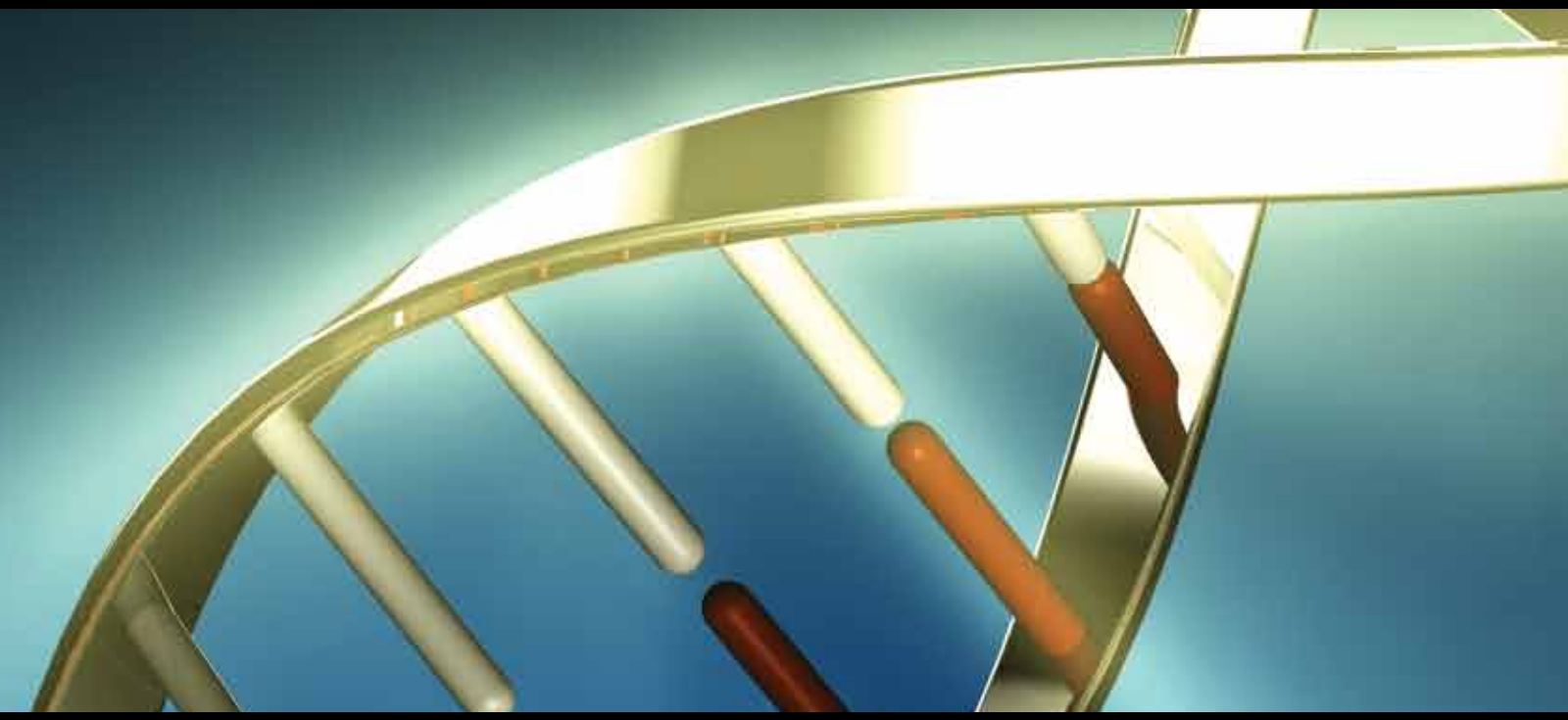
Genetics Research International

The Epigenetics of Emerging and Nonmodel Organisms

Guest Editors: Vett K. Lloyd, Jennifer A. Brisson, Kathleen A. Fitzpatrick, Lori A. McEachern, and Eveline C. Verhulst



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Editorial Board

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Editorial

The Epigenetics of Emerging and Nonmodel Organisms

Vett K. Lloyd,[1] Jennifer A. Brisson,[2] Kathleen A. Fitzpatrick,[3] Lori A. McEachern,[4] and Eveline C. Verhulst[5]

[1] *Department of Biology, Mount Allison University, 63B York Street, Sackville, NB, Canada E4L 1G7*
[2] *School of Biological Sciences, University of Nebraska, Lincoln, NE 68588, USA*
[3] *Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada V5A 1S6*
[4] *Department of Physiology & Biophysics, Dalhousie University, 5850 College Street, Halifax, NS, Canada B3H 4R2*
[5] *Evolutionary Genetics Group, Department of Life Sciences, University of Groningen, P.O. Box 11103, 9700 CC Groningen, The Netherlands*

Correspondence should be addressed to Vett K. Lloyd, vlloyd@mta.ca

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Genetic model organisms have gifted researchers with a breathtakingly detailed understanding of the most intimate aspects of their genomes, cells, and development. And yet there is a problem—model organisms have been selected because they have simple life histories and happily inhabit laboratories. In short, they make a virtue of being boring. But the diversity of the natural world is not fully captured by yeast, flies, or mice. To truly appreciate the variety of biological mechanisms underlying this remarkable diversity, one must study the often inconvenient but fascinating non model organism. Experimental and descriptive approaches in non-model organisms have become more tractable with reduced genome-sequencing costs and the transferability of techniques and tools developed in model organisms, elevating some of them from non-model to emerging model organism status.

