Ecotoxicology (2011) 20:607–624
DOI 10.1007/s10646-011-0634-0

Epigenetics and its implications for ecotoxicology

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Accepted: 5 March 2011 / Published online: 22 March 2011

Epigenetics is the study of mitotically or meiotically heritable changes in gene function that occur without a change in the DNA sequence. Interestingly, epigenetic changes can be triggered by environmental factors. Environmental exposure to e.g. metals, persistent organic pollutants or endocrine disrupting chemicals has been shown to modulate epigenetic marks, not only in mammalian cells or rodents, but also in environmentally relevant species such as fish or water fleas. The associated changes in gene expression often lead to modifications in the affected organism's phenotype. Epigenetic changes can in some cases be transferred to subsequent generations, even when these generations are no longer exposed to the external factor which induced the epigenetic change, as observed in a study with fungicide exposed rats. The possibility of this phenomenon in other species was demonstrated in water fleas exposed to the epigenetic drug 5-azacytidine. This way, populations can experience the effects of their ancestors' exposure to chemicals, which has implications for environmental risk assessment. More basic research is needed to assess the potential phenotypic and population-level effects of epigenetic modifications in different species and to evaluate the persistence of chemical exposure-induced epigenetic effects in multiple subsequent generations.

Keywords DNA methylation · Transgenerational effects · Invertebrates · Environmental toxicology

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Introduction

Epigenetics is the study of heritable changes in gene function that occur without a change in the DNA sequence which forms the genetic code, hence the prefix 'epi', from the Greek word meaning 'above, beyond'. Several reviews have dealt with the subject, each with a specific line of approach: e.g. molecular targets of epigenetic mechanisms, transgenerational inheritance of epigenetic modifications, consequences of epigenetic changes for safety assessment, animal models to study epigenetics, epigenetic impacts of adverse environmental effects in mammals (Rosenfeld 2010; Choudhuri et al. 2010; Jablonka and Raz 2009; LeBaron et al. 2010; Bollati and Baccarelli 2010). Toxicant exposure can perturb the epigenetic status of cells and organisms, as known from several studies on cell lines or rodent models. Since a perturbed epigenetic status can entail changes in gene expression levels, which may have effects on organisms and populations, the study of epigenetics is also highly relevant for ecotoxicologists. In ecotoxicology however, epigenetic research is still very limited. Therefore, the aims of the current review are (1) to give a brief background of epigenetics and epigenetic mechanisms and (2) to summarize and discuss the current literature on environmental impacts on epigenetics, with a focus on toxicant exposure, highlighting the present knowledge and research needs related to epigenetics in ecotoxicology.

Epigenetics

Definition

Various authors have provided a definition for the constantly evolving science of epigenetics. The term was

introduced by Conrad Waddington (1939) to describe 'the causal interactions between genes and their products, which bring the phenotype into being'. This was mainly used in the context of unfolding the genetic program for development. In the last decade of the twentieth century, scientists realized that epigenetics had broader implications and two new definitions of epigenetics were proposed (Holliday 1994): 'The study of the changes in gene expression which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression' and 'Nuclear inheritance which is not based on changes in DNA sequence'. The first definition is rather broad, stating nothing about molecular mechanisms, but stressing the importance of mitotic inheritance, which is the inheritance of an epigenetic status between cells after normal mitotic cell division. In the second definition, the evolution in the genetic field since Waddington's paper is obvious: it points to mechanisms not related to changes in the sequence of the four known DNA bases. Based on Holliday's two definitions, a new definition was put forward by Riggs et al. (1996). Variants of this working definition are still used in current textbooks and by many scientists in the field (Allis et al. 2007; Berger et al. 2009). It is also this definition that will be used in this review: 'Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence'.

Mechanisms

Several molecular mechanisms are known to be involved in epigenetics. Next to the three important and well-studied systems that are described below, new molecular aspects of epigenetic control have very recently been discovered and are subject of intensive research nowadays, e.g. cytosine hydroxymethylation and nucleosome-space occupancy (Tahiliani et al. 2009; Cui et al. 2010).

DNA methylation

DNA methylation in eukaryotes occurs through the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to the 5-position of cytosine, to form 5-methylcytosine (Fig. 1a). In mammals this mainly occurs in a CpG (deoxycytidine linked with a phosphate group to deoxyguanosine) dinucleotide context, while in plants methylation is also found at CZG or CZZ sites (with Z = A, C or T for deoxyadenosine, deoxycytidine or thymidine, respectively, with all neighboring nucleosides linked by a phosphate group) (Zhang 2008). Methylated cytosines can be deaminated and transformed into thymidine. As a result of this C to T transition mutation, which is difficult to repair, methylated CpGs are expected to decrease in abundance with evolutionary time, resulting in CpG deficiency. The ratio of observed to expected CpGs can be used to predict methylated and unmethylated genomic regions (Wang and Leung 2008). The enzymes responsible for DNA methylation are DNA methyltransferases or DNMTs. In humans, three groups of DNMTs are described. DNMT1 is the maintenance methyltransferase. DNMT3 enzymes establish methylation patterns in a totally unmethylated CpG context (de novo methylation). DNMT2, while possessing a weak DNA methyltransferase activity, is actually also involved in tRNA methylation (Jurkowski et al. 2008; Jeltsch et al. 2006). Adenine methylation is known to occur in bacteria (Collier 2009), however, discussion of this mechanism is beyond of the scope of this review.

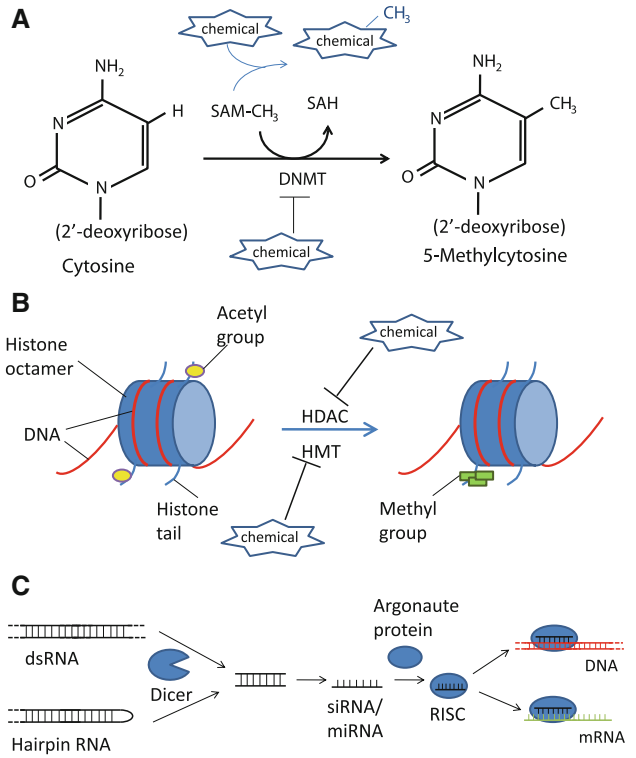

Fig. 1 Schematic diagram of three main epigenetic mechanisms. **a** DNA methylation by the transfer of a methyl group ($-CH_3$) to the 5-position of cytosine, to form 5-methylcytosine. SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine, DNMT: DNA-methyltransferase. Chemicals may interact by e.g. using SAM as a methyl donor in their metabolisation or inhibiting the action of DNMTs. **b** Histone deacetylation and histone methylation represent histone tail modifications. HDAC: Histone deacetylase, HMT: histone methyltransferase. Chemicals may interact with the action of both HDACs and HMTs. **c** Non-coding RNA molecules such as microRNA (miRNA) and small interfering RNA (siRNA) are formed from double-stranded RNA (dsRNA) or hairpin RNA molecules. The RNA-induced silencing complex (RISC) is targeted to complementary messenger RNA (mRNA) or DNA and interferes with translation or transcription

