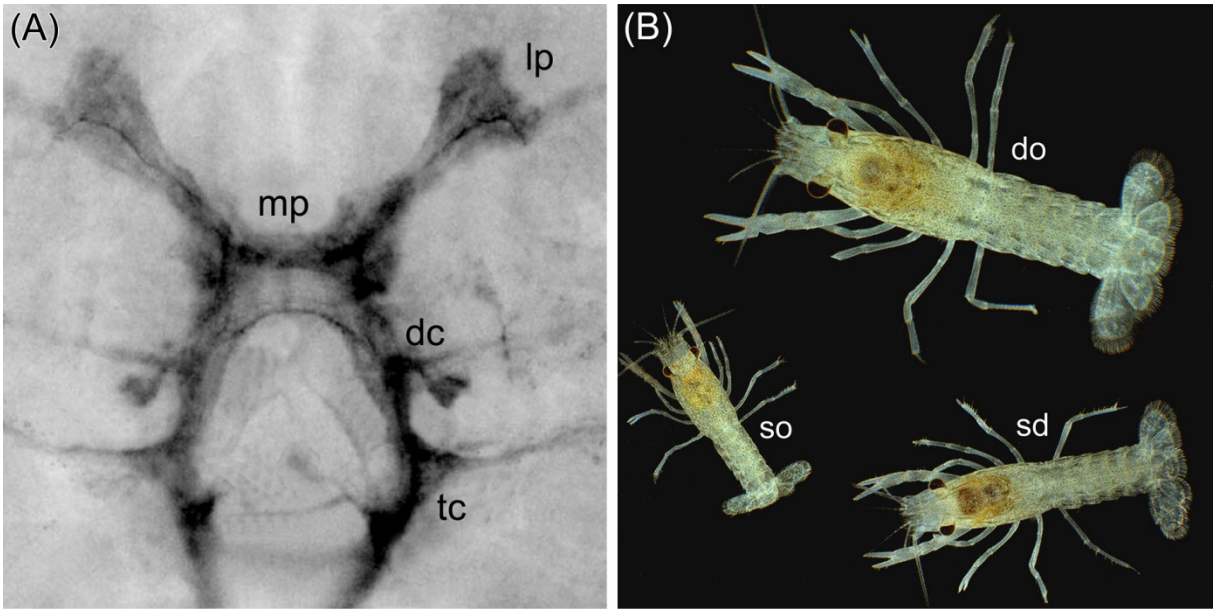

Figure 5. Brain development in marbled crayfish and differences in behaviour and growth among clutch-mates. (**A**) Synapsin-labelled brain of marbled crayfish at 60% embryonic development. dc, deutocerebrum; lp, lateral protocerebrum; mp, medial protocerebrum; tc, tritocerebrum (from Vilpoux *et al.* 2006). (**B**) Development of differences in behaviour and growth among communally raised clutch-mates. Five genetically identical and size-matched clutch-mates were kept together for 34 days. At the end of the experiment, the group had differentiated into one dominant (do) with aggressive behaviours, two subdominants (sd) with aggressive and avoiding behaviours and two subordinates (so) with avoiding behaviours. The dominant grew much faster than the subdominants and subordinates although food was available in excess and not monopolized (from Vogt *et al.* 2008).

Marbled crayfish is also suitable to investigate the genetic and epigenetic underpinning of agonistic behaviours (Figure 5B). Basic data on the agonistic behaviour of marbled crayfish are provided by Vogt *et al.* (2008) and Farca Luna *et al.* (2009). Of particular interest are the genes of the serotonin system that are involved in aggression control in crayfish and other species including man (Panksepp *et al.* 2003; Duke *et al.* 2013)

4.9 Evolution of clonal genomes

Knowledge on the acquisition of mutations over lifetime and between generations is relevant for many fields of biology including disease development, environmental adaptation and the evolution of species. This issue is best studied by comparison of whole genome sequences throughout life and across generations in asexually reproducing species, starting from a single specimen. Papadopoulos *et al.* (1999) investigated clonal evolution in the bacterium *Escherichia coli* over 10,000 generations under laboratory condition by using restriction fragment length polymorphisms. They found relatively high dynamics in this evolutionarily short time span. I am not aware of such studies on the whole genome scale in asexually reproducing eukaryotes.

The monoclonal marbled crayfish now enables studying of clonal evolution on the whole genome scale, albeit not for thousands of generations. Considering a genome size of 3.5 Gb and an estimated SNP substitution rate per generation of 10^{-8} like in humans (Weber-Lehmann *et al.* 2014) a difference of approximately 35 SNPs can be expected between mother and progeny. Such an analysis has not yet been done for marbled crayfish. However, Gutekunst (2017) found 6-49 homonomous single nucleotide substitutions in eight marbled crayfish from three laboratory colonies and five wild populations when compared to the reference genome. He concluded from these results that the mutation rate in marbled crayfish is similar to that of other animals. In future, whole genomes should be compared among mothers and offspring, among clutch-mates and among specimens from multiple geographic sites and ecosystems. A particularly interesting target for the latter is the numerous populations of Madagascar (Vogt 2015a, 2017a; Gutekunst 2017), which have evolved from a single introduction around the year 2003 (Jones *et al.* 2009).

It is controversially discussed whether somatic mutations have their origin mainly in early development or in later life stages (Vijg 2014). In any way, they lead to genetic mosaicism of the body possibly influencing diseases and ageing. The mutation rate in different postembryonic tissues is expected to vary considerably due to differences in cell renewal. In marbled crayfish, the cells divide rather synchronously during the first ten rounds of cleavage but in the adults the tissues vary significantly with respect to cell turnover (see section 5.4). Therefore, comparison of whole genomes between blastomeres and organs with different cellular turnover like the hepatopancreas, abdominal musculature and brain should give an idea on the temporal and spatial hotspots of somatic mutations in an individual. Whole genome and whole methylome sequencing of single cells is possible with recently developed technique (Gawad *et al.* 2016; Clark *et al.* 2017).

Understanding the evolution of clonal genomes is also important for cancer research (Lyko 2017). Tumours evolve by a reiterative process of clonal expansion, genetic diversification and clonal selection within the adaptive landscapes of tissues (Greaves and Malev 2012; Aparicio and Caldas 2013; Sottoriva *et al.* 2015), resembling genetic expansion and adaptation of clonal organisms in their ecosystems (Amend *et al.* 2016). Therapeutic interventions decimate cancer clones but may provide selective pressure for the expansion of resistant variants. This inherently Darwinian character of cancer lies at the heart of therapeutic failure but perhaps also holds the key to more effective control (Greaves and Malev 2012). The evolution of the genome in clonal organisms and long-range dispersal of the clones to new geographical regions may serve as an ecological paradigm for understanding tumour development and metastasis in humans, which prompted the group of Frank Lyko at the DKFZ in Heidelberg to start a study on clonal genome evolution in marbled crayfish (Lyko 2017). First data are available as described in the previous section. At first glance bacteria seem much better suitable for this purpose because of their short generation time but the genome of bacteria is differently organized and regulated when compared to human tumour cells and horizontal gene transfer considerably biases the distribution of mutations (Shapiro 2016).

4.10 Evolution of functionally diverse gene families

New genomes are often exploited for the investigation of the evolution of universal genes like the *hox* or *wnt* genes that have similar functions in all animal phyla (e.g. Kao *et al.* 2016). Gene families that have evolved highly diverse functions in different phyla are only rarely investigated. An example of interest to which the marbled crayfish genome could contribute is the astacins, a Zink-endopeptidases family (EC 3.4.24.21) that was first detected in the noble crayfish *Astacus astacus*. The astacins include more than 100 members and have functions diverse as digestion of food, processing of extracellular matrix components, patterning,

morphogenesis and hatching (Bond and Beynon 1995; Park *et al.* 2010). They were found from hydra to humans (Sterchi *et al.* 2008). Some astacins like the tolloids seem to be universally present in animals whereas others like the meprins are restricted to distinct taxa.

Freshwater crayfish comprise at least two members of the astacin family, the eponymous digestive enzyme astacin and a hatching enzyme (Geier and Zwilling 1998; Möhrlen *et al.* 2001). However, there are probably more astacins encoded in the genome of marbled crayfish as may be deduced from genomic analyses of *Caenorhabditis elegans* and *Drosophila melanogaster* (Sterchi *et al.* 2008; Park *et al.* 2010). The fruit fly possesses 16 genes of the astacin family and the nematode has 40, the functions of which are only partly identified (Sterchi *et al.* 2008; Park *et al.* 2010). The crayfish astacin is a collagenolytic digestive enzyme with unique properties as explained above (Stöcker *et al.* 1995). The hatching enzyme serves for weakening of the rigid egg envelope before hatching so that it can be mechanically disrupted during eclosion (Geier and Zwilling 1998). Homologous hatching enzymes are also known from echinoderms, fishes and frogs.