One area of biology into which non-model organisms promise to provide significant insight is the area of epigenetics. Epigenetics focuses on how internal and external environments interact with the genome to produce the phenotype, and non-model organisms arguably present a larger range of phenotypes than model organisms. In this issue, we present recent research into the epigenetics of non- and emerging-model organisms. The papers in this series highlight several common themes: experimental approaches to studying epigenetics in non-model organisms, epigenetics as a mediator of environmental changes in morphology and development, and epigenetic contributions to individual and population diversity.

How to Study Epigenetics in Non- and Emerging-Model Organisms. Despite the ecological and evolutionary importance of non-model organisms, an obvious disadvantage is the absence of genetic and epigenetic tools available for these organisms. This issue is addressed by W. A. MacDonald, who examines the question of whether one of the best-studied aspects of epigenetics, genomic imprinting, is evolutionarily conserved. His conclusion that the basic epigenetic mechanisms, if not the target genes, are conserved, allows the extrapolation of findings from model organisms to non-model organisms. This approach is taken by studies on polychaetes (G. Gibson et al.) and *Daphnia* (N. F. Robichaud et al.) in this special issue. L. A. McEachern further explores the potential of transgenic epigenetic studies in non-model organism research. This underutilized but powerful and sophisticated approach to studying epigenetics involves transferring a potential epigenetic control sequence from one organism to another for detailed molecular analysis. On a practical level, G. Prantera and S. Bongiorni examine new experimental approaches used to dissect one of the first epigenetic processes described, chromosome imprinting in mealybugs, and K. R. Shorter et al. describe the manifold resources for the study of the deer mouse *Peromyscus*, by the *Peromyscus* genome center.

Epigenetics as a Mediator of Environmentally Driven Changes in Morphology and Development. A mouse looks like a mouse—one tail, four feet, two ears, one nose, and so forth. A stressed mouse still looks like a mouse. The same is true

for fruit flies, nematodes, and a great many other animals. However, there are some animals, which in response to environmental cues, alter their morphology so dramatically that they would not be recognized as the same species as their unstressed, and sometimes genotypically identical, counterparts.

When exposed to predators, various *Daphnia* species can grow cuticular elaborations that would be the envy of any punk rocker: long pointy helmets, tail spikes, neck teeth, and so forth. In some cases, these can double the size of the animal, making them unpalatable to the predator. Similarly, environmental cues can change the sex of an individual, turning what would normally be a parthenogenetically reproducing female into a male, thereby allowing sexual reproduction. Morphological changes are also seen in aphids; under stress, the normally boxy-bodied sap-sucking machine can produce offspring that develop into a svelte winged form with a much smaller streamlined body with entirely different musculature. In this special issue, K. D. M. Harris et al. and D. G. Srinivasan and J. A. Brisson review what has been explored with respect to the epigenetic basis of these dramatic morphological differences in *Daphnia* and aphids, respectively.

Epigenetically mediated morphological differences are also observed in the genus *Onthophagus*, in which male beetles may grow large horns or not, depending on development time and consequent body size, and also display a corresponding plasticity in mating behaviour. Array experiments have identified candidate genes differentially expressed in the two "morphs", and in this issue, S. Valena and A. P. Moczek discuss how differential DNA methylation may be involved in these expression changes. Similarly, some polycheate worms can alter their development to produce either small planktonic larvae optimized for dispersal or large yolk-filled larvae, which tend to settle near the parents. G. Gibson et al. provide the first information on the epigenetic basis of these polyphenisms. This theme is continued in the review of epigenetics in social insects, in which S. A. Weiner and A. L. Toth describe some interesting connections between differential DNA methylation and caste polyphenisms in social insects.

Epigenetic Contributions to Individual and Population Diversity. Epigenetic states can be inherited and thus can contribute to the evolutionary process. However, the role of epigenetic diversity in promoting selectable phenotypic diversity at the population level has not yet been well studied in animals. Here, A. W. Schrey et al. use methylation-sensitive-amplified fragment length polymorphism markers to measure epigenetic variation in introduced populations of house sparrows. With these results the authors hypothesize that this epigenetic variation may play a role in maintaining phenotypic diversity following the reduced genetic diversity that occurs with a genetic bottleneck. Using similar methods, R. Massicotte and B. Angers investigate the flexibility of the phenotype using the static genotype of a clonally reproducing fish. They report on different epigenetic modifications under different environmental conditions such as water pH. These two studies demonstrate that epigenetic modifications in natural populations and their relationship to the phenotype is a rich area for further exploration.

Vett K. Lloyd
Jennifer A. Brisson
Kathleen A. Fitzpatrick
Lori A. McEachern
Eveline C. Verhulst

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Review Article

Mealybug Chromosome Cycle as a Paradigm of Epigenetics

Giorgio Prantera[1] and Silvia Bongiorni[2]

[1] *Department of Ecology and Biology, University of Tuscia, Via San Camillo de Lellis, 01100 Viterbo, Italy*
[2] *Department for Innovation in Biological, Agro-food and Forest systems, University of Tuscia, Via San Camillo de Lellis, 01100 Viterbo, Italy*

Correspondence should be addressed to Giorgio Prantera, prantera@unitus.it and Silvia Bongiorni, bongiorni@unitus.it

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Recently, epigenetics has had an ever-growing impact on research not only for its intrinsic interest but also because it has been implied in biological phenomena, such as tumor emergence and progression. The first epigenetic phenomenon to be described in the early 1960s was chromosome imprinting in some insect species (*sciaridae* and *coccoideae*). Here, we discuss recent experimental results to dissect the phenomenon of imprinted facultative heterochromatinization in Lecanoid coccids (mealybugs). In these insect species, the entire paternally derived haploid chromosome set becomes heterochromatic during embryogenesis in males. We describe the role of known epigenetic marks, such as DNA methylation and histone modifications, in this phenomenon. We then discuss the models proposed to explain the noncanonical chromosome cycle of these species.