Levels and patterns of DNA methylation can differ substantially between different species. The nematode worm *Caenorhabditis elegans* does not possess a conventional DNA methyltransferase and completely lacks detectable DNA methylation (Bird 2002). The fruit fly *Drosophila melanogaster* was also long thought to have an unmethylated genome. However, now it is known that it possesses a DNA-methyltransferase-like gene. A low level of methylation was observed in its genome: 0.1–0.4% of the cytosines were methylated, with the highest levels noted in early-stage embryos. An additional peculiarity is that the methylation in *D. melanogaster* is mostly found in CpT and CpA dinucleotides, as opposed to the CpG dinucleotide context of vertebrate DNA methylation (Tweedie et al. 1999; Lyko 2001). In the genome of the honey bee *Apis mellifera* and the wasp *Nasonia vitripennis*, homologs to all three types of DNMTs were found and CpG methylation has been observed in several genes. However, the overall DNA methylation level has not been quantified yet (Wang et al. 2006; Schaefer and Lyko 2007; The *Nasonia* Genome Working Group 2010). CpG methylation has also been detected in DNA of the water flea *Daphnia magna* (Vandegehuchte et al. 2009a). In this species, 0.22–0.35% of the cytosines are methylated under standard conditions (Vandegehuchte et al. 2009b). Other invertebrate genomes display moderate CpG methylation levels, often in a pattern known as mosaic methylation: methylated cytosines concentrated in large domains, separated by domains with unmethylated DNA. In the sea urchin *Strongylocentrotus purpuratus* and the sea squirt *Ciona intestinalis,* roughly half of the genome consists of DNA domains in which most of the CpGs are methylated, while the remaining DNA is methylation-free (Tweedie et al. 1997; Suzuki et al. 2007). In vertebrates, the methylation level is high: approximately 5% of the cytosines are methylated in DNA of mammals and birds and roughly 10% in that of fish and amphibians (Field et al. 2004). This methylation is spread evenly throughout almost the entire genome with the notable exception of short CpG-rich regions named CpG islands, found at the 5′ position of a large number of genes and which are generally unmethylated (Suzuki and Bird 2008). These different methylation distributions suggest that DNA methylation may have different functions in different organisms.

In vertebrates, DNA methylation is generally associated with silencing of genes or maintaining a silenced state. In the past decade a consensus view had appeared that DNA methylation is a mediator of transcriptional silencing. This was mainly based on studies in CpG islands of gene promoters in mammalian DNA. However, recent data on vertebrate DNA, as well as some less known studies of invertebrate DNA methylation, suggest a broader role of this epigenetic mark. Gene body methylation (as opposed to promoter methylation) of the *Ddah2* gene was shown to be a biomarker for embryonic stem cell differentiation to neural stem cells (Backdahl et al. 2009). Remarkable in this study is that DNA methylation was positively correlated with gene expression. In different clones of the aphid *Myzus persicae*, gene body methylation was shown to play a role in the transcription of E4 genes, coding for insecticide-detoxifying esterase proteins. As in the study of Backdahl et al., methylated genes were expressed, while loss of the methylation was correlated with a loss of transcription (Field 2000). Bongiorni et al. (1999) demonstrated that unmethylated chromosomes of the mealybug *Planococcus citri* were inactivated by heterochromatinization, while methylated chromosomes remained active. In the genome of the honey bee *Apis mellifera*, CpG methylation is suggested to be used as an epigenetic mechanism to maintain transcription of 'housekeeping genes', necessary for conserved core biological processes in virtually every type of cell (Foret et al. 2009). A newer hypothesis for the functional role of DNA methylation, supported by the above described data, is the prevention of transcription initiation (Zilberman 2008; Suzuki and Bird 2008). This preserves the idea that DNA methylation is a transcriptional repressor, but allows intragenic methylation in an active transcription unit: i.e. it does not preclude transcription as such, but rather its (spurious) initiation. Moreover, this concept is in line with the cyclical DNA methylation/demethylation which has been described in transcriptional cycling of a gene in a human cell line (Metivier et al. 2008).

Histone tail modifications or the histone code

The genome is organized in chromatin, consisting of DNA and associated proteins. The building blocks of chromatin are nucleosomes, which consist of approximately 180–190 base pairs, of which the majority is coiled twice around an octamer of histone (H) proteins (four times two molecules of H2A, H2B, H3 and H4). The linker histone protein H1 at the outside of the nucleosome serves to further compact the chromatin (Pruss et al. 1995). The structure of these histones is not static. Their mutual affinity as well as their affinity for DNA and for other chromatin associated proteins is determined by post-translational modifications of their protruding amino-terminal tails. These modifications may include acetylation, ubiquitination, phosphorylation or methylation (Fig. 1b). Together, they form a histone code, which affects gene transcription and chromatin structure (Lennartsson and Ekwall 2009). These modifications are performed by specialized enzymes such as histone acetyl transferases (HAT), histone methyl transferases (HMT) and histone deacetylases (HDAC). Active histone marks, e.g. acetylation of H3 on lysine 9 (H3K9) or dimethylation of

H3K4, lead to chromatin decondensation and thus result in the formation of euchromatin, creating conditions for gene transcription. Conversely, condensed heterochromatin lacks this histone acetylation and is enriched in trimethylated lysine 9 and lysine 27 on H3 (Bartova et al. 2008). Two important types of proteins can be found in association with specific histone modifications: the trithorax group proteins and the polycomb group proteins, which are associated with transcriptionally active euchromatin and transcriptionally silent heterochromatin, respectively (Schuettengruber et al. 2007).

It is known that there is interaction between histone modifications and DNA methylation, although the precise mechanisms have not been completely discovered. In a study with knock-out mice lacking a DNA methyltransferase, it was shown that histone methylation in certain genomic regions was markedly decreased (Henckel et al. 2009). This demonstrated that DNA methylation is involved in the establishment and/or maintenance of histone methylation in those regions. Methylated DNA can also cause histone deacetylation, through binding with proteins such as MeCP2, which interact with histone deacetylases (Nan et al. 1998). Fuks (2005) on the other hand, suggested that DNA methylation is a secondary epigenetic modification by DNA methyltransferase enzymes which are attracted to locations where histone H3 is methylated at lysine 9. It has also been shown that the methylation of H3K27 was necessary for the recruitment of DNA methyltransferases to methylate DNA in the promoters of certain genes (Vire et al. 2006). Future research will further elucidate the interactions and crosstalk between the histone code and the DNA methylation machinery.

Non-coding RNA (ncRNA)

Gene silencing through small (approximately 20–30 nucleotides) non-coding RNA molecules most probably originated as a protection system against invasive sequences such as transposons and viruses (Zilberman and Henikoff 2005). 'Non-coding' refers to the fact that these RNA strands do not function as messenger RNA, transfer RNA or ribosomal RNA. The process of gene expression regulation by small ncRNA molecules is also known as RNA interference or RNAi (Yu 2008). The two main categories of these regulatory small RNAs are microRNA (miRNA) and short interfering RNA (siRNA). These small RNAs are excised from larger, double-stranded or hairpin-shaped precursor molecules by a Dicer enzyme. They associate with effector proteins (Argonaute proteins) to form an RNA-induced silencing complex (RISC), which is targeted to complementary nucleic acid sequences (Fig. 1c). Silencing of genes can happen through transcriptional or translational inhibition, heterochromatin formation or DNA/RNA degradation (Carthew and Sontheimer 2009).