In the mouse and human genomes, six to seven astacin family genes have been found (Sterchi *et al.* 2008). The best investigated vertebrate astacins are the meprins, which are confined to this animal group. Meprins are either membrane bound or secreted and can hydrolyse biologically active peptides, cytokines, extracellular matrix proteins and cell-surface proteins (Sterchi *et al.* 2008; Prox *et al.* 2015). They are of medical importance because they are involved in several human diseases including Crohn's disease and ulcerative colitis and possibly contribute to tumour progression and metastasis as well. The marbled crayfish genome is expected to extent knowledge on this very old, functionally highly diverse and structurally most interesting astacin family.

The marbled crayfish genome may also contribute new data to the evolutionary history of human disease genes. The key molecular pathways of patterning of body axes, organogenesis, wiring of the nervous system and control of cell proliferation are highly conserved in animals. When they are disrupted similar defects are often observed in vertebrates and invertebrates. About 77% of 714 tested human disease genes had homologues in the fruit fly (Reiter *et al.* 2001), and meanwhile, the suitability of *Drosophila* as a model for the study of human genetic diseases is well accepted. The same kind of research could now also be done with marbled crayfish, which is closer to humans than fruit fly with respect to mode of development, body size and life span.

4.11 Genetic basis of resistance to environmentally-induced and age-related cancer

Decapod crustaceans are highly resistant to both environmentally-induced and age-related cancer. There are only 16 incidences reported on tumour-like growths for the decapods, some of them being of dubious validity (Vogt 2008c, 2016). This low number of tumour incidences can neither be explained by short life spans nor low intensities of pathological investigations. Many decapod species live longer than ten years and American lobsters even reach ages of about 70 years (Vogt 2012a). The commercially important decapods are regularly investigated for their health status in the course of fisheries research, aquaculture biosecurity programmes, seafood quality controls and environmental monitoring. As a result, more than 200 different diseases (not incidences) are known for the Decapoda, most of them being infectious diseases (ref. in Vogt 2008c, 2016).

Incidences of tumours are much lower in decapod crustaceans than in fish although they live in the same environments and have similar life spans. For example, skin tumours in marine fishes occur in 1-30% of specimens, depending on species and population (Mawdesley-Thomas 1971). In humans and the vertebrate models mouse and zebrafish the cancer rate increases rapidly at higher age. For example, 15-30% of laboratory mice and rats develop tumours in the second half of their 1.5-2 years life span, depending on strain

(Anisimov *et al.* 2005). Zebrafish, which have a life span of 2-3 years, show neoplasias in approximately 10–15% of the 1.5-2 year old specimens (Spitsbergen and Kent 2003). Marbled crayfish have approximately the same life span as mouse and zebrafish but I have never found tumours although I have intensely searched for them (Vogt 2008c). The same holds for other crayfish and shrimp species I worked with. These differences in tumour frequency make marbled crayfish a valuable model to unravel the causes and mechanisms of cancer resistance.

Why are decapod crustaceans more resistant to tumour formation than vertebrates? There may be differences in the presence and activity of oncogenes and tumour suppressor genes, detoxification of environmental carcinogens and elimination of neoplastic cells by the immune system. There is not much knowledge on these issues but a few examples support the later two possibilities. When crayfish and lobsters were exposed to the model carcinogen benzo[*a*]pyrene they showed differences in catabolism when compared to mice (Figure 6), preventing the production of DNA adducts and subsequent tumour formation (James *et al.* 1995; Jewell *et al.* 1997). Histopathological investigation of a crab revealed that neoplastic cells and emerging tumours can be phagocytosed by cells of the immune system, indeed (Sparks and Morado 1987).

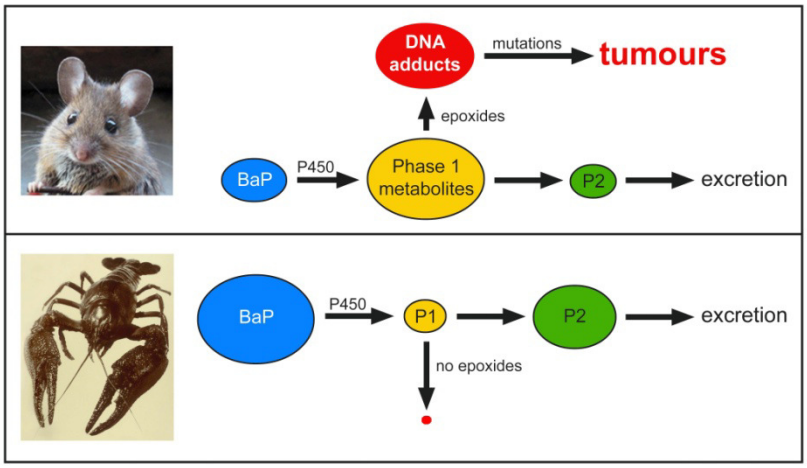

Figure 6. Differences between mouse and crayfish in environmentally induced tumour formation. Both species can accumulate high amounts of the model carcinogen benzo[*a*]pyrene (BaP) in the liver and hepatopancreas, respectively. However, in crayfish phase-1 metabolites are more slowly formed from these stores by cytochrome P450 and more rapidly converted into phase-2 metabolites (P2) that are excreted with the urine. Consequently, the formation and accumulation of DNA adducts that promote tumour formation in mammals remain negligible in crayfish and tumours do not develop (data from Jewell *et al.* 1997).

The availability of the genome of marbled crayfish now enables further testing of these hypotheses. The first hypothesis could be tackled by screening of the genome for the presence and activity of oncogenes and tumour suppressor genes. Oncogene candidates include genes involved in regulation of cell growth, replication, differentiation, proliferation inhibition and apoptosis (Croce 2008; Aktipis *et al.* 2015) and tumour suppressor gene candidates include genes coding for p53, pRb, ST5, etc (Levine 1993; Lee and Muller 2010; Abreu Velez and Howard 2015). Screenings of the genome and transcriptome of marbled crayfish are expected to reveal if carcinogenic mutants of proto-oncogenes are present and if tumour suppressor genes are active. The second hypothesis could be tested by genomic and transcriptomic

investigation of the cytochrome P450 and further enzymes involved in the detoxification of environmental carcinogens.

Another interesting aspect of the resistance of decapods to cancer, particularly age-related cancer, is the co-evolution of indeterminate growth and life-long telomerase production without adverse side effects (Vogt 2016). The telomeres form the ends of the chromosomes and in the determinately growing mammals they are shortened with each round of cell division, resulting in replicative senescence of the cells and gradual senescence of the individual (Shawi and Autexier 2008). Interestingly, replicative senescence is believed to provide a barrier for tumour progression. The idea behind is that cancer cells must require multiple mutations to become malignant and that premalignant cells may be stopped by replicative senescence before having accumulated enough mutations (Rossi *et al.* 2008; Gomes *et al.* 2011). However, this kind of protection against cancer in humans is at the cost of gradual senescence.

The acceleration of senescence by telomere shortening could principally be counteracted by telomerase, a reverse transcriptase, which adds new repeats to the ends of the chromosome. However, in mammals the tissues of the adults usually lack telomerase activity. This enzyme is only upregulated during oncogenesis making it one of the hallmarks of cancer (Hanahan and Weinberg 2000; Rossi *et al.* 2008). Paradoxically, treatment with telomerase is proposed as a possible method to combat ageing in humans while telomerase inhibition is suggested as a possible cancer therapy (Shawi and Autexier 2008; Arndt and MacKenzi 2016). In contrast to mammals, the decapods seem to lack this conflict because they maintain telomere length throughout life without adverse side effects like cancer (Godwin *et al.* 2011). Telomere maintenance is achieved by sustained telomerase expression over lifetime, as shown by Klapper *et al.* (1998) for the long-lived American lobster *Homarus americanus*.

The life-long production of telomerase without boosting cancer could now be investigated in more detail in marbled crayfish by genomics and transcriptomics approaches. The results may help researchers to find out how to prevent telomere shortening without promoting cancer, stimulating the development of new anti-ageing and anti-cancer strategies for humans at best.