1. Epigenetics

The first appearance of the term epigenetics can be ascribed to Conrad Waddington, who stated in 1942 that "*epigenetics is the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being*" [1]. In the modern view, epigenetics encompasses all those hereditary (genetic) phenomena not depending on the DNA sequence itself but on some functionally relevant molecular signatures which are imposed over the sequence ("*epi*" in Greek means "over"). All the systems involved in gene expression regulation are based on interactions between proteins and DNA. Some mechanisms inhibit or activate the expression of a single gene, acting on the promoter region, and thus reflect the structural organization of the gene itself (gene regulation). However, the *epi*-genetic systems can regulate phenotypic expression regardless of the gene sequence and are transmitted from one cell generation to the next one or from the parents to their progeny. These systems modulate the functional behavior of chromosomal regions, entire chromosomes, or even whole sets of chromosomes [2]. According to Denise Barlow "*epigenetics has always been all the weird and wonderful things that cannot be explained by genetics.*" Epigenetic phenomena occur in all the kingdoms from yeast to metazoans and plants. Some are limited to just one or few species. For example, RIP (rearrangement induced premeiotically) [3] and MIP (methylation induced premeiotically) [4] were reported in fungi, where they seem to protect the genome from transposable elements. The term paramutation, on the other hand, was coined to describe a heritable change in gene expression of an allele imposed by the presence of another specific allele, which occurs only in plants [5]. Paramutation seems to require the physical interaction between the two homologous alleles [6], as does the quite similar transvection phenomenon described in *Drosophila* by Lewis in 1954 [7]. Other phenomena are universal, at least in eukaryotes. These include, for example, the double-stranded RNA-mediated posttranscriptional gene silencing (PTGS).

Classical genetics has always considered the two parental copies of a gene functionally equivalent in determining the offspring phenotype, regardless of their origin. Genomic imprinting identifies, instead, the epigenetic process by which specific genes, single chromosomes, or entire haploid chromosome sets exhibit a differential functional behaviour, that is, dependent upon their parental origin [8–10]. The

first evidences for the existence of genomic imprinting (and indeed the first use of this term in a genetic sense) came from the early works by Helen Crouse, in 1960s, on the fungus gnat *Sciara coprophila* [11, 12], and the subsequent studies on *Coccidae* [13, 14], showing that reciprocal crosses are not always equivalent (reviewed in [15, 16]). Nonetheless, those findings were seen as curiosities in their time, until imprinting evidence was uncovered in the mouse, in mid-1980s [17, 18].

A strong impetus to genomic imprinting studies came from the demonstration that failure of imprinting is responsible for severe syndromes in humans (reviewed in [19, 20]). For example, some human syndromes are caused by the transmission of both homologs from a single parent (uniparental disomy) [21].

The elaboration of the parent-of-origin-specific epigenetic information proceeds through three steps, namely, establishment, maintenance, and erasure (Figure 1) (reviewed in [22, 23]). During gametogenesis a genome-wide erasure of the parent-specific epigenetic "marks" occurs, followed by the establishment of the signatures specific for each sex. After fertilization, the differential epigenetic marks, carried by the two parental pronuclei, are maintained and faithfully transmitted through the subsequent mitotic divisions during development. The imprinting marks specific to each parental allele are then "read" by the cellular machinery and translated into a differential, parent-of-origin-specific functional behavior. Genomic imprinting represents a paradigmatic example of epigenetic regulation, found not only in insects and mammals but also in yeast and plants [24, 25].

Hereafter we will describe the unusual chromosome system of the Lecanoid coccids (mealybugs), and the molecular machinery which is used by males of these insects to perform one of the most striking epigenetic phenomena: the imprinted facultative heterochromatinization of the entire paternal haploid chromosome set.

2. The Mealybug Chromosome System

Coccid insects are very small, most species are less than one centimeter in length. This group of Hemiptera exhibits "sexual dimorphism." The body shape of females is globose and flattened, with the fusion of the head to thorax. Other segmental boundaries are often not clearly visible. Females are always wingless and frequently neotenic; they are covered with protective secretions such as wax, lacquer or, silk. Males are much smaller than females and have an elongated body with wings.

Coccid species are identified based on male and female morphology, as well as on the karyotype. At the beginning of last century, two large groups were identified on the basis of morphological criteria the Margaroididae and the Lecanodiaspidoidae [26]. The karyocytological analysis confirmed the validity of this subdivision [27]. The Margaroididae retain the XX-XO mechanism of sex determination. In the

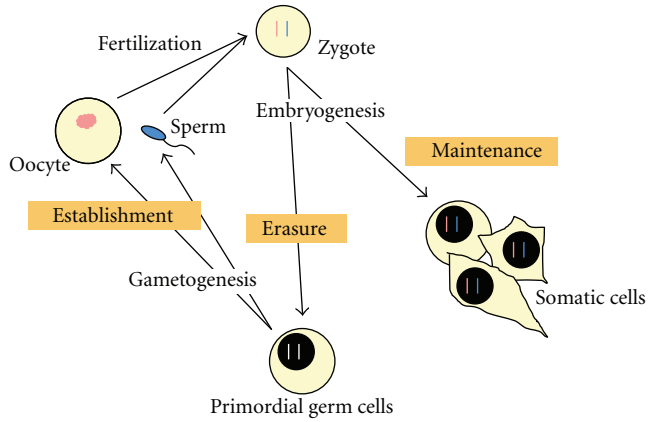


Figure 1: The "life cycle" of parental imprinting. The elaboration of the parent-of-origin-specific epigenetic information during animal development is achieved in three steps, namely, establishment, maintenance, and erasure. The paternal genome is illustrated in blue, the maternal genome in pink.