The RNA silencing system is also known to interact with other epigenetic mechanisms. In association with RNA polymerase, RISC can attract or activate DNA methyltransferases (DNMTs), resulting in methylation of DNA. This is a known mechanism of siRISC silencing in plants, but also occurs in mammals (Lippman and Martienssen 2004). RNAi is also suggested to play a role in the protection of genomes against long-term epigenetic defects, targeting remethylation of specific sequences (Teixeira et al. 2009). In the yeast *Saccharomyces pombe* it was discovered that the siRISC, also termed RNA-induced transcriptional silencing complex (RITS), guides a histone methyltransferase to methylate H3K9, ultimately resulting in heterochromatin formation. This process also takes place in plants and mammals (Wassenegger 2005).

Environmental factors and epigenetics

Introduction

Several types of environmental factors are known to affect the epigenetic status of organisms and changes in all known epigenetic mechanisms can be triggered by environmental cues. Recently, alterations in DNA methylation and gene expression due to space flight, a complex environmental condition involving e.g. cosmic radiation, microgravity and space magnetic fields, were demonstrated in the rice plant *Oryza sativa* (Ou et al. 2009). Maternal care was shown to affect DNA methylation, histone acetylation and behavior of the offspring in a study of the licking-grooming behavior of maternal rats toward their pups (Weaver et al. 2004).

The notion that diet can influence epigenetics originated in studies with children born during the Dutch Hunger Winter in the Second World War. Adults who were exposed to the famine as children in utero exhibited less DNA methylation at a locus in the insulin like growth factor gene and a larger incidence of coronary heart disease and obesity, compared to their non-exposed siblings (Heijmans et al. 2008). Laboratory studies with rats and primates demonstrated that unbalanced prenatal nutrition can induce histone acetylation changes or persistent, gene-specific epigenetic changes which affect gene transcription (Aagaard-Tillery et al. 2008; Lillycrop et al. 2005).

Also in non-mammalian species, nutrition can dramatically affect the phenotype by inducing epigenetic changes. In a honey bee (*Apis mellifera*) colony, fertile queens and sterile workers develop from genetically identical larvae. Larvae destined to become queens are fed with royal jelly, while worker larvae get a less complex diet. Kucharski

et al. (2008) demonstrated, in workers versus queens, different degrees of DNA methylation at several loci. Moreover, by silencing the DNA methyltransferase Dnmt3 in newly-hatched larvae, they induced the development to queens in the majority of these larvae. Thus, these results suggest that DNA methylation stores epigenetic information, which can be altered by nutrition and which has a profound effect on the development and the adult phenotype of the honey bee. The impact of nutrition on epigenetics can be relevant to ecotoxicological testing, where organisms exposed to chemicals often need to be fed during the exposure. However, to date this is an unexplored area of research.

Transgenerational epigenetic effects

A very interesting aspect of epigenetic changes is the fact that they can, in some cases, be inherited by subsequent generations which are not exposed to the environmental factor that caused the change. Several recent reviews have described this phenomenon, each from a slightly different point of view and with own interpretations of 'transgenerational transfer' or 'inheritance' (Jablonka and Raz 2009; Ho and Burggren 2010). In this section, some studies on the transgenerational transfer of epigenetic marks and their alterations due to environmental factors will be discussed.

DNA methylation profiles are transferred through somatic cell divisions by the action of maintenance DNA methyltransferases. In sexually reproducing organisms, epigenetic variations in DNA methylation should survive the complex process of meiosis in order to be transmitted to the next generation. In multicellular organisms, they should also be maintained after gametogenesis and embryogenesis, resisting the significant restructuring of cells and chromatin which takes place at these developmental stages. In mammalian development, DNA is progressively demethylated during the preimplantation period, after which remethylation takes place (Bocock and Aagaard-Tillery 2009). Developmental DNA methylation changes have also been demonstrated in the fruit fly *Drosophila melanogaster* and the zebrafish *Danio rerio* (MacKay et al. 2007). There is evidence that changed DNA methylation patterns can be transmitted over generations in spite of these developmental alterations, but at present it is not clear how this occurs mechanistically (Bonduriansky and Day 2009). Some authors argue that gametic inheritance of an epigenetic change induced by exposing a gestating female to environmental stress can only be proven if this change is still present in the F_3 generation. Indeed, the primordial germ cells creating the F_2 generation are already present in the F_1 embryo inside the pregnant F_0 female (Youngson and Whitelaw 2008). However, if an epigenetically induced phenotype in the non-exposed F_2 generation is observed after exposure of F_0 pregnant females, epigenetic patterns must have been transferred between cells in the F_2 generation. In that respect, this can also be called a transgenerational epigenetic effect.

The occurrence of transgenerational epigenetic effects has inspired several authors to study the impact of epigenetics on evolution. In a somewhat provocative commentary, Martienssen (2008) used the term 'adaptive Lamarckian evolution', referring to epigenetic mechanisms that may enable the environment to direct evolution at a short time scale. In a breakthrough study with *Arabidopsis*, Johannes et al. (2009) created a panel of epigenetic Recombinant Inbred Lines, with little DNA sequence differences, but contrasting DNA methylation profiles. Stable inheritance of multiple parental DNA methylation variants was demonstrated over at least eight generations, associated with heritability of flowering time and plant height. This research demonstrated the relevance of incorporating epigenetic information in population genetics studies. It should be clear that epigenetics does not deny or reject classic Darwinian evolution or genetics, but rather adds an extra dimension to it. This is nicely illustrated in a study of wild populations of the Spanish violet *Viola cazorlensis*, showing epigenetic differentiation of different populations at the DNA methylation level, which was correlated with adaptive genetic divergence demonstrated by a population-genomic analysis (Herrera and Bazaga 2010). In the context of ecotoxicology, a knowledge gap that requires dedicated study concerns the potential of chemicals-induced epigenetic modifications to be stably transmitted over several generations. This could lead to epigenetic micro-evolution, with e.g. local adaptation to stress, as suggested by Morgan et al. (2007). In population studies of micro-evolution due to chemical stress exposure, it would be useful to screen for epigenetic modifications, next to studying genetic adaptation.

A special model organism for studying (transgenerational) environmental effects on DNA methylation is the viable yellow agouti (A^{vy}) mouse. In the A^{vy} locus of this model system genome, a transposon is associated with the promoter region of the agouti gene, which codes for coat color. When the agouti gene is fully expressed, these inbred mice have yellow fur and suffer from obesity, diabetes and increased tumor susceptibility. However, expression of this gene depends on the degree of methylation in the A^{vy} locus. When this locus is hypermethylated, mice will be brown or 'pseudoagouti'. A hypomethylated A^{vy} locus will result in a yellow coat color, while partial methylation is reflected in slightly or more densely 'mottled' fur color, depending on the degree of methylation. As such, this model allows for direct phenotypic assessment of the epigenetic DNA methylation state at the A^{vy} locus (which is called a metastable epiallele). Interestingly, mice that were

fed a diet enriched with the methyl donors folic acid, vitamin B12, choline and betaine exhibited a shift in phenotype in their offspring due to increased DNA methylation at the A^{vy} locus (Waterland and Jirtle 2003). Similar results were obtained in another mouse model, where methylation of the $Axin^{Fu}$ metastable epiallele determines the shape of the tail: from straight to very kinky. Feeding female mice a methyl donor-rich diet resulted in a clear shift towards the straight tail phenotype in the offspring (Waterland et al. 2006).