5. Benefits of marbled crayfish for functional, environmental and evolutionary epigenetics

Answering the big questions of epigenetics requires elucidation and substantiation in the existing and new model systems (Allis *et al.* 2015). The most intensely used models of epigenetics are *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio* and *Mus musculus* (Blewitt and Whitelaw 2013; Kelly 2014; Chernyavskaya *et al.* 2016; Karnay and Elefant 2017). These models are not equally useful for studying the various questions of epigenetics. For example, the worm and fruit fly lack DNA methylation and are therefore unsuitable to investigate this important facet of epigenetics. Intensely analyzed genome-wide methylomes are available for about 20 animal species, mainly insects and vertebrates (ref. in Vogt 2017b). The marbled crayfish methylome is the first high-resolution methylome of a monoclonal, obligatory apomictic species, facilitating investigations on the relationship of epigenotype and phenotype and other fundamental questions of epigenetics. In the following chapters I will focus on DNA methylation, because histone modifications and microRNAs, the two other important epigenetic mechanisms (Jenuwein and Allis 2001; Sato *et al.* 2011), are not yet investigated in marbled crayfish.

5.1 Role of DNA methylation in gene regulation

There is considerable evidence that DNA methylation is involved in gene regulation of animals but presently there is no universal scheme recognizable. Instead, the functions of DNA methylation seem to vary with animal group and context (Jones 2012; Schübeler 2015; Vogt 2017b). Some animals even lack DNA methylation and must regulate their genome otherwise (Raddatz *et al.* 2013). Recent genome-scale mapping of methylation in various animals revealed variation of methylation marks at the transcription start sites, regulatory elements, gene bodies and repeat sequences (Jones 2012; Schübeler 2015). It is now well established that genes can be activated and silenced by demethylation and remethylation of promoters (Liu *et al.* 2016b). DNA methylation can also stably repress genomic elements underpinning imprinting, X-chromosome inactivation and silencing of transposons. The role of gene body methylation, which is shared by all animals with methylated genomes, has remained elusive. It has been suggested to serve for fine tuning of gene expression and alternative splicing (Lyko *et al.* 2010; Maor *et al.* 2015).

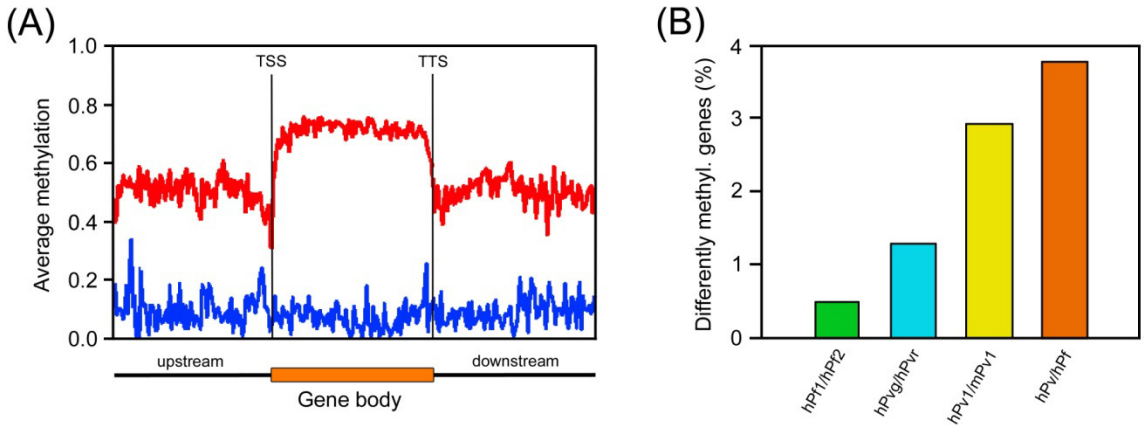

Figure 7. Variation of DNA methylation between genes, tissues, individuals and species. (**A**) Comparison of average methylation along unexpressed (blue) and moderately expressed (red) housekeeping genes of marbled crayfish, starting 4 kb upstream of the transcription start site (TSS) and ending 4 kb downstream of the transcription termination site (TTS) (redrawn and modified after Falckenhayn 2016). (**B**) Comparison of gene body methylation differences between tissues, individuals and species. Given is the percentage of genes with an absolute methylation difference > 0.2. Compared are the hepatopancreases of two *Procambarus fallax* in growth phase (hPf1/hPf2), the hepatopancreases of two *P. virginalis* in growth and reproduction phase (hPvg/hPvr), the hepatopancreas and abdominal musculature of the same *P. virginalis* specimen (hPv1/mPv1) and the hepatopancreases of a *P. fallax* and a *P. virginalis* in growth phase (hPv/hPf). The numbers of genes analysed were 6347, 5148, 6333 and 6303, respectively (data from Falckenhayn 2016).

Deciphering the functions of DNA methylation in gene regulation is necessary to advance our understanding of the epigenetic contributions to ageing, diseases and other biological phenomena (Bird 2002; Robertson 2005; Suzuki and Bird 2008; Jung and Pfeifer 2015; Schübeler 2015). The marbled crayfish is suitable to contribute to this topic because it allows comparison of the epigenetic signatures of genes that are permanently active, permanently silenced, periodically active and inactive, or active in a short period of life only. Good examples of constitutive, almost permanently transcribed genes are the housekeeping genes (McCulloch *et al.* 2012). They are suitable to investigate the involvement of DNA methylation in fine-tuning of gene activity. Comparison of housekeeping genes in marbled crayfish revealed low methylation of gene bodies in unexpressed genes and higher

methylation levels in moderately expressed genes (Figure 7A) (Falckenhayn 2016), corroborating that gene body methylation may fine-tune these genes.

Good examples of facultative genes that are only activated on demand are the two members of the astacin family described above, the astacin and the hatching enzyme. These genes allow the investigation of the role of DNA methylation in on-off-switching of gene activity. Their genomic structures have been investigated in detail for noble crayfish *Astacus astacus* (Stöcker and Zwilling 1995; Geier and Zwilling 1998). Both enzymes are present in marbled crayfish. The hatching enzyme is apparently synthesized in a transient dorsally located embryonic organ and is secreted in a short period of time before hatching. In all other tissues and all other life stages it is not expressed (Geier and Zwilling 1998). Comparison of the hatching enzyme gene in embryos shortly before hatching and in later life stages should demonstrate how the methylation pattern looks like in active and silenced genes.

The endopeptidase astacin is synthesized in the hepatopancreas and stored together with the other digestive enzymes in the stomach lumen as described above. Emptying of the stomach with a pipette stimulates refilling during the next 2-4 hours as shown for noble crayfish (Vogt *et al.* 1989). Thus, the astacin gene can be experimentally induced. Moreover, when feeding stops like in the days around ecdysis, enzyme production stops as well. In organs other than the hepatopancreas the astacin gene is permanently silent. Comparison of the methylation signatures of the astacin gene among different organs of marbled crayfish and different functional states of the hepatopancreas should reveal whether periodic and permanent silencing are associated with different methylation marks.

5.2 Epigenetic determinants of cell specificity

All cells and tissues of an organism have the same DNA but are markedly different with respect to structure and function. In humans, an estimated 400 cell types originate from the single genome of the zygote (Vickaryous and Hall 2006). If the tissue specific differences are not the result of genomic diversity then they must be caused by differential expression of the genome, i.e. by epigenetic mechanisms. DNA methylation is one of the effectors of tissue specificity in mouse and humans (Yagi *et al.* 2008; Ziller *et al.* 2013; Lokk *et al.* 2014; Roadmap Epigenomics Consortium *et al.* 2015). For example, Lokk *et al.* (2014) subjected 17 somatic tissues from four humans to functional genome analysis and identified a great number of tissue-specific differently methylated regions (DMRs). Many of the genes carrying these DMRs had tissue specific functions.

There is only a single study published on tissue specificity of DNA methylation in invertebrates. Suzuki *et al.* (2013) compared sperm and muscle cells of sea squirt *Ciona intestinalis* and found significant differences in global levels of 5-methylcytosine between both tissues but close similarities in gene body methylation. These results suggest that DNA methylation is anyhow involved in the generation of tissue specificity but that the effective mechanism is not gene body methylation. On the other hand, the authors demonstrated a positive correlation of gene body methylation and expression in housekeeping genes, resembling the results obtained with marbled crayfish (Falckenhayn 2016).