Lecano-Diaspidoidae there are no differentiated sex chromosomes but these species possess a very complex and intriguing chromosome system. In the male line of Diaspidoids the whole paternal chromosome complement is discarded from midcleavage embryo cells; while in Lecanoid (mealybug) males, the whole paternally derived chromosome set undergoes heterochromatinization and the males become functionally haploid, a condition known as parahaploidy (Figure 2). After fertilization, all the embryo chromosomes are euchromatic. However, in female embryos all the chromosomes retain the euchromatic state, whereas in embryos destined to develop into males, the whole haploid set of paternal chromosomes becomes heterochromatic after the 7th cleavage division (Figure 2) [28]. This implies that, at least in males, the parental origin of the two chromosome sets must be distinguishable until blastoderm stage, when the heterochromatinization process specifically acts upon the paternally derived chromosomes. The process of heterochromatinization may thus be fruitful in the investigation of the behavior of epigenetic marks before and across the onset of heterochromatinization (see Section 3 for details).

The mealybug chromosome system exhibits the characteristics of a genuine imprinting phenomenon [29]. The imprint is established in the gametes, maintained through the embryonic and adult somatic cell divisions, and erased in the germline (Figure 1). In somatic cells, the heterochromatic paternal chromosomes cluster and form a chromocenter that makes it very easy to distinguish male from female embryos. The chromocenter is noticeable in the nuclei of most tissues except the Malpighian tubules and the gut, where facultative heterochromatin reverts to a euchromatic state [30, 31]. The maternal euchromatic chromosomes are always distinguishable from the paternal ones until metaphase, when they too reach a high degree of condensation. Based on these features, paternal chromosome inactivation in male mealybugs represents, together with X-chromosome

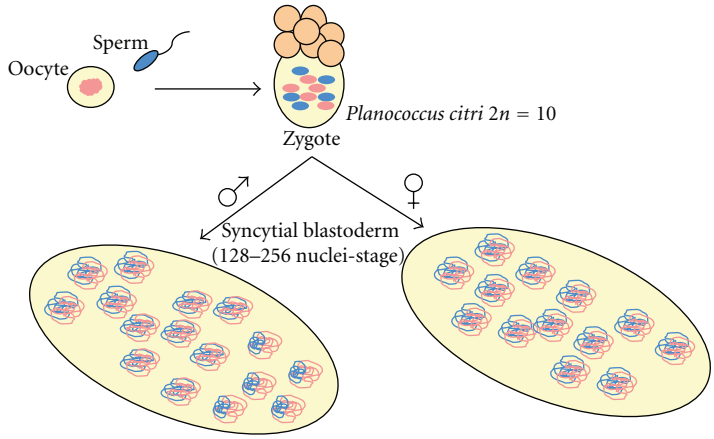


Figure 2: Male and female *P. citri* embryos at syncytial blastoderm (7th mitotic division, 128–256-nuclei stage). In the male embryo (left), it is possible to observe different stages of heterochromatinization. Heterochromatinization proceeds as a wave from one pole (bottom right) of the embryo, where nuclei show a fully developed chromocenter, toward the other one (top left), where nuclei still lack a chromocenter. Heterochromatinization selectively affects the paternal chromosomes (blue). In the female embryo (right), paternal and maternal (pink) chromosomes present the same degree of compaction and remain active.

inactivation in female mammals, the most clear and large-scale example of facultative heterochromatinization. Facultative heterochromatinization may be defined as the developmentally regulated and tissue-specific *cis*-spreading of a heterochromatic state onto a euchromatic region, with a remodeling of the chromatin conformation that eventually leads to inactivation of all the genes it harbors. Distinct from constitutive heterochromatin, facultative heterochromatin is not composed of specific DNA sequences and in general involves only one of the two homologous sites; in these aspects it represents a true epigenetic phenomenon.