The naturally occurring DNA-methylation-dependent variable phenotypes of A^{vy} and $Axin^{Fu}$ mice can be transmitted to the subsequent generation. Morgan et al. (1999) demonstrated that the phenotype of an A^{vy} dam influenced the phenotype distribution of the offspring. For the $Axin^{Fu}$ mouse, Rakyan et al. (2003) also demonstrated that the phenotype of the parent influenced the phenotypic distribution of the offspring. In this case, both the maternal and the paternal phenotype had an effect. The fact that mature sperm had the same methylation state at $Axin^{Fu}$ as the somatic tissue was consistent with the transgenerational inheritance of epigenetic marks. The above described phenotype shift in the F_1 offspring of F_0 females which received nutritional methyl donor supplementation during pregnancy was shown to be transferred to the F_2 generation, although methyl donor supplemented food was only given to F_0 organisms (Cropley et al. 2006). The results of this study were later questioned by Waterland et al. (2007). These authors claim that diet-induced hypermethylation at the A^{vy} epiallele is not inherited transgenerationally, based upon the absence of a cumulative effect of dietary methyl donor supplementation across generations. However, diet type and timing, as well as the initial epigenetic state of the A^{vy} allele differed between those studies. The results of both studies have recently been interpreted as follows: a methyl-donor supplemented diet can affect a female's propensity to transmit the A^{vy} epiallele, although this does not appear to engender novel epialleles at the A^{vy} locus (Bonduriansky and Day 2009). A study based on the design of Waterland et al. (2007), but without continuous methyl donor supplementation throughout generations, would be informative in this respect. Not only dietary supplementation, but also dietary restriction can induce transgenerational epigenetic effects. Burdge et al. (2007) reported altered methylation patterns in gene promoters of adult male F_1 and F_2 offspring from mother rats fed on a protein-restricted diet during pregnancy.

Exciting work on transgenerational epigenetic effects has been performed with plants. Lang-Mladek et al. (2010) recently showed that temperature and UV-B stress cause changes in the histone occupancy and histone acetylation on H3 in a reporter gene in *Arabidopsis*. These alterations were transmitted to non-stressed progeny, but limited to areas consisting of a small number of cells and to a restricted number of generations. Other epigenetic mechanisms were also shown to be affected in untreated progeny of *Arabidopsis* exposed to stress: DNA methylation was altered in non-exposed progeny of salt-, heat-, cold-, UV- or flood-stressed *Arabidopsis* plants (Boyko et al. 2010). Using mutants, these authors showed that this was dependent on ncRNA processing enzymes. Also in this study, effects did not persist in successive generations. In a study with Norway spruce, Yakovlev et al. (2011) set up a common garden experiment, as suggested by Bossdorf et al. (2008), to determine the effect of temperature during zygotic embryogenesis on bud phenology and cold acclimation. By studying the transcriptome in suppressive subtracted cDNA libraries, they found that three genes involved in siRNA pathways showed differential transcription in progeny of plants with a cold versus warm treatment during embryogenesis. These siRNAs were suggested to be involved in the epigenetic memory phenomenon resulting in different performance in progeny with an identical genetic background. An interesting aspect of this study is that different families of spruce differed in their epigenetic memory response, making the case for a genetic basis of this epigenetic system.

Epigenetic changes due to exposure to chemicals

Cancer research is probably the most intensely studied field in epigenetics. The pattern of DNA methylation changes substantially when cells become cancerous: the tumor genome becomes globally hypomethylated, while local and discrete regions at the promoter region of tumor-suppressor genes undergo intense hypermethylation (Ropero and Esteller 2009). Exposure to non-genotoxic carcinogens has been shown to induce DNA methylation changes. One well-known example is phenobarbital, which was shown to reduce the overall methylation level in the liver of a tumor-sensitive mouse strain, while increasing DNA methylation in GC-rich regions of hepatic DNA (Counts et al. 1996; Watson and Goodman 2002). Wu et al. (2010) recently reported that maternal exposure to the non-genotoxic carcinogen DEHP (Di-2-(ethylhexyl) phthalate), which is widely distributed in the environment due to its use as plasticizer, caused increased DNA methylation in testes of fetal and newborn mice. This was associated with the induction of testicular dysgenesis syndrome. Newbold et al. (2006) described an increased incidence of tumors in mice that were prenatally or neonatally exposed to the synthetic estrogen diethylstilbestrol (DES). This was also observed in the offspring of early DES exposed female mice mated with unexposed sires. Transgenerational transfer of altered DNA methylation was suggested to be involved in the increased tumor rate in non-exposed offspring, since DES

exposure was shown to induce altered methylation in several uterine genes (Li et al. 2003; Bromer et al. 2009).

Based on such studies with rodents or mammalian cells, Aniagu et al. (2008) investigated the effects of exposure to the putative non-genotoxic carcinogens 17β-oestradiol (E_2) and hexabromocyclododecane (HBCD) and the demethylating agent 5-aza-2′-deoxycytidine (5-AdC) on global DNA methylation levels in liver and gonads of the three-spine stickleback *Gasterosteus aculeatus*. This is one of the few studies that have addressed the epigenetic impact of environmental exposure to chemicals in species different from humans, primates or rodents. An overview of these studies, which will be further discussed in this section, can be found in Table 1. HPLC was used to measure 5-methyl-2′-deoxycytidine levels in the stickleback DNA. HBCD caused no significant effects. E_2 exposure caused a non-significant decrease in DNA methylation in female livers, while a significant increase in DNA methylation in gonads of exposed males was observed. Unexpectedly, 5-AdC, which is a known inhibitor of DNA methyltransferases and which was used in this study as a positive control for demethylation, also caused an increase in DNA methylation in male gonads, while the expected decrease in DNA methylation was observed in female liver samples. The authors speculate that a rebound hypermethylation following cytotoxicity or a secondary response to factors released from other tissues in which hypomethylation may have taken place could be the cause of this unexpected hypermethylation. A large knowledge gap remains to be filled regarding the (transgenerational) epigenetic effects of non-genotoxic carcinogens in ecotoxicologically relevant species.

Air pollution can induce alterations in global DNA levels. In sperm of mice exposed to polluted air near steel plants or a highway, an increase of global methylation was reported by Yauk et al. (2008). These authors also observed DNA strand breaks and they suggest that this caused increased DNMT activity—DNMTs are known to be upregulated during DNA damage and bind with high affinity to many DNA lesions—resulting in the observed hypermethylation. Tarantini et al. (2009) described reduced DNA methylation levels in Alu and LINE-1 repeats (monitored as an indicator for global DNA methylation) in leukocytes from workers in a steel plant who were exposed to well defined levels of PM_{10} (Particulate Matter with aerodynamic diameters < 10 μm). These results are in accordance with the observation of decreased LINE-1 DNA methylation in human blood samples after airborne exposure to black carbon or $PM_{2.5}$ (Baccarelli et al. 2009).

An interesting unifying hypothesis for explaining hypomethylation due to either nutritional factors or environmental chemical exposure was recently proposed by Lee et al. (2009). These authors suggested that the impairment of S-adenosyl-methionine (SAM, Fig. 1a) synthesis may be at the basis of these methylation reducing pathways. A diet low in methyl donors inhibits the methyl addition to homocysteine to form methionine, which is one of the building blocks of SAM. However, homocysteine can also be used to form glutathione (GSH). Glutathione is conjugated with toxic chemicals or their metabolites in the phase II detoxification pathway. Exposure to toxicants may increase the need for GSH, exploiting homocysteine for GSH synthesis and not for SAM synthesis, resulting in lower SAM levels and hypomethylation. Wang et al. (2009) reported a dose-dependent decrease in global DNA methylation in the liver of the false kelpfish *Sebastiscus marmoratus* after a 48-day exposure to waterborne tributyltin, triphenyltin or a mixture of both. This was induced by altering SAM and SAH (S-adenosyl homocysteine) concentrations, and especially the SAM:SAH ratio, rather than affecting DNMT1 expression. TBT is indeed known to decrease cellular GSH concentrations (Huang et al. 2005). Impaired SAM synthesis may also be the reason for a decreasing trend of global DNA methylation at Alu repeats with increasing concentrations of persistent organic pollutants (POPs), which was observed in a study of blood samples from Greenlandic Inuit (Rusiecki et al. 2008).