In most marbled crayfish investigated so far, the hepatopancreas and abdominal musculature had different global 5mC values. The greatest difference between these organs in an individual was 20.8% suggesting that DNA methylation is involved in tissue determination (Vogt *et al.* 2008). Likewise, the expression of Dnmt1, Dnmt3 and Tet varied significantly among the hepatopancreas, heart, claw musculature and abdominal musculature of an individual (Falckenhayn 2016). However, comparison of gene body methylation of more than 5,000 genes, mostly housekeeping genes, revealed no clear-cut picture. One the one hand, there was a 2.88% methylation difference between hepatopancreas and abdominal muscle of the same specimen, which is considerably higher than the difference of 0.4% between the

hepatopancreases of identically raised individuals (Figure 7B) (Falckenhayn 2016). On the other hand, the difference between hepatopancreas and gills was only 0.66% (Falckenhayn 2016).

The data obtained so far in marbled crayfish suggest a relationship of DNA methylation and tissue specification but gene body methylation of the housekeeping genes is apparently not the major determinant. In humans, DNA methylation differences among tissues were strongly associated with gene regulatory promoters and enhancers (Zhang *et al.* 2013), which have not yet been investigated in marbled crayfish. Moreover, the methylation pattern of tissue specific genes should be investigated and compared to the housekeeping genes.

5.3 Dynamic changes of DNA methylation between life stages and functional states

DNA methylation was earlier regarded as being relatively constant in a given individual throughout life. However, methylation of genes can apparently vary between distinct life periods such as embryonic development, juvenile period and adulthood (Martin 2009; Hackett and Surani 2012; Schuhmacher 2017). It can even vary between different functional states of adults as shown for the workers of honey bee that switch between nursing and foraging phases (Herb *et al.* 2012). Reverting foragers back to nurses re-established methylation levels for the majority of genes and provided evidence of reversible epigenetic changes associated with behaviour and function.

The marbled crayfish seems well suitable to investigate the relationship between DNA methylation and life period or functional state in depth because in its 2-3 years of life it passes through distinct embryonic, juvenile and adult periods and regularly alternating functional states in the adult life (Vogt *et al.* 2004, 2008; Vogt 2010). The embryos develop within the egg shells on the maternal pleopods and live exclusively from the yolk for about a month (Vogt and Tolley 2004). The juvenile phase lasts a few months and is characterized by rapid linear growth. The adult period, which lasts for one to three years, is characterized by regular alternation between reproduction and growth phases interspersed by moults (Vogt *et al.* 2008). The reproduction and growth phases last several weeks each and the moulting phases last a few days. In the different life periods and adult functional phases, specific pathways must be activated that either catabolize the yolk in developing embryo, promote rapid growth in juveniles, provide the oocytes of reproducing females with yolk, enlarge the organs in the adult growing phases or synthesize the new cuticle during moulting, respectively. Thus, certain sets of facultative genes must be specifically activated and silenced. In contrast, the housekeeping genes are thought to remain active all the time being only upregulated and downregulated to some degree.

First comparison of gene body methylation in 6333 genes (mainly housekeeping genes) in marbled crayfish revealed methylation differences in 1.2% of genes between reproductive phase and growth phase (Figure 7B) (Falckenhayn 2016). The specimens compared were raised under the same environmental and nutritional conditions. This first result does not allow far-reaching conclusions yet but it shows the principal suitability of this approach to clarify whether the DNA methylation pattern is changed between alternating functional phases. Future studies should focus more on facultativly than consitutively expressed genes.

5.4 Role of epigenetic mechanisms in tissue regeneration and ageing

The role of epigenetic mechanisms in adult stem cell regulation, tissue homeostasis, regeneration and ageing of organs is less well investigated than their involvement in embryonic stem cell regulation and cell differentiation (Hu and Rosenfeld 2012; Beerman and Rossi 2015; Avgustinova and Benitah 2016). In humans, the speed of cell renewal differs much among the adult organs. Some tissues like the intestinal epithelium are renewed within a

few days, some tissues like liver, lung or bones are renewed within weeks, months and years, and other like most areas of the brain are not renewed at all. In adult animals, there are principally three possibilities of tissue enlargement and regeneration, namely by true adult stem cells, division of mature tissue cells and immigration of stem cells or dedifferentiated cells from external sources. Recent investigations revealed that in mammals both DNA methylation and hydroxymethylation are involved in the regulation of adult stem cells (Cheng *et al.* 2015). DNA hydroxymethylation has already been demonstrated in tissues of marbled crayfish aside of DNA methylation (Table 1) but the level was two orders of magnitude lower than in mouse (Falckenhayn 2016).

Indeterminate growers such as marbled crayfish enlarge their organs during the entire lifetime and offer thus interesting systems of adult tissue growth and homeostasis (Vogt 2012b; 2016). Freshwater crayfish can additionally regenerate their limbs. In this section, I will discuss the usefulness of three organs of marbled crayfish for investigating the involvement of epigenetic mechanisms in adult stem cell regulation, tissue homeostasis, regeneration and ageing, namely the hepatopancreas, olfactory antennule and walking leg. These organs differ significantly in the speed of cell turnover.

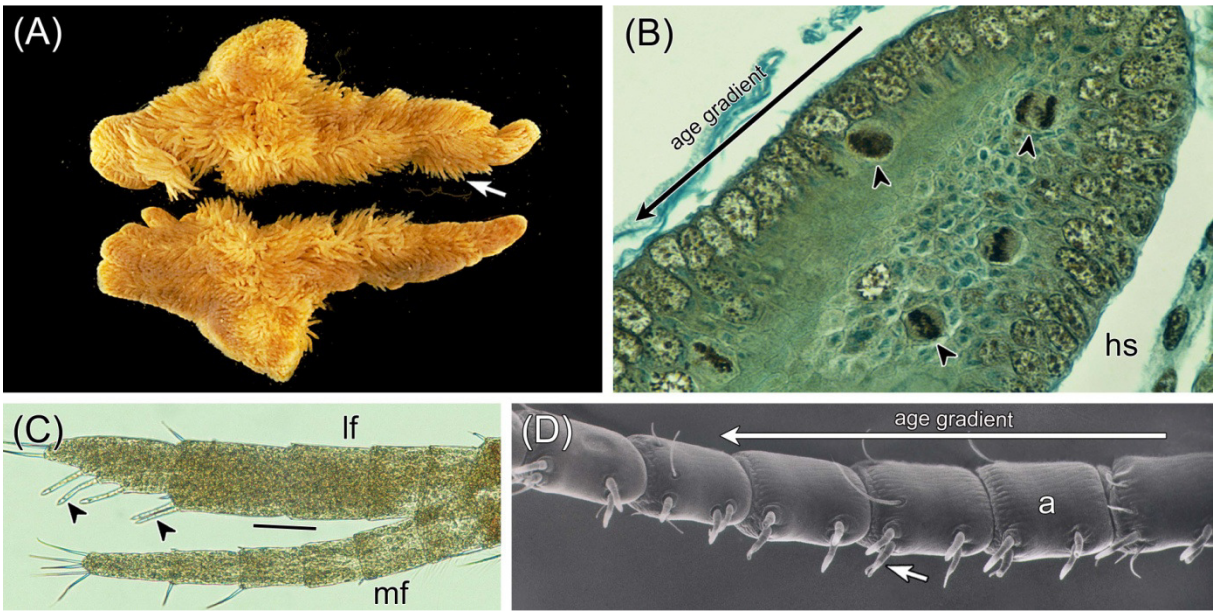

Figure 8. Suitable organs of marbled crayfish for investigating the epigenetic signatures of adult stem cells and ageing cells. (**A**) Crayfish hepatopancreas composed of two half organs and numerous blindly ending tubules (arrow) (from Vogt 2002). (**B**) Stem cell niche at blind end of hepatopancreas tubule of marbled crayfish showing mitotic stages (arrowheads). The new cells are pushed downstream by the next feeding-induced mitotic pulses establishing a distinct age gradient along the tubule. hs, haemal space (from Vogt 2008a). (**C**) Antennule of stage-2 juvenile of marbled crayfish with olfactory aesthetascs (arrowheads) on lateral flagellum (lf). Bar indicates region where new aesthetascs will be added during the next moult. mf, median flagellum (from Vogt and Tolley 2004). (**D**) Middle part of lateral flagellum of adult showing pair-wise arrangement of aesthetascs (arrow) on annuli (a) and distinct age gradient from proximal to distal.