The paternal origin of the heterochromatic set was established by Brown and Nelson-Rees (1961) [32]. These authors irradiated *Planococcus citri* males with X-rays prior to mating and then scrutinized their male offspring. Due to their holocentric nature, the chromosome fragments are not lost during paternal spermatogenesis and embryo development so the authors could demonstrate that the radiation-induced chromosomal aberrations were present only in heterochromatic haploid set of the sons. In contrast, in the male offspring of X-ray-treated females, only the euchromatic chromosomes were damaged. Using an analogous strategy, the same authors demonstrated the genetic inactivity of the heterochromatic set [32]. The parahaploid male progeny of X-ray-treated males exhibited normal vitality, whereas the survival of the diploid daughters decreased with increasing X-ray dose. This apparent paradox can be easily explained if one considers that, in the sons, any paternally-transmitted mutation was harbored by heterochromatic chromosomes and hence was not expressed, while in female progeny, any dominant lethal mutation was expressed. Nevertheless, the heterochromatic haploid chromosome set is not completely genetically inert in males since at least three different effects were found that could be ascribed to some residual activity of the paternal genome. First, the survival of male offspring of heavily X-ray-treated males (60.000 to 90.000 rep) depended on the amount of heterochromatic material, since the loss of heterochromatic fragments reduced the vitality [33]. The second effect was related to fertility: 100% of the male offspring of irradiated males (30,000 rep) survived, but a large percentage was sterile, and the frequency of sterile individuals increased with the radiation dose [33]. The third effect can be deduced by the observation that, in the male progeny of interspecific crosses, the heterochromatic set from one species could not be substituted for that (of an equivalent amount) of another [34]. Moreover, the activity of heterochromatic rDNA loci was demonstrated by the observation that in male cells ribosomal genes located on heterochromatic chromosomes were associated with nucleoli and nascent rRNA [35].

In mealybugs, the meiosis is atypical since the meiotic divisions are inverted. In male and female mealybugs, the first meiotic division is equational, with separation of sister chromatids, while the second one is reductional with segregation of the homologs (*Inverted meiosis*) [36, 37]. However, in females the remaining meiotic events are canonical, since homologous chromosomes undergo crossing over and independent assortment. During male meiosis, each spermatogonial precursor cell produces a cluster of synchronously dividing spermatogonia. Each spermatogonium divides four times to produce a cyst of 16 primary spermatocytes which then undergo the two meiotic divisions (Figure 3) [30]. The reductional second meiotic division is characterized by a nonindependent assortment of chromosomes, with the maternal euchromatic set segregating from the paternal heterochromatic one through a monopolar spindle (Figure 4) [37]. The two meiotic divisions thus generate a quadrinucleate spermatid with two nuclei containing the maternally derived euchromatic chromosomes and other two nuclei containing the paternally derived heterochromatic ones. Only spermatids containing the euchromatic chromosomes differentiate into sperm, while the heterochromatic products fail to form mature sperm and slowly degenerate *in situ*. The final result is the formation of a 64-nuclei cyst, where

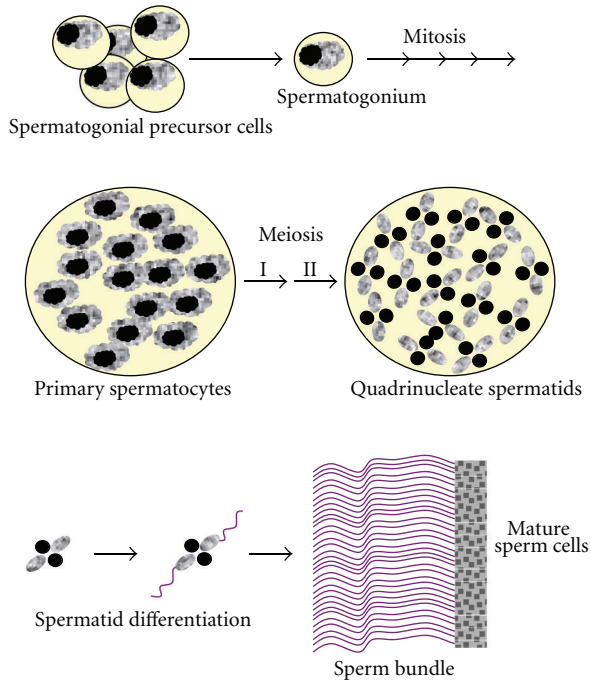


Figure 3: *P. citri* spermatogenesis. Each spermatogonial precursor cell produces a cluster of synchronously dividing spermatogonia, and after four mitotic divisions a cyst of 16 primary spermatocytes is obtained. Primary spermatocytes undergo an inverted type of meiosis, characterized by a nonindependent assortment. Meiosis produces a quadrinucleate cell with 2 elongated spermatids containing only the euchromatic chromosomes (gray staining), and 2 picnotic spermatids containing only the heterochromatic chromosomes (dark staining). Only euchromatic spermatids differentiate into 32 mature sperms.

only the 32 "euchromatic" spermatids start the elongation process that ends with the production of 32 mature sperm (Figure 3). As a consequence of this extreme meiotic drive, only the maternally derived euchromatic chromosomes are transmitted to the progeny.

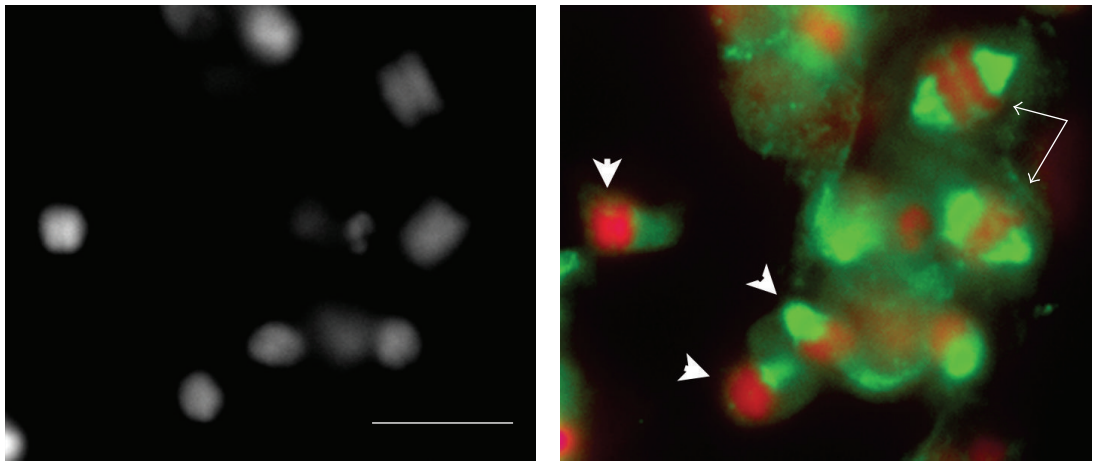
In summary, mealybug males exhibit not only two relevant epigenetic phenomena, chromosome, imprinting, and facultative heterochromatinization on a genome-wide scale, but also a dramatic deviation from canonical meiosis represented by inverted meiosis, nonindependent chromosome assortment and extreme meiotic drive.