One of the earliest papers discussing the effects of exposure to chemicals on an epigenetic system in an environmentally relevant organism also dealt with organic pollutants (Shugart 1990). This study reported persistent hypomethylation in the DNA of bluegill sunfish upon exposure to benzo[a]pyrene, metabolites of which are known to be conjugated with GSH (Jernström et al. 1996). However, other mechanisms were suggested to cause the hypomethylation: the onset of DNA hypomethylation correlated with DNA strand breakage, which suggested an inefficient methyltransferase system. In a later phase, hypomethylation corresponded to the appearance of benzo[a]pyrene-DNA adducts, which is known to inhibit the maintenance of DNA methylation.

Environmental exposure to several metals is known to induce epigenetic changes. Nickel (Ni) is a known carcinogenic, but its genotoxicity is low. Lee et al. (1998) demonstrated that Ni induces overall genomic DNA hypermethylation in a transgenic hamster cell line, although the DNA methyltransferase enzyme activity was inhibited by Ni exposure in vitro and in vivo. This lead to the model that Ni^{2+} directly interacts with heterochromatin, replacing Mg^{2+} in the phosphate backbone, which results in increased chromatin condensation. The enhanced chromatin condensation capacity of the Ni^{2+} ions would then attract and condensate neighboring DNA that was previously in a euchromatin state. This newly formed heterochromatin can subsequently be targeted by de novo DNA methyltransferases. In cell lines, crystalline forms of Ni are

Table 1 Studies which addressed the epigenetic impact of environmental exposure to chemicals in non-model species (i.e. other than mouse, rat, primate, human or *Arabidopsis*)

Species	Chemical	Exposure concentration, Exposure time	Epigenetic mark studied	Main result	References
Lepomis macrochirus (bluegill sunfish)	Benzo[a]pyrene	1 μg/l, 40 days + 45 days post-exposure in clean medium	Global DNA methylation in liver	Trend of hypomethylation from day 2 to post-exposure	Shugart (1990)
Ursus maritimus (polar bear)	Mercury (Hg)	0.11–0.73 ppm Hg in brains of wild polar bears (age 2–22)	Global DNA methylation in brain	Trend of decreasing DNA methylation with increasing [Hg] in brain ($p = 0.07$) for male bears	Pilsner et al. (2009)
Sebastiscus marmoratus (false kelpfish)	Tributyltin (TBT), triphenyltin (TPT) and mixture of both	TBT: 1, 10 and 100 ng/l as Sn, TPT: 1 and 10 ng/l as Sn, mixture: 0.5 ng/l each, 5 ng/l each as Sn, 48 days	Global DNA methylation in liver	concentration-dependent decrease in 5-methylcytosine content in liver DNA for all treatments	Wang et al. (2009)
Carassius auratus (goldfish)	Copper (Cu), zinc (Zn), lead (Pb), cadmium (Cd) and mixture of these metals	(1) Cu: 100 μg/l, Zn: 50 μg/l, Pb: 20 μg/l, Cd: 10 μg/l, mixture of above, 48 h (2) mixture of Cu:Zn:Pb:Cd in ratio 10:5:2:1: conc. of 0, 180, 270 and 540 μg/l, 48 h	Global DNA methylation in liver	Significant increase of DNA methylation, more pronounced for Cd and Pb than for Cu and Zn (1), concentration-dependent for the mixture (2)	Zhou et al. (2001)
Trifolium repens (white clover) and *Cannabis sativa* (industrial hemp)	Mixtures of nickel (Ni^{2+}), cadmium (Cd^{2+}) and chromium (Cr^{6+})	(1) 25 mg kg^{-1} $K_2Cr_2O_7$, 25 mg kg^{-1} $NiCl_2$, 25 mg kg^{-1} $CdSO_4$ in soil, 2 weeks (2) 50 mg kg^{-1} $K_2Cr_2O_7$, 100 mg kg^{-1} $NiCl_2$, 100 mg kg^{-1} $CdSO_4$ in soil, 2 weeks	Global DNA methylation and methylation changes in specific fragments in roots	Concentration-dependent significant global hypomethylation; most demethylation sequence-specific	Aina et al. (2004)
Gasterosteus aculeatus (three-spine stickleback)	17β-oestradiol (E_2), hexabromocyclododecane (HBCD) and 5-aza-2′-deoxycytidine (5-AdC)	E_2: 100 ng/l, 22–23 days HBCD: 30 and 300 ng/l, 30 days	Global DNA methylation in liver and gonads	E_2: significantly increased DNA methylation in male gonads HBCD: no effects	Aniagu et al. (2008)
Danio rerio (zebrafish)	17α-ethynilestradiol (EE2)	100 ng/l, 14 days	DNA methylation in specific CpG sites in the 5′ flanking region of the vitellogenin I gene in liver and brain of 5–7 months old zebrafish	Significant methylation decrease in 2 and 3 CpG sites in liver of males and females, respectively	Stromqvist et al. (2010)
Daphnia magna (water flea)	Cd	180 μg/l, 4 days exposure + previous generation 10 days	DNA methylation in two genomic fragments	No changes in DNA methylation	Vandegehuchte et al. (2009a)
Daphnia magna (water flea)	Zn	388 μg/l, 1–3 generations of 21–22 days (F_0 to F_2, post-exposure in clean medium after F_1 and F_2)	Global DNA methylation	Hypomethylation in F_1 offspring of F_0 exposed, not propagated into F_2	Vandegehuchte et al. (2009b, 2010a)

Table 1 continued

Species	Chemical	Exposure concentration, Exposure time	Epigenetic mark studied	Main result	References
Daphnia magna (water flea)	5-azacytidine (5-AC), 5-aza-2′-deoxycytidine (5-AdC), genistein, biochanin A and vinclozolin	21-day chronic tests: 5-AC: 1.2–28 mg/l, 5-AdC: 0.52–12.8 mg/l, genistein: 0.09–3.4 mg/l, biochanin A: 0.11–4.9 mg/l, vinclozolin: 0.15–0.43 mg/l; multigeneration tests: 1–3 generations (F_0–F_2) of 15–17 days, post-exposure in clean medium after F_0; 5-AC: 2.9 mg/l (F_0), 2.3 mg/l (F_1); genistein: 4.7 mg/l (F_0–F_2); vinclozolin: 0.54 mg/l (F_0), 0.45 mg/l (F_1), 0.18 mg/l (F_2)	Global DNA methylation	Vinclozolin and 5-AC induced significant hypomethylation in F_0; with 5-AC this effect was transferred to non-exposed F_1 and F_2 offspring	Vandegehuchte et al. (2010b)
Taraxacum officinale (common dandelion)	Jasmonic acid (JA) and Salicylic acid (SA)	0.25 ml and 0.75 ml of a 10 mM JA or SA solution applied on 5 and 7 weeks old F_0 plants, respectively; DNA analysis on 8 week old plants, F_1 control generation from seeds after 10–13 weeks F_0 culture	DNA methylation polymorphisms	Increased variation in locus-specific DNA methylation, many modifications transmitted to non-exposed F_1	Verhoeven et al. (2010)

more readily delivered to the nucleus than soluble Ni. Exposure to crystalline Ni forms, e.g. by inhaling small particles, would thus form a greater hazard than exposure to soluble Ni forms, which would be dissipated by dissolution (Costa et al. 2005). Opposed to this heterochromatin inducing effect of Ni, a study by Zhou et al. (2009) demonstrated an increase in H3K4 tri-methylation, an epigenetic mark associated with euchromatin, in human carcinoma cells upon exposure to Ni.