The hepatopancreas of crayfish is the main organ of metabolism performing intestinal, hepatic and pancreatic functions (Vogt 2002; Gherardi *et al.* 2010). It is composed of dozens to hundreds of blindly ending tubules (Figure 8A), depending on age of the specimen. These tubules fuse together to form collecting ducts that finally terminate in the stomach. The

hepatopancreatic stem cells or E-cells are confined to stem cell niches at the distal ends of the tubules (Figure 8B). They give rise to three mature cell types with different cytological features and functions, the nutrient absorbing R-cells, the digestive enzyme synthesizing F-cells and the fat emulsifier producing B-cells (Vogt 2002; Gherardi *et al.* 2010).

The exclusive location of the stem cells at the blind ends of the hepatopancreas tubules and the propagation of their descendants in one direction only produces a distinct age gradient along the tubules. The E-cells divide in a late phase of each digestive cycle to replace discharged epithelial cells (Vogt 1994, 2002). The entire organ is probably renewed in less than two weeks as can be deduced from pulse-chase experiments with the crayfish *Procambarus acutus acutus* (Davis and Burnett 1964). Information on the role of DNA methylation and hydroxymethylation in adult stem cell regulation could be obtained by comparing E-cells with mature cells. Potential alterations of the DNA methylation pattern during cell ageing could be revealed by comparing the mature cells along the tubular age gradient.

The olfactory aesthetascs on the antennules (Figure 8C, D) are characterized by highly symmetric arrangement, distinct age gradient and life-long persistence. They first appear in juvenile stage 2 (Figure 8C) (Vogt and Tolley 2004). Starting from juvenile stage 3, new annuli and sensory units are added proximally to the existing ones at each moult (Vogt 2008b). As an example, they increased in number from 5 per antennule at juvenile stage 2 to 77 in an adult of 4.7 cm body length (Vogt 2016). The new aesthetascs are produced by progenitor cells that originate from small cells of the antennular epidermis (Vogt 2016). These small cells are either true stem cells or dedifferentiated epidermis cells. Each mature aesthetasc contains about 100 olfactory receptor neurons and some auxilliary cells. The axons of the new receptor neurons grow towards the olfactory lobe of the mid-brain to be wired into the neuronal network, resulting in regular enlargement and structural refinement of this brain area (Gherardi *et al.* 2010). Comparison of the aesthetascs of different regions of the antennule should provide information on whether biological age is reflected by the methylation pattern.

Crayfish and other decapods can actively autotomize damaged legs at a pre-formed fracture plane, which is an adaptive response to escape from a predator's grip. Regeneration starts with the formation of a blastema, which consists of mitotically active epidermis cells and undifferentiated cells that immigrate in large quantities (Hopkins *et al.* 1999). The immigrant cells may be multipotent blood cells or dedifferentiated cells from various organs. The regenerative blastema produces different tissues such as epidermis, musculature, connective tissue and nervous tissue. It would be interesting to compare the DNA methylation and hydroxymethylation pattern of the blastema cells, the early differentiating cells and the fully differentiated tissues of the regenerated limb. Of interest is also the comparison of the methylation patterns between original and regenerate, reflecting changes during ageing.

Epigenetic data on regeneration in marbled crayfish limbs are not yet available but there are first genetic data. The gene encoding for Baboon, a type I TGF-β superfamily receptor involved in the activin pathway, controls growth but not patterning of the new leg during regeneration (Shinji *et al.* 2016). Knockdown by RNAi of the *Smox* gene, which encodes a downstream transcription factor in the activin pathway, resulted in the formation of complete but smaller pereopods after autotomy, indicating that activin signaling via Smox functions in regulation of pereopod size (Shinji *et al.* 2017). The authors assumed that the *Baboon-Smox* system is an evolutionary conserved molecular signaling system involved in regeneration in both protostomes and deuterostomes.

Investigation of the involvement of epigenetic mechanisms in tissue homeostasis, regeneration and ageing in marbled crayfish may be of some interest for regenerative medicine (Weiss 2014; Stoltz *et al.* 2015). It might also contribute to the relatively new and controversial idea of the "epigenetic clock". This concept suggests using the cumulative effect

of the epigenetic system as a novel biological age determinant that may be of general value in developmental biology, cancer and ageing research (Horvath 2013; Schuhmacher 2017; Stubbs *et al.* 2017).

5.5 Transgenerational inheritance of epigenetic signatures

The transgenerational inheritance of epigenetic marks that are acquired during an individual's life is one of the hottest and most controversial topic of modern biology (Jablonka and Raz 2009; Crews and Gore 2014; Allis and Jenuwein 2016). Marbled crayfish could now contribute some new aspects to this theme from the perspective of parthenogenetic animals, which have not been considered so far. Since the possibility of transgenerational epigenetic inheritance (TEI) is closely linked with the fate of epigenetic marks in the germ cells I will discuss both issues in the following.

In the sexually reproducing mammals, DNA methylation marks are largely erased and reprogrammed on a global scale concomitant with restoration of developmental pluripotency. This reprogramming takes place a first time in the primordial germ cells and a second time in the zygote and early cleavage stages (Seisenberger *et al.* 2012; Petell *et al.* 2016). In zebrafish, the methylomes of the gametes are less intensely demethylated and there are significant differences between males and females (Jiang *et al.* 2013; Potok *et al.* 2013). The paternal methylome is largely maintained throughout early embryogenesis, whereas the maternal methylome is maintained only until the 16-cell stage and then progressively reprogrammed by losses and gains of methylation markers (Jiang *et al.* 2013). There is no information yet on such processes in asexually reproducing animals. However, despite the absence of potential triggers of global demethylation like fertilization they must anyhow restore pluripotency in the embryonic stem cells.

Investigation of this topic in marbled crayfish requires some knowledge on the development of the primordial germ cells, oocytes, zygotes and early cleavage stage, which is already available (Vogt and Tolley 2004; Alwes and Scholtz 2006; Vogt 2016). Hatchlings harbour one primordial germ cell in each of the paired ovarian anlagen (Figure 9A). In the following juvenile stages, these germ cells multiply and give rise to the oogonia (Figure 9B). Later, the oogonia are grouped into several germaria that become distributed throughout the ovary. These oogonia produce then pre-vitellogenic oocytes that leave the germaria and settle in oogenetic pouches. There, the oocytes become ensheathed by follicle cells and grow by the accumulation of yolk (Figure 9C). The freshly spawned eggs (equivalents of the zygote of sexual reproducers) are attached to the pleopods, where they undergo cleavage and further embryonic development (Figure 9D-F). All of these embryonic stages are readily accessible.

Comparison of DNA methylation between primordial germ cells, oogonia, growing oocytes, mature oocytes, freshly spawned eggs and the following cleavage stages should reveal whether the DNA methylation pattern is erased and reestablished as in mammals, reorganized by the parallel losses and gains of methylation marks like in zebrafish, or not changed at all. Comparison between marbled crayfish and *Procambarus fallax* should identify potential differences between sexually reproducing and parthenogenetic relatives.

First investigation of various embryonic stages of marbled crayfish with immunostaining, Dot plot analysis and mass spectrometry revealed the presence of 5-methylcytosine from the 256-cell stage (Grimmer 2015). Earlier stages revealed no reliable results due to technical problems. 5-hydroxymethylcytosine, which is often regarded as a marker of pluripotency (Cheng *et al.* 2015) was detected from the 1024-cell stage (Grimmer 2015). The methylation and demethylation enzymes Dnmt1, Dnmt3, and Tet were dynamically expressed during early embryonic development (Grimmer 2015) indicating the involvement of DNA methylation in differentiation and organogenesis. These first data demonstrate that marbled crayfish is well suitable for studying DNA methylation dynamics during parthenogenetic development.