3. The Mechanisms of Imprinted Facultative Heterochromatization in Mealybug

We carried out an extensive scrutiny of the epigenetic mechanisms in the mealybug *P. citri* and found that the machinery underpinning imprinted facultative heterochromatinization involves HP1-like and HP2-like proteins, as well as specific posttranslational histone modifications [28, 38–42]. Chromatin remodeling events have been commonly indicated as a mechanism by which eukaryotic cells regulate most of the epigenetic phenomena (reviewed in [43]), though the relevance of histone modifications as the carrier of epigenetic memory has been questioned [44]. Chromatin remodeling involves the interplay of many different posttranslational modifications of histones and of a number of nonhistone proteins. Histone modifications play a central role in the regulation of gene expression, and this led some authors to postulate the existence of a "histone code" as a regulatory code modulating the potentialities of the genetic code [45, 46]. Though the crosstalk of histone modifications does actually influence chromatin function, their combinations probably do not identify a true code.

The interplay between the heterochromatin protein HP1 [47] and the lysine 9 trimethylated isoform of the histone H3 (K9H3me3) has been shown to be pivotal for the assembly of silent chromatin domains [48–50]. The human (SUV39H1), the murine (Suv39h), and the Drosophila (SU(VAR)3-9) histone methyltransferases (HMTases), that selectively di- and trimethylate the histone H3 at lysine 9, generate a binding site for HP1 family proteins [48–50]. Moreover, in mammals, yeast, and Drosophila, it has been also shown that HP1 is, in turn, associated with the K9H3 HMTase, suggesting a self-maintenance model for the propagation of heterochromatic domains in native chromatin, that may well be responsible for epigenetic memory [51–54]. Facultative heterochromatinization in the nuclei of male mealybugs does not occur simultaneously in all cells of the 7th cleavage embryo but takes place as a wave, beginning at one end of the embryo and spreading to the other (Figure 2) [28]. HP1-like distribution in *P. citri* embryos was investigated using an antibody against *Drosophila* HP1 (C1A9 antibody [47]) [28]; this antibody recognized a protein of similar mass (29 kDa) which shared the *Drosophila* HP1 epitope [10, 28]. The establishment of a well-formed chromocenter in male embryo nuclei was preceded by the appearance of aggregates of HP1-like immunostaining that then continued to decorate the male-specific heterochromatin [28]. These results led us to hypothesize that the *P. citri* HP1-like might play a causative role in facultative heterochromatin formation (Figures 5 and 6). This hypothesis was confirmed by cloning the *P. citri* HP1-like gene [40] which was found to coincide with *pchet2,* a chromodomain-containing gene identified by Epstein and collaborators in 1992 [55]. The *pchet2* sequence was used to construct double-stranded interfering RNA that was employed to knockout *pchet2* expression in coccid embryos. The knockout resulted in the inhibition of facultative heterochromatin formation [40]. In fact, the lack of chromocenter development following PCHET2 depletion made it very difficult to distinguish male embryos. The role of PCHET2 was also confirmed in some adult tissues in which the reversion of heterochromatinization occurs. In gut tissues, for example, the loss of chromocenters was accompanied by the dispersion of the PCHET2 signal (Figure 6).

The distribution of the histone modifications K9H3me3 and K20H4me3 in *P. citri* nuclei was also coincident with facultative heterochromatin [38, 39]. Moreover, the immunological detection of these histone modifications in male embryos preceded the appearance of facultative heterochromatin. Significantly, *pchet2* knockout led to the loss of immunostaining for both K9H3me3 and K20H4me3 [40].



(a) (b)

Figure 4: *P. citri* male meiosis. Meiotic sector from a testis of a third instar male. (a) DAPI-staining; (b) the same tissue patch, stained for DNA (pseudocolored in red) and for meiotic spindle, by an antibody against α-tubulin (pseudocolored in green). The DNA staining shows the metaphase I (arrow) and metaphase II (arrowheads) plates that can be distinguished on the basis of their different sizes (see Bongiorni et al., 2004, [37]). The meiotic spindle immunostainingstaining shows that meiosis II metaphase plates are associated with a monopolar spindle. Bar represents 10 μm.