Cadmium (Cd) exposure for 1 week caused hypomethylation in a rat liver cell line, while a 10 week exposure period induced DNA hypermethylation (Takiguchi et al. 2003). Cd was shown to inhibit DNA methyltransferase activity, possibly caused by binding with the DNA binding domain of the enzyme, which can explain the initial hypomethylation. The hypermethylation after longer Cd exposure co-occurred with an increased DNA methyltransferase activity. This was suggested to be a compensatory effect: because of the initial inhibition of the enzyme activity, DNA methyltransferase genes are overexpressed, leading to the observed hypermethylation. This is consistent with other studies, in which short-term (24–48 h) and long-term (2 months) Cd exposure induced hypomethylation and hypermethylation, respectively, in human cells (Huang et al. 2008; Jiang et al. 2008).

Another metal for which exposure has been associated with epigenetic effects is arsenic (As). As is methylated during its metabolism, with S-adenosyl-methionine acting as a methyl donor. Thus, As metabolism interferes with the one-carbon metabolism pathway, which is essential for DNA methylation. In a study with rat liver cell lines chronically exposed to As, global hypomethylation was indeed observed and shown to be dose- and time-dependent. Depressed levels of S-adenosyl-methionine were observed concurrently (Zhao et al. 1997). In a recent study with immortalized human urothelial cells exposed to As, results from DNA methylation microarrays demonstrated non-random alterations in DNA methylation at multiple promoters and CpG rich sites in the genome (Jensen et al. 2009). These aberrations were both hypo- and hypermethylation events. The authors demonstrated that As did not affect DNMT activity, but the mechanism by which these site-specific changes occur remains to be found. Similar to Ni, arsenate exposure was also shown to induce histone trimethylation at H3K4 in human carcinoma cells (Zhou et al. 2009). Finally, Marsit et al. (2006) showed that arsenic exposure significantly altered miRNA expression profiles in exposed human lymphoblastoid cells, resulting in a global increase of miRNA expression.

Lead (Pb) is a metal which has only recently been studied in an epigenetic context. Wu et al. (2008) reported a decreased DNA methyltransferase activity (1) in mouse cortical neuronal cells exposed for 24 h to Pb and subsequently aged 1 week and (2) in brain cells from 23-year old monkeys that were exposed to Pb during the first 400 days of their lives. In accordance with this inhibitory effect of Pb, Pilsner et al. (2009) demonstrated an inverse relationship between Pb concentrations in maternal bone tissue, which are good bio-indicators of Pb exposure, and global DNA methylation in umbilical cord blood samples in a study group of Mexican people. The mechanism of Pb interaction with DNA methylation is unknown.

Following a similar approach, but in an ecotoxicological context, the same group of authors used a luminometric methylation assay (LUMA) to study global DNA methylation in brains of polar bears that were hunted in Greenland during 3 years (Pilsner et al. 2010). In male bears, they observed an inverse relationship between DNA methylation and brain mercury (Hg) exposure levels, of which the significance level was $p = 0.07$. There was a large variation in DNA methylation levels in the studied population of 47 bears, also between animals with comparable Hg concentrations in the brain. In this type of field studies, other unknown factors may also influence DNA methylation. The observed trend suggested a possible Hg-induced hypomethylation in male bears. However, further studies are required to confirm this finding.

A unifying process to account for the effect of different metals on DNA methylation may be metal-induced oxidative stress. Metals are known to catalyze and increase the production of reactive oxygen species, which may create oxidative DNA damage. This can inhibit the action of DNA methyltransferases and as such lead to hypomethylation (Baccarelli and Bollati 2009). This process was initially thought to be a potential cause of the metal concentration-dependent global decrease in DNA methylation observed in a study with white clover *Trifolium repens* and industrial hemp *Cannabis sativa* exposed to mixtures of nickel, chromium and cadmium (Aina et al. 2004). Interestingly, however, the hypomethylation in these plants was not random but involved specific DNA sequences, which reproducibly lost methylation in all replicates per treatment. Moreover, hypermethylation events were also detected at specific loci. These results showed that epigenetic responses to metal stress are more diverse than what can be explained by metal-induced reactive oxygen species. This also follows from the increased overall DNA methylation observed in the liver of the goldfish *Carassius auratus* when exposed to copper, zinc, lead, cadmium or a metal mixture; with lead and cadmium inducing a larger change than copper and zinc (Zhou et al. 2001). DNA methylation changes may be part of the defense mechanism against metal stress. Identifying the genes affected by these epigenetic modifications and linking them to alterations at the transcriptional or proteomic level is needed to elucidate the nature of metal-induced changes in DNA methylation.

A key study demonstrating the transgenerational effects of toxicant exposure was performed by Anway et al. (2005). These authors exposed pregnant rats (F_0 generation) to the fungicide vinclozolin or the pesticide methoxychlor, which are both endocrine disrupting chemicals. Male F_1 offspring of these rats exhibited a reduced spermatogenic capacity (lower sperm counts and mobility). These effects were observed in three subsequent generations of non-exposed male offspring (F_2 to F_4) and were associated with aberrant DNA methylation patterns in sperm. Transgenerational transfer of DNA methylation was suggested to be the mechanism of transmission of these effects, through altering the transcriptome (Anway et al. 2008). Next to the effect on fertility, vinclozolin exposure of gestating F_0 females also induced an increased tumor incidence rate and increased frequencies of different disease states (e.g. of the kidney and immune system) in aging F_1 to F_4 males (Anway et al. 2006). In later studies it was shown that F_1 to F_4 male offspring also suffered from several abnormalities in the ventral prostate. The prostate transcriptome of affected F_3 males showed differential expression compared to control males for 259 genes, which was suggested to be the consequence of the altered DNA methylation pattern (Anway and Skinner 2008). Female F_1 to F_3 progeny of vinclozolin exposed pregnant rats also exhibited increased adverse effects compared to control rats, including kidney abnormalities, tumor development and uterine hemorrhage and/or anemia during late pregnancy. While outcross experiments demonstrated that effects in males were passed on through the male germline, effects in females were only observed when both parents were descendants of vinclozolin-exposed rats (Nilsson et al. 2008). Epigenetic mechanisms were not studied in these female rats, but were suggested to have caused the transgenerational disease states.

The vinclozolin studies evoked some controversy, which was fed by the recent retraction by the same research group of a paper describing DNA sequences with altered DNA methylation in the sperm of offspring of vinclozolin-exposed rats. These sequence results, which were generated solely by the first author, could not be reproduced (Chang et al. 2006, 2009). However, the original results from Anway et al. (2005) were not involved in this retraction. In this work, 25 DNA fragments were reported to be subject to altered methylation upon in utero exposure to vinclozolin of F_1 rats. Two of these fragments were more thoroughly investigated and also showed altered DNA methylation patterns in F_2 and F_3 rats. In a follow-up test with oral administration of vinclozolin to pregnant rats, which was deemed more relevant in terms of risk assessment, no effects were observed in F_1 to F_3 offspring (Schneider et al. 2008). Lower toxicant concentrations at the site of action compared to the concentrations after intraperitoneal injection in the study of Anway et al. (2005) may play a role in the different outcomes of both studies. In another verification study with the same intraperitoneal injection of vinclozolin to pregnant rats as in Anway et al. (2005), no effects on spermatogenesis in F_1 rats and no alterations in DNA methylation of F_1 and F_2 rats were measured (Inawaka et al. 2009). This different finding may be due to a different genetic background of the rats. In a repeat experiment, the outbred status of the rat line, as well as the exposure timing and duration were found to be crucial to the effects (Skinner et al. 2010). Interestingly, in a recent study with mice, which were administered a dose of 50 mg/kg/day vinclozolin during pregnancy, transgenerational effects on the DNA methylation status of some imprinted genes in sperm were observed in the F_1, F_2 and F_3 generations (Stouder and Paoloni-Giacobino 2010). Although the intensity of the effect gradually reduced over the generations, this study confirms the induction of transgenerational effects on DNA methylation due to in utero vinclozolin exposure.