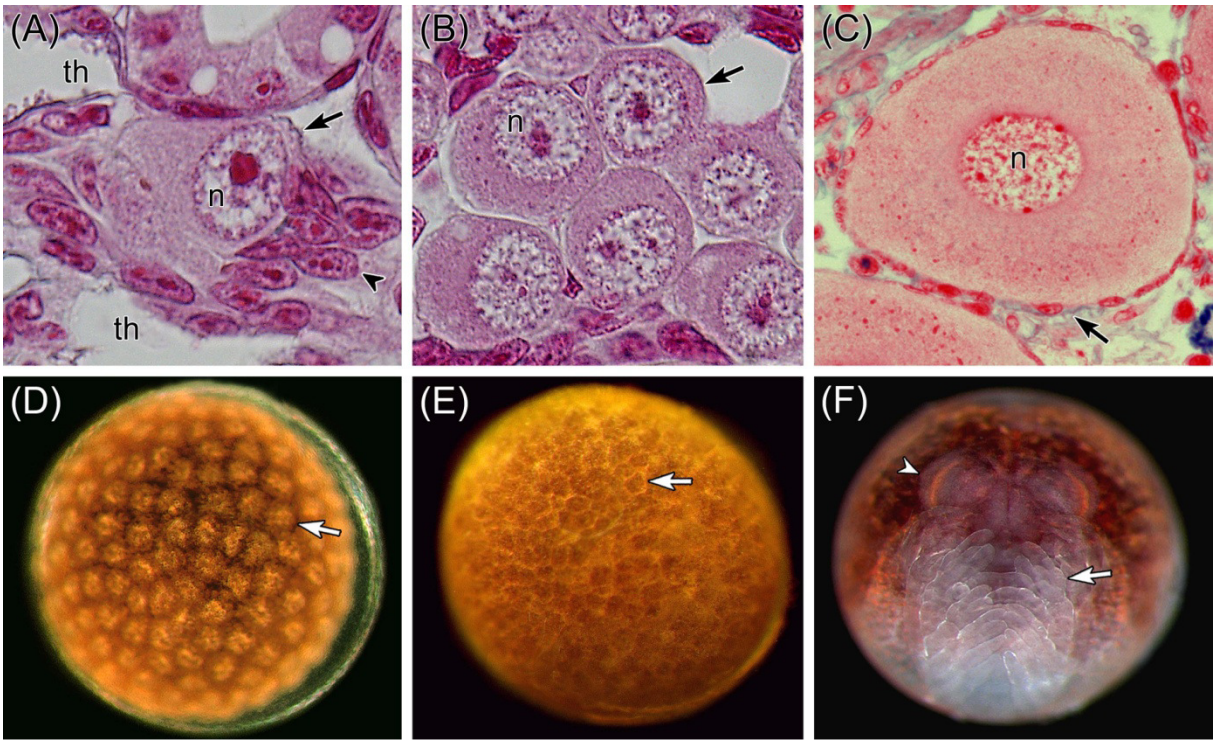

Figure 9. Key stages of germline and embryonic development in marbled crayfish. (**A**) Ovarian anlage in hatchling consisting of large primordial germ cell and surrounding matrix cells (arrowhead). The germ cell is not yet completely ensheathed by the ovarian envelope and has still direct contact (arrow) to the large thoracic haemal sinus (th). n, nucleus (from Vogt 2015a). (**B**) Ovary of stage-5 juvenile showing group of oogonia (arrow) (from Vogt 2007). (**C**) Vitellogenic oocyte ensheathed by follicle cells (arrow) (from Vogt *et al.* 2004). (**D**) 128-nuclei embryo showing energids without cell membranes on surface (arrow). (**E**) 256-cell embryo showing delimination of cells by membranes (arrow). (**F**) Embryo at 65% development with complete set of segments showing differentiation of eyestalks (arrowhead) and appendages (arrow).

The idea of TEI is not yet generally accepted. Earlier, this possibility was regarded as a myth by most researchers for two reasons: firstly, it had a Lamarckian touch, which was forbidden in biology, and secondly, the erasure of methylation marks in mammalian models seemed to make their inheritance to the next generation impossible. However, the latter argument is not applicable to animals that can reproduce asexually via fragmentation and budding like sponges, cnidarians, planarians or bryozoans and it is questionable for parthenogenetic animals. Meanwhile, there are convincing examples of TEI of DNA methylation signatures, histone modifications and non-coding RNAs published for plants and animals, including mammals (Rassoulzadegan and Cuzin 2015; Robertson and Richards 2015; Burggren 2016; Wang *et al.* 2017). Interestingly, there are significant differences among animal groups and even between males and females of the same species (Wang *et al.* 2017).

As a representative of the parthenogenetically reproducing animals, marbled crayfish can now contribute new aspects to this big question of biology, which has far-reaching implications for evolution (see chapter 5.10) and human health and diseases (Denham 2017; Wang *et al.* 2017). Long-term experiments with epigenetically manipulated strains (see chapter 6) in the laboratory under different stable and unstable environmental conditions should clarify, whether epigenetic signatures can be inherited across generations and under which conditions they are stabilized over generations. Both supposedly depend on the epigenetically altered DNA sequence itself, the environmental conditions and the cost of

resetting (Herman *et al.* 2014; Kronholm 2017). Ideally, only those epigenetic profiles should be preserved over many generations that enhance fitness in a constant environment or facilitate adaptation to new environmental challenges. Otherwise they should be reset.

5.6 Relationship between epigenetic and genetic variation

Epigenetic marks are on the nucleotides that built up the DNA double helix, and therefore, epigenetic variation is dependent on genetic variation but there is apparently also a certain degree of independence (Liebl *et al.* 2013; Skinner *et al.* 2014; Leung *et al.* 2016). For example, house sparrow *Passer domesticus*, which was introduced to Kenya in the 1950s, has since evolved significant epigenetic variation but retained rather low genetic variation (Liebl *et al.* 2013).

The epigenetic marks on the DNA can apparently be changed stochastically or by environmental induction (Feinberg and Irizarry 2010; Becker *et al.* 2011; Verhoeven and Preite 2014; Skinner 2015; Leung *et al.* 2016; Vogt 2017a). These epimutations are the basis for the generation of epigenetic variation from the same genetic template that may contribute to phenotypic variation. Epimutations are much more frequent than genetic mutations, for example 3×10^{-4} methylation gains or losses per CpG and generation compared with 7×10^{-9} base substitutions per site and generation in the model plant *Arabidopsis thaliana* (Van der Graaf *et al.* 2015). One may conclude from these data that the generation of phenotypic diversity by epigenetic mechanisms occurs with much higher speed when compared to genetic mutation. However, it is largely unknown how many single nucleotide polymorphisms or single base methylation polymorphisms are required to change phenotypic traits. Moreover, it is not yet established how stable epialleles are and under which conditions they are transgenerationally inherited.

The relative independence of epigenetic variation from genetic variation can best be investigated in genetically identical but phenotypically diverse monoclonal species like the marbled crayfish. Comparison of the genomes and epigenomes of brooded clutch-mates from the maternal pleopods that have not yet fed and experienced different environments should provide reliable information on the frequency of random genetic mutations and epimutations in a single generation. Exposure to different environmental conditions and toxicants should identify the environmental factors that are able to induce epimutations.

5.7 Contribution of epigenetics to behavioural variation and social hierarchy

Clonal animals can develop different behaviours despite genetic identity and identical rearing. This was shown for marbled crayfish (Vogt *et al.* 2008; Vogt 2015a) and Amazon molly *Poecilia formosa* (Bierbach *et al.* 2017). Social hierarchies in marbled crayfish are particularly prominent under stress conditions, for example when shelters are scarce (Vogt *et al.* 2008; Farca Luna *et al.* 2009). Usually, the biggest individual of a group is the dominant showing aggressive behaviours whereas the subordinates show evading behaviours (Farca Luna *et al.* 2009). When a dominant was placed in a group of considerably larger specimens it became soon subordinate and its formerly aggressive behaviours were replaced by avoiding behaviours. It would be interesting to see if the epigenetic signatures and expression patterns of genes involved in aggression (Panksepp *et al.* 2003) differ between dominants and subordinates and if such differences can be experimentally reversed.

Interestingly, the dominants in marbled crayfish groups of the same age grew much faster than the subordinates (figure 5B). This phenomenon is well known for sexually reproducing crayfish species and was explained by genetic differencs in aggression and food conversion efficacy and monopolization of food by the dominants (Reynolds 2002). In the genetically

uniform marbled crayfish, such growth differences must rely on epigenetic rather than genetic variation. Juveniles show initially no agonistic behaviours and start to establish social hierarchies from juvenile stage 7 when their claws become suitable for fighting (Vogt *et al.* 2008). This was shown in a 34-days experiment with five size-matched stage-6 clutch-mates that were grouped in a 30 x 20 x 20 cm aquarium equipped with a net only. In the first days of the experiment there were no behavioural differences but after a while dominants and subordinates emerged. First, the dominant and subordinates had similar sizes but with time the dominant grew much faster than the subordinates (Figure 5B) although food was not monopolized and all specimens fed regularly (Vogt *et al.* 2008). Dominants and subordinates may vary in the epigenetic patterns and expression of genes that maintain reinforcing circuitries involving behaviour, metabolism and neurohormonal regulation. The respective organs to be examined are the brain, hormone glands, hepatopancreas and musculature.