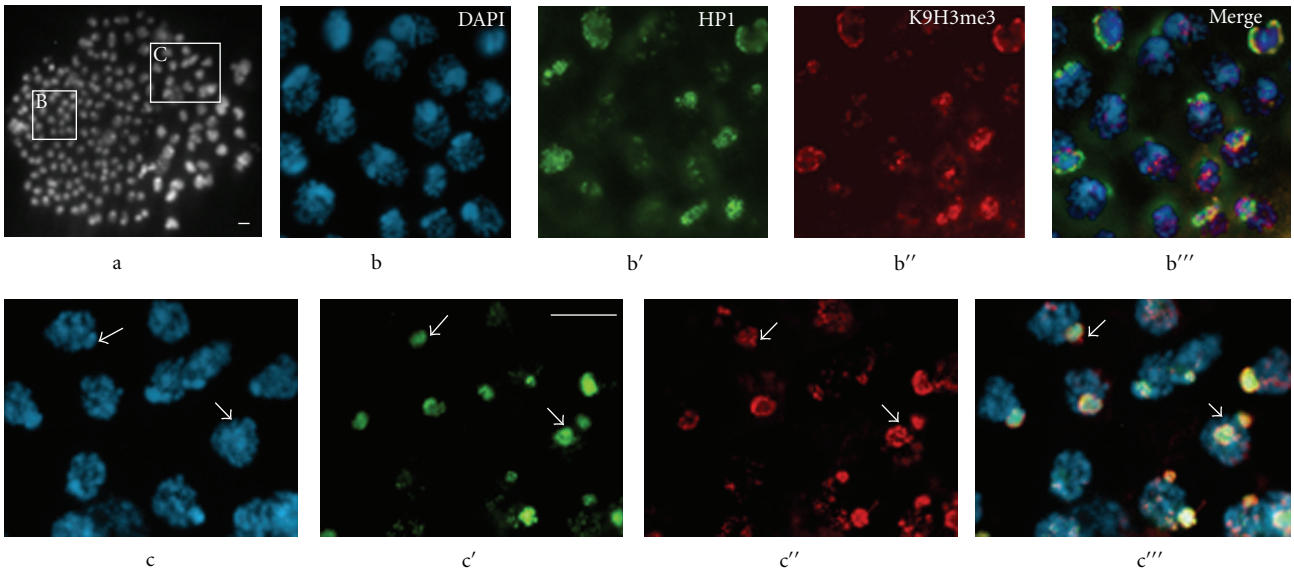


Figure 5: Pattern of epigenetic marks in *P. citri* cleavage embryos. Localization of C1A9 (anti-HP1) and anti-K9H3me3 antibodies to nuclei in midcleavage embryos (128–256-nuclei embryos) undergoing facultative heterochromatinization. (a) Whole embryo: the wave of facultative heterochromatinization is spreading from the bottom left corner toward the top right corner. Boxed area (B) shows nuclei that have completed heterochromatinization and contain DAPI-positive chromocenters (see magnified image in b), whereas boxed area (C) shows nuclei still undergoing heterochromatinization, many of which have no overt DAPI-positive chromocenters (arrows in c). The nuclei in b are labeled with the anti-HP1 antibody (b′) and the anti-K9H3me3 antiserum (b″). The merged image in b‴shows colocalization of DAPI-positive chromocenter, HP1 and K9H3me3 staining. The DAPI-stained nuclei in c were stained with anti-HP1 antibody (c′) and the anti-K9H3me3 antiserum (c″). Although the pattern of K9H3me3 staining is more spread out in these nuclei compared with those that have completed heterochromatinization (compare c″to b″) the merged image in c‴shows that the K9H3me3 pattern largely colocalizes with HP1 staining. C1A9 antibody recognizes the *P. citri* HP1-like, PCHET2. Bars, 10 μm. Reprinted from Bongiorni et al. 2007 [40].