In an (eco)toxicological context, the concentration-effect relation should not be neglected. Anway et al. (2006) reported that a dose of 50 mg vinclozolin/kg/day produced 'more variable' effects than the dose of 100 mg/kg/day used in their transgenerational study. Other factors, related to the exposure to the substance, e.g. the half live and application rate/time of a pesticide, are also important when this type of effects are translated to field situations. In an interesting exercise, Dalkvist et al. (2009) modeled the long-term impact of a vinclozolin-like pesticide on vole populations in a landscape setting, taking into account the epigenetic transgenerational effect. Results demonstrated that animal ecology and behavior (e.g. habitat preference) were equally important as toxicology (e.g. No Observed Effect Level) to determine population-level impacts.

A series of recent publications reported on epigenetic and transgenerational effects of toxic stress in the water flea *Daphnia magna*, an important ecotoxicological model species. DNA methylation was detected at CpG sites in two unknown genomic fragments. Exposure to an elevated Cd concentration for two generations, which resulted in decreased reproduction, did not affect the DNA methylation at these sites (Vandegehuchte et al. 2009a). Subsequently, these authors quantified DNA methylation in *D. magna* exposed to zinc in a three-generation study. In the F_1 generation offspring of F_0 generation Zn exposed daphnids, a slight but significant decrease in DNA methylation was observed. This demonstrated that environmental conditions can affect DNA methylation in this species. However, this effect did not propagate into the next generation (Vandegehuchte et al. 2009b). The effect of this Zn exposure on gene transcription was also studied using custom cDNA microarrays. The transcription of a

large number of genes was affected by the Zn exposure, both in the exposed daphnids and in their non-exposed offspring. A large proportion of common genes were similarly up- or downregulated in the exposed and non-exposed F_1 daphnids, suggesting a possible effect of the DNA hypomethylation. Interestingly, two of these genes can be mechanistically involved in DNA methylation reduction. The similar transcriptional regulation of two and three genes in the F_0 and F_1 exposed daphnids on one hand and their non-exposed offspring on the other hand, could be the result of a one-generation temporary transgenerational epigenetic effect. A stable transgenerational epigenetic effect on gene transcription through the three generations, however, was not observed (Vandegehuchte et al. 2010c; Vandegehuchte et al. 2010a). It should be stressed though, that gene-specific links between DNA methylation and transcription could not be made in these studies, since only global methylation was measured. With the arrival of a *D. magna* genome assembly, which is work in progress by the *Daphnia* Genomics Consortium (http://daphnia.cgb.indiana.edu), locus-specific DNA methylation analyses will enable deciphering the transcriptional consequences and potential phenotypic implications of altered DNA methylation.

Finally, the effect of substances known to affect DNA methylation in mammals was evaluated using *D. magna* multigeneration experiments. Vinclozolin and 5-azacytidine, a demethylating drug, were shown to induce DNA hypomethylation in the first exposed generation (F_0). Opposed to the results of the above described studies with rats, the vinclozolin-induced effect did not propagate into the next generation. In the case of 5-azacytidine exposure, however, this effect was transferred to the two subsequent non-exposed generations, coinciding with a significantly reduced juvenile growth (Vandegehuchte et al. 2010b). These results demonstrate the possibility of transgenerational inheritance of environment-induced epigenetic effects in non-exposed subsequent generations of *D. magna*, which is an important principle. Key issues for the ecotoxicological implications of this, which need further study, are the causality between DNA methylation and the reduced growth phenotype, the ecological relevance of this reduced growth and the potential more subtle site-specific effects on DNA methylation at exposure concentrations lower than 2.9 mg/l.

Another recent report on multigenerational epigenetic effects described DNA methylation changes in the dandelion *Taraxacum officinale* exposed to different kinds of environmental stress (Verhoeven et al. 2010). Similar to parthenogenetic *D. magna*, apomictic *T. officinale* offer the advantage of detecting epigenetic variation in the absence of genetic variation. Nutrient limitation, salt stress and the application of plant hormones each resulted in an increased epigenetic variation in terms of locus-specific DNA methylation. Many of these modifications in DNA methylation were transmitted to the offspring which was raised in an unstressed environment, and which also exhibited significantly affected traits. It would be most interesting to investigate the transmission of these effects into subsequent generations and to test the effects of environmental exposure to chemicals other than plant hormones in this species.

These type of studies incite to investigate the epigenetic effects of substances with known transgenerational effects, such as endocrine disrupting chemicals. Brown et al. (2009) reported reduced embryonic survival in the rainbow trout *Oncorhynchus mykiss* due to exposure of males to the endocrine disrupting chemical 17α-ethynilestradiol (EE2) during late spermatogenesis. These authors discussed that this was potentially mediated by an epigenetic mode of action. However, they did not evaluate the molecular mechanism of EE2 toxicity.

Adult zebrafish (*Danio rerio*) were exposed to EE2 by Stromqvist et al. (2010) in order to study the effect on DNA methylation in specific CpG sites in the 5′ flanking region of the vitellogenin I gene in liver and brain. This specific gene was selected because of the known induction of vitellogenin by environmental estrogen exposure in zebrafish. Pyrosequencing revealed that the DNA methylation levels in the liver at all three investigated sites for females and at two sites for males were significantly reduced upon EE2 exposure, suggesting that demethylation may be involved in vitellogenin induction. The authors recommend future ecotoxicological studies on epigenetic changes in fish. It would be very interesting to determine the DNA methylation in an expanded zebrafish experiment with a next exposed and non-exposed generation and to include the analysis of phenotypic effects. EE2 is known to induce developmental effects at lower concentrations in offspring from EE2-exposed zebrafish, compared to concentrations causing effects in the parental generation (Soares et al. 2009). If multigenerational DNA methylation changes are involved, this may also impact non-exposed offspring.

The soy isoflavone genistein, also a known endocrine disruptor, has been shown to reduce DNA methyltransferase activity, but the detailed mechanism of how it affects DNA methylation has yet to be determined (Majid et al. 2010; Ross 2009). Day et al. (2002) demonstrated that male mice fed a genistein-enriched diet showed increased DNA methylation at specific genes in the prostate. Dietary genistein supplementation in female mice also lead to increased methylation at the A^{vy} metastable epiallele in offspring, which was associated with a shift in coat color, similar to what was observed with methyl donor supplemented food (Dolinoy et al. 2006). In a study with the ecotoxicological model species *Daphnia magna* exposed to

chemicals with a known effect on DNA methylation, however, no global DNA methylation changes were observed upon genistein exposure via water (Vandegehuchte et al. 2010b).