5.8 Relationship of epigenetic variation and disease susceptibility

There is increasing evidence that epigenetic factors can influence the susceptibility to diseases aside from genetic factors (Jirtle and Skinner 2007; Hitchins 2010; Brookes and Shi 2014). Marbled crayfish may help to investigate this issue in depth. A good model disease is the crayfish plague, which is caused by the oomycete *Aphanomyces astaci*. This disease is latently carried by the American Cambaridae species but breaks out only under conditions of severe stress. In contrast, the crayfish plague is absolutely lethal for the European Astacidae and Australian Parastacidae (Svoboda *et al.* 2017). In the last century, this catastrophic disease has wiped out most of the native European crayfish populations after its introduction with the cambarid *Faxonius limosus*.

Lake Moosweiher in Southern Germany harbours vivid populations of marbled crayfish and the sexually reproducing *Faxonius limosus*. *Aphanomyces astaci* is present in this lake and is carried by 9% of marbled crayfish and 4% of *F. limosus* (Keller *et al.* 2014). In the sexually reproducing *F. limosus* the differences in infection rate may be explained by genetic differences among individuals but in the isogenic marbled crayfish this explanation is not applicable. Instead, differences in the susceptibility to *A. astaci* must be due to epigenetic variation among individuals. Other populations of marbled crayfish also showed variations in crayfish plague susceptibility: in a pond in Klepzig (Germany) and a Dutch laboratory the degree of infection was 9% and 70%, respectively (Keller *et al.* 2014).

First data on the suspected relationship between epigenetic variation and disease susceptibility could be gained by comparison of the DNA methylation profiles of immune defence genes between healthy and crayfish plague-infected marbled crayfish. The experimental modification of plague promoting and suppressing candidate DMRs by CRISPR/Cas9 (see chapter 6) could then refine the obtained picture. Reversing disease promoting epigenetic marks is of great interest in biomedicine (Santos-Rebouças and Pimentel 2007) and marbled crayfish may help to figure out how this could be done in practice.

5.9 Contribution of epigenetic phenotype variation to environmental adaptation

There is evidence from literature that epigenetic variation may help animals to adapt to environmental challenges by broadening the range of phenotypes in a population (Skinner *et al.* 2014; Leung *et al.* 2016; Vogt 2017a). This strategy seems particularly advantageous for clonal lineages, small invasive groups and genetically depauperate populations and may explain controversial ecological issues like the invasion paradox (Sax and Brown 2000) and the general purpose genotype (Massicotte and Angers 2012). The monoclonal marbled crayfish seems particularly suitable to investigate this topic in detail because it has invaded

different geographical regions in Europe, Madagascar and Japan since 2003 and has adapted to a wide spectrum of habitats (Jones *et al.* 2009; Chucholl 2016; Vogt 2017a).

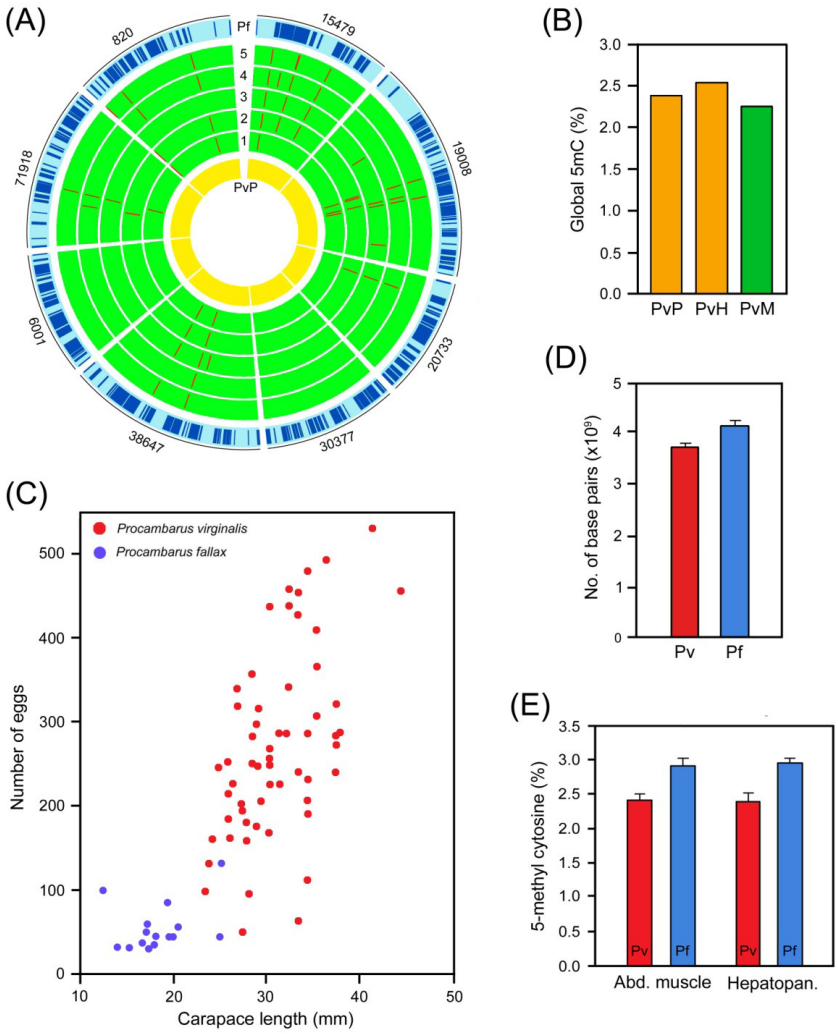

Figure 10. Genetic and epigenetic underpinning of environmental adaptation and speciation. (**A**) Comparison of homozygous single nucleotide variations (vertical bars) in eight arbitrarily chosen genomic scaffolds of a total length of 1.5 Mb among differently adapted *Procambarus virginalis* (PvP, 1-5) and *P. fallax* (Pf). There are pronounced differences between *P. virginalis* (yellow and green) and *P. fallax* (blue) but high similarity between marbled crayfish adapted to laboratory conditions (PvP, specimen from Petshop lineage = reference genome) and natural habitats in Madagascar (1-5). The data suggest that species differences are based on genetic differences whereas ecological adaptation is not (redrawn and modified after Gutekunst 2017). (**B**) Comparison of global DNA methylation between marbled crayfish from laboratory lineages (PvP, Petshop; PvH, Heidelberg) and Lake Moosweiher (PvM). The laboratory raised specimens show slightly higher values than the wild specimen (data from Vogt *et al.* 2015). (**C**) Comparison of size and fecundity of *P. virginalis* from Malagassy swamps and *P. fallax* from Floridan swamps. Marbled crayfish grow bigger and are much more fecund than slough crayfish (data from Hendrix and Loftus 2000 and Jones *et al.* 2009). (**D**) Comparison of the haploid genome size between *P. virginalis* and *P. fallax*. Marbled crayfish has lost ~10% of genomic sequence during speciation (redrawn and modified after Vogt *et al.* 2015 and Falckenhayn 2016). (**E**) Comparison of global DNA methylation between *P. virginalis* and *P. fallax*. Marbled crayfish has ~20% lower methylation values than slough crayfish (redrawn and modified after Vogt *et al.* 2015).

Marbled crayfish raised under the stringent laboratory conditions outlined in chapter II.2 reached maximum total lengths of 8.8 cm and maximum weights of 20 g (Vogt 2010), whereas wild specimens reached maximum sizes of 11 cm and weights of 32 g (Chucholl and Pfeiffer 2010), resulting from better living conditions and food. Comparison of the complete mitochondrial genomes (Figure 1C), several microsatellite loci (Vogt *et al.* 2015) and 1.5 million bp long stretches of nuclear DNA (Figure 10A) from laboratory raised and wild specimens showed no or very little genetic variation, suggesting that environmental adaptation in marbled crayfish is not the result of genetic variation. Instead, it may have been achieved by epigenetic variation resulting from differences in DNA methylation, histone modifications and microRNAs. First comparison of global DNA methylation between marbled crayfish from the Heidelberg and Petshop lineages and Lake Moosweiher revealed higher values in laboratory-raised than wild specimens (Figure 10B) (Vogt 2017a). More meaningful information on the involvement of DNA methylation in environmental adaptation is expected from the comparison of the entire methylomes, which is presently done in the Lyko group at the DKFZ in Heidelberg, including the multiple populations of Madagascar. Other epigenetic mechanisms like histone modifications and microRNAs that can generate phenotypic variation should be analysed as well. Respective whole-genome approaches are available (Roh *et al.* 2004; Chen *et al.* 2016).

5.10 Role of epigenetic mechanisms in evolution and speciation

There is some evidence, mainly from plants, that epigenetic mechanisms contribute to speciation (O'Neill *et al.* 1998; Madlung and Wendel 2013; Vogt 2017a). Marbled crayfish seems well suitable to investigate this topic in depth because it originated from *Procambarus fallax* by polyploidization, which is generally associated with prominent rearrangements of the genome and methylome (ref. in Vogt 2017a). *P. fallax* and marbled crayfish differ considerably in growth and fecundity, DNA-content and global DNA-methylation. Marbled crayfish grow considrably bigger than *P. fallax* and produce significantly more eggs per clutch (Figure 10A). Comparison of the genomes of both species revealed a reduction of the haploid genome size from 4.1 Gb in *P. fallax* to 3.7 Gb in marbled crayfish (Figure 10D) (Vogt *et al.* 2015; Falckenhayn 2016). Nevertheless, each body cell of marbled crayfish contains higher total amounts of DNA due to triploidy. Global DNA methylation is in average about 20% lower in marbled crayfish than in slough crayfish as revealed for whole specimens and individual organs (Figure 10E).

The separate evolution of new species requires reproductive isolation from the parent species. Epigenetic mechanisms can promote reproductive isolation as was shown for several plant species (ref. in Lafon-Placette and Köhler 2015) and the deer mouse *Peromyscus maniculatus* species complex (Vrana 2007). In the latter, the reproductive barrier is provided by epigenetic imprinting of genes involved in placentation. Marbled crayfish females readily copulated with males of *P. fallax* in laboratory experiments but the offspring were always pure marbled crayfish (Vogt *et al.* 2015). The exact mechanism of reproductive isolation between parent species and neo-species is not yet known.

In literature, empirically determined interspecies differences of epigenetic patterns are mostly interpreted as the result of genetic differences between species (e.g. Seymour *et al.* 2014; Hagmann *et al.* 2015), interpreting epigenetic changes as followers of genetic changes. However, some authors consider possible that under specific conditions epigenetic changes can come first and lead to genetic changes on the long run, being leaders rather than followers in evolution (Nanjundiah 2003; Jablonka and Lamb 2014; Skinner *et al.* 2014; Kronholm and Collins 2016; Vogt 2017a, b). Klironomos *et al.* (2013) demonstrated by modelling that when natural selection acts on epigenetic variation in addition to genetic variation, populations adapt faster and adaptive phenotypes can arise before any genetic changes occur.

The relationship of epigenetic variation, genetic variation, adaptation and selection could empirically be tested by comparing closely related species of different evolutionary age and environmental adaptation (Kronholm 2017; Vogt 2017a). Young and less well adapted species should show more epigenetic variation related to environmental adaptation, while older populations should show an adaptation with a genetic basis. The species pair *Procambarus virginalis* (evolutionarily young, ongoing invasions) and *P. fallax* (evolutionarily old, well adapted) is well suitable to investigate this issue in depth. If epigenetically determined phenotypes could be inherited, selected and genetically fixed over time, then our understanding of evolution would be revolutionized.

6. Production of genetically and epigenetically edited strains for research

Knockout and knockin of genes allows direct studying of gene functions. Several gene manipulating techniques were already applied in freshwater crayfish (Sarmasik *et al.* 2000; Pamuru *et al.* 2012; Sagi *et al.* 2013). The most promising technique for future gene editing in marbled crayfish is the recently developed CRISPR/Cas9 tool (Sander and Young 2014). This technique proved already highly effective in producing heritable knockouts and knockins in other crustaceans like water flea *Daphnia pulex* (Nakanishi *et al.* 2014), sandhopper *Parhyale hawaiensis* (Martin *et al.* 2016b) and shrimp *Exopalaemon carinicauda* (Gui *et al.* 2016). CRISPR/Cas9 is also useful to add and remove methylation marks on the DNA (Liu *et al.* 2016b; McDonald *et al.* 2016; Xu *et al.* 2016) but this application has not yet been applied in crustaceans. Thanks to the availability of a genome and methylome both approaches could now be adopted for marbled crayfish.

The most suitable life stages for genetic and epigenetic manipulations in marbled crayfish are the early embryonic stages between the zygote and 128-nuclei stage and the hatchlings. In the early embryonic stages the nuclei are not yet separated from each other by cell membranes (Figure 8C), and therefore, microinjected agents should reach all nuclei equally. Thus, all cells of the developing individual including the future germ cells should carry the induced mutation or epimutation, avoiding mosaicism. So far, marbled crayfish embryos have neither been microinjected nor cultured from the zygote to the hatching stage. However, a microinjection system for decapod embryos was recently established for shrimps (Gui *et al.* 2016) and *in vitro* culture through the entire embryonic development was achieved in other crayfish species (Henryon and Purvis 2000). These techniques should now be adopted for marbled crayfish.

Microinjection of hatchlings is an alternative to microinjection of early embryos to circumvent potential problems with the rigid egg envelopes and *in vitro* culture of the embryos. Hatchlings possess one primordial germ cell on each body side that is not yet completely ensheathed by the ovarian matrix (Figure 8A). These germ cells should be easily accessible for manipulating agents injected into the neighbouring thoracic sinus and all offspring should be equally transgenic. This approach would require one more generation than microinjection in early embryos to obtain transgenic specimens. Microinjection of adult females is less recommended because only a part of the offspring becomes transgenic as shown by Sarmasik *et al.* (2001) for the closely related *Procambarus clarkii*. Marbled crayfish have the advantage over sexually reproducing animal models that the transgenes would be inherited in true-to-type form if not excised by DNA repair mechanisms.

7. Conclusions

(1) Marbled crayfish is a novel research model, popular pet and highly invasive species. It was so far mainly used for research in morphology, physiology, development and neurobiology. The availability of a draft genome, transcriptome and genome-wide methylome

now enables studying of the genomic and epigenetic aspects of topical issues of biology and medicine.

(2) My motivation to write this article was to encourage colleagues from various disciplines to consider this promising model for investigating puzzling problems in their field. The article is hoped to provide a solid data and references base and some stimulating ideas for future research. It was my special concern to provide newcomers with detailed information on the advantages and biological peculiarities of this species to minimize mistakes in study design and data interpretation.

(3) Marbled crayfish is an obligately apomictic parthenogen. All laboratory lineages and wild populations are of the same origin and monoclonal, making this crustacean unique among the animal models. Its main advantage is the production of large numbers of offspring genetically identical to the mother allowing extensive experimentation on the relationship of genotype, epigenotype and phenotype.

(4) The size of the genome of marbled crayfish is close to the human genome and in the order of magnitude of mammals, fish and some species-rich invertebrate groups such as crustaceans, molluscs and hemimetabolic insects. The estimated number of genes is close to the published values of mouse and humans making marbled crayfish a good animal representative for genomics research.

(5) First experimental data suggest that marbled crayfish is well suitable to investigate the genetic specification of decapods and the genetic basis of reproduction, immune defence and moulting in arthropods. It is also suitable to investigate more general tropics like the genetic underpinning of omnivory, adaptation to fresh water, sexual system shifts, behavioural variation and cancer resistance, and the evolution of functionally diverse gene families and clonal genomes. To me, the genetic underpinning of resistance to environmentally induced and age related cancer is the most interesting issue because its understanding may initiate the development of new cancer therapies in humans at best.

(6) The level of global DNA methylation in marbled crayfish is close to values in humans and the mammalian models but an order of magnitude higher than in most insects. The methylation and demethylation machinery of marbled crayfish consists of an ancestral pattern of single copies of Dnmt1, Dnmt3 and Tet but these enzymes are apparently highly effective. Marbled crayfish shares crucial features of DNA methylation such as CpG methylation, gene body methylation and repeat methylation with many other animals. Thus, the marbled crayfish methylome seems to be a good animal representative for epigenetics research.

(7) First experimental data indicate that marbled crayfish is well suitable to investigate the involvement of epigenetic mechanisms in gene regulation, cell type identity, stem cell determination, regeneration, ageing, disease susceptibility, environmental adaptation and evolution. To me, the most interesting epigenetics theme to which marbled crayfish could contribute is the role of epigenetic phenotype variation in ecological adaptation and evolution.

(8) Future epigenetics research with the marbled crayfish should be extended to other mechanisms like histone modifications and microRNAs. Particularly promising is genome and epigenome editing with the CRISPR/Cas9 technique that enables direct studying of the function of genes and epigenetic modifications, which is of fundamental relevance in biology.

(9) Research of the last years confirmed the particular suitability of marbled crayfish for addressing puzzling questions of epigenetics. If things go well, marbled crayfish may one day gain the same importance for epigenetics that *Drosophila* has for genetics.

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