Nanomaterials and epigenetic changes

In the last decades, the production and use of nanomaterials has increased dramatically. The special properties that these materials possess because of their unique physical and chemical structure have also raised questions related to their possible impact on human health and the environment. In recent years, epigenetics has been introduced in the field of nanotoxicology. Choi et al. (2008) demonstrated global histone hypoacetylation in human breast carcinoma cells exposed to cadmium telluride quantum dot nanoparticles at very low concentrations. This was linked to decreased gene transcription. Global hypoacetylation was suggested to induce a global reduction in gene transcription, including anti-apoptotic genes. That could be an explanation for the observed cell death after quantum dot exposure. However, some anti-apoptotic genes were shown to be upregulated in the presence of the quantum dots. The authors suggest further studies to determine the global reconfiguration of chromatin in the exposed cells.

Exposure to silicon-dioxide (SiO_2) nanoparticles at concentrations up to 10 μg/ml induced a dose-dependent hypomethylation in the DNA of human HaCaT cells (Gong et al. 2010). Interestingly, SiO_2 microparticles at the same concentration of 10 μg/ml did not induce a significant effect on DNA methylation. This observation coincided with a dose-dependent decrease in the expression at mRNA and at protein level of the DNA methyltransferases DNMT1 and DNMT3a and the methyl-binding domain protein MBD2. The authors refer to their study as 'an an initial step in the unexplored field of epigenetic changes by nanoparticles'. Knowledge from this field should be taken into account in the developing research discipline of nanoecotoxicology, where no epigenetic research has been performed yet (Baun et al. 2008).

Epigenetics and risk assessment of chemicals

The above-described vinclozolin studies highlight potential consequences of environmental chemical exposures for the health of non-exposed future generations. This may be relevant for human health and toxicology, which has been described in popular media in expressions like 'what you eat or drink today could affect the health of your great-grandchildren (Watters 2006)'. However, this concept could also be of importance for ecotoxicology and environmental risk assessment of chemicals, as indicated by the transgenerational effect of 5-azacytidine in *Daphnia*. Indeed, if chemical exposure to one generation can have effects on multiple subsequent non-exposed generations, the risk assessment of these chemicals should incorporate this time interval between effects and exposure in previous generations (Fig. 2).

The fact that exposure to chemicals can induce epigenetic changes, combined with the occurrence of transgenerational epigenetic effects has raised concern about the role of epigenetics in chemical safety/risk assessment. In 2009, the Health and Environmental Sciences Institute of the International Life Sciences Institute organized a workshop in Research Triangle Park (North Carolina) to discuss the state of the science regarding epigenetic changes, with the aim of assessing the knowledge needed to incorporate an epigenetic evaluation into safety assessments (Goodman et al. 2010). The workshop report concludes that a great deal needs to be learned prior to taking the science-based decision to incorporate epigenetics into safety assessment. This also holds true for the environmental risk assessment of chemicals. Several recommendations for future studies were made during that workshop. The choice of an appropriate exposure route and relevant exposure doses/concentrations is a very important aspect when evaluating epigenetic effects of chemical exposure. It is also key that any changes detected in an epigenetic system are rationally linked to adverse phenotypical effects. In the framework of chemical safety or risk assessment, ideally there should be information about (1)

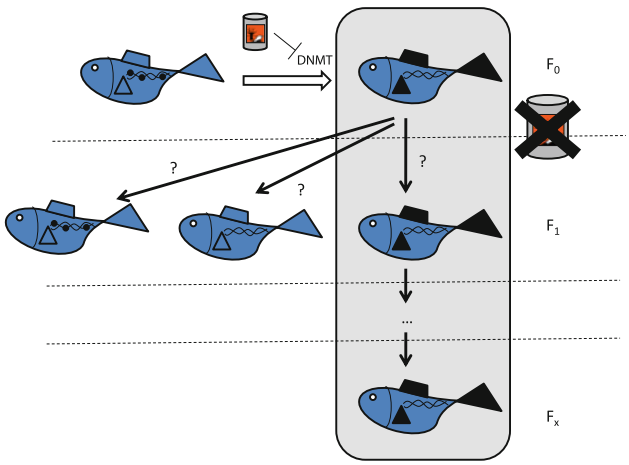

Fig. 2 Conceptual figure indicating that exposure to chemicals can induce epigenetic changes, e.g. demethylation of DNA (represented by the removal of black dots from the schematic DNA double helix) by inhibiting the action of DNA methyltransferases (DNMT) and phenotypic effects (schematically represented by the black fins). These epigenetic changes and/or phenotypic effects may or may not be transmitted to the next generation, which is not exposed to the chemical. If these changes persist for multiple generations (*shaded frame*), non-exposed organisms would experience effects of their ancestors' exposure, which would have profound implications for environmental risk assessment of chemicals

molecular mechanisms, (2) the epigenetic changes resulting from this mechanism and (3) the integrated phenotypic effects of these changes. Basic research, including the determination of reference epigenomes, experiments with positive and negative controls and the setting of threshold and cutoff values are required to fill data gaps with respect to these information needs. Similar conclusions and recommendations were made in a review on epigenetics and chemical safety assessment focused on human health risks by LeBaron et al. (2010). These authors also argued that the current toxicological testing battery is expected to identify any potential adverse effects, including those resulting from epigenetic changes. The question of species cross-relevance, which is also highly relevant to ecotoxicological studies, was emphasized in this review. Studies comparing the methylation status of imprinted genes (considered to be the most conserved) in mice versus man showed that there is a high level of discordance and that species are highly dissimilar. Hence, epigenetic effects observed in one species cannot be assumed to occur in all other species.

Up to now, evolutionary aspects or effects of chemical exposure in future non-exposed generations are not considered in the ecological risk assessment process. It is not known how much uncertainty this implies for the current environmental risk assessments. However, the limited number of reports reviewed above (Table 1) are a cause for concern. Before recommendations can be made to incorporate epigenetics into the ecological risk assessment process, more fundamental research is needed to address (1) the occurrence of chemical-induced epigenetic modifications at environmentally realistic exposure concentrations in ecotoxicologically relevant species, (2) the phenotypic and population-level effects of these modifications and (3) the transmission of these changes to subsequent non-exposed generations.

Concluding remarks

It is now clear that environmental exposure to chemicals can affect the epigenetic status of several species, not only rodent models, but also ecotoxicologically relevant fish, plants and invertebrates (Table 1). Moreover, certain studies demonstrated epigenetic alterations in the non-exposed offspring of exposed organisms. This is a new concept in ecotoxicology. If chemical exposure to one generation can have effects on multiple subsequent non-exposed generations, the risk assessment of these chemicals should incorporate this time interval between effects and exposure in previous generations. However, major knowledge gaps in most of the reported ecotoxicological studies are (1) the potential causal links between chemical-induced epigenetic changes and phenotypic effects and (2) the persistence of these changes and their higher-level effects into multiple subsequent generations. In that respect, the evaluation of classic ecotoxicological endpoints will remain necessary, but should be complemented by molecular research. The wider availability of relatively new technologies such as next-generation sequencing can help to understand the functional consequences of epigenetic modifications, e.g. through a genome-wide investigation of affected genes in case of global demethylation, and to design focused experiments to determine higher-level impacts of epigenetic changes.

The advent of epigenetics in all areas of biological sciences means that ecotoxicologists should consider altering experimental protocols to include developmental exposure, epigenetic analysis and transgenerational studies, as suggested by Legler (2010). With this, basic ecotoxicological concepts such as bioavailability, exposure routes and relevant concentration/effect relations should be taken into account. With the current knowledge it is too early to assess the full impact of epigenetics on ecotoxicology or ecological risk assessment of chemicals. However, the state of the science in this field evolves at a rapid pace and results presented in this review are anticipated to encourage the scientific community to further the understanding of epigenetics in an ecotoxicological context.

Acknowledgments The authors wish to thank two anonymous reviewers and the editor dr. Richard Handy for their constructive comments which improved the manuscript.

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