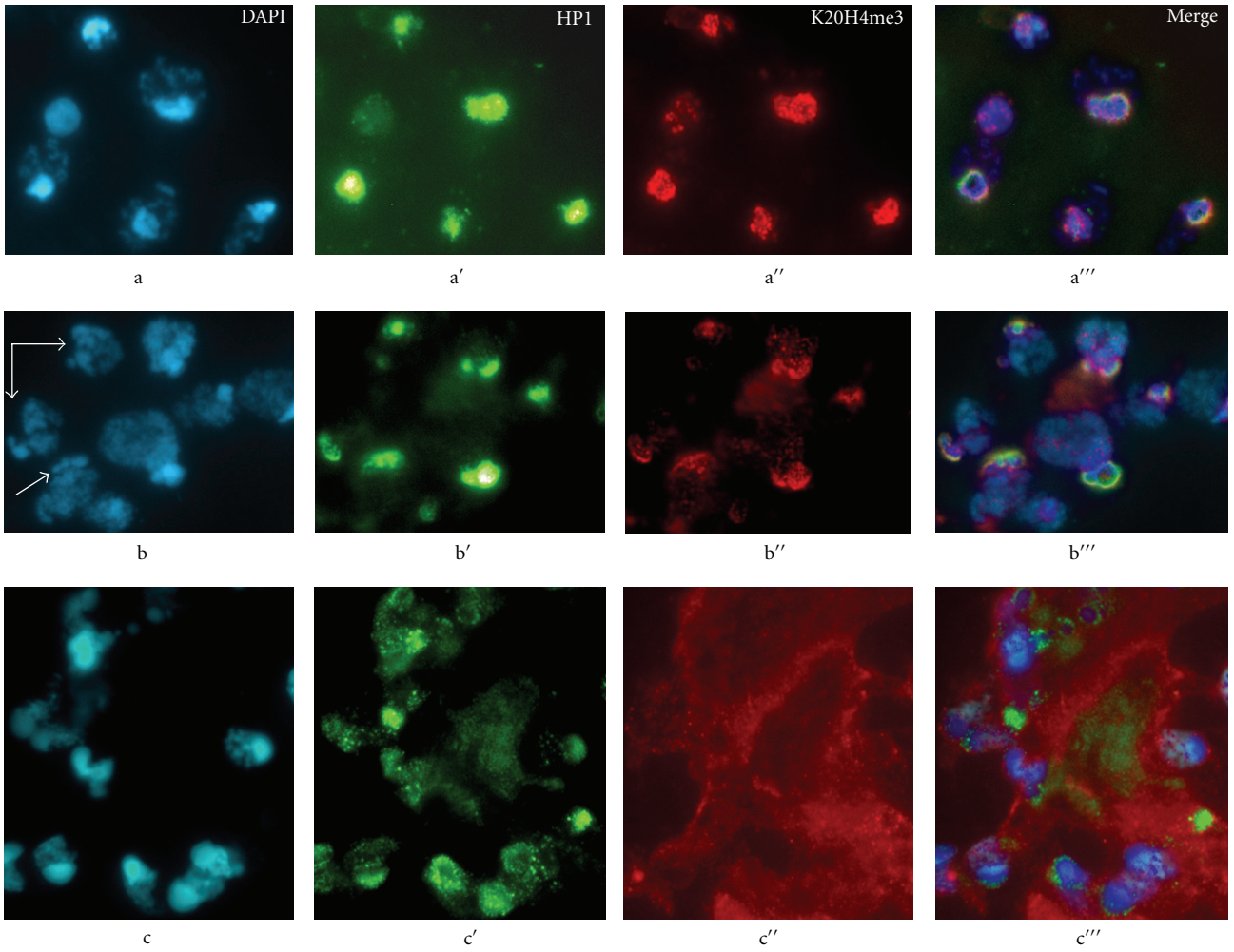


Figure 6: Localization of K20H4me3 in *P. citri* nuclei undergoing either facultative heterochromatinization or developmental de-heterochromatinization. (a) DAPI-stained nuclei from a midcleavage embryo (128–256-nuclei embryo) that underwent facultative heterochromatinization. A clear DAPI-stained chromocenter can be seen in each nucleus. The same nuclei were labeled with C1A9 (anti-HP1) antibody (a′) and with the anti-K20H4me3 antiserum (a″); the merged image in a‴ shows coincidence of DAPI-positive chromocenters with HP1 and K20H4me3 staining. The nuclei in b are from another area of the same embryo that has yet to complete heterochromatinization and several have no overt DAPI-positive chromocenters. The DAPI-stained nuclei in b were simultaneously stained by anti-HP1 antibody (b′) and by the anti-K20H4me3 antiserum (b″). Whereas the K20H4me3 staining is more dispersed in these nuclei compared with those that have completed heterochromatinization (compare b″ to a″), the merged image (b‴) shows that the K20H4me3 pattern largely colocalizes with HP1 staining. (c) DAPI-stained nuclei from cells of adult tissues that undergo developmental reversal of heterochromatinization. HP1 staining is dispersed and has a grainy appearance over the nuclei (c′) that, instead, lack any K20H4me3 staining which is rather dispersed over the cytoplasm (c″). (c‴) Merged image. Bars, 10 μm. Reprinted from Bongiorni et al. 2007 [40].

Interestingly, a study on the inactivation of the human X chromosome showed a similar colocalization of HP1 with K9H3me3 and K20H4me3 histone modifications on the inactive X [56]. The K9H3me3-HP1-K20H4me3 pathway is thus an evolutionarily conserved mechanism for epigenetic silencing of large chromosomal domains by facultative heterochromatinization.

The pattern of acetylation of histone H4 (AcH4), a histone modification that has been associated with active chromatin was investigated in *P. citri* by Ferraro and collaborators [57], who found that the male-specific heterochromatic chromocenter is devoid of this modification, as is also the case for the inactive X chromosome in female mammals [58].

Interestingly, all the factors implied in mealybug facultative heterochromatin assembly are already associated with constitutive heterochromatin. The same is true of heterochromatin protein HP2 that was isolated as a constituent of *D. melanogaster* constitutive heterochromatin. Using an antibody against the Drosophila HP2, we demonstrated that it also decorates the *P. citri* male-specific heterochromatin [41].

The imprinting cycle features (see the last paragraphs of Section 1) focused the search for its molecular mechanisms on DNA methylation, whose characteristics (establishment,

maintenance, and erasure) fulfilled the requirements of imprinting cycle in mammals [23] (Figure 1). In chromosomal domains, where imprinted genes lie, sequence elements have been identified that are essential to the imprinted gene expression. These "imprinting control elements" (ICEs) are rich in CpG dinucleotides (many correspond to CpG islands), which exhibit parent-of-origin-specific differential DNA methylation. Following fertilisation, allele-specific methylation marks are maintained throughout development and modulate the imprinted differential expression of the alleles [59]. These regions of differential methylation (DMRs) may be either at the boundary between reciprocally imprinted genes or in the promoter of antisense silencing RNAs [60, 61]. In mealybugs, the role of DNA methylation in imprinting was first studied by Scarbrough and collaborators in 1984 [62]. These authors showed the presence of methylated cytosines in the male genome of *P. calceolariae* and measured, by HPLC, the total amount of methylated cytosines in males (0.68 + 0.02%) and females (0.44 + 0.04%) [62]. However, these studies failed to directly correlate DNA methylation and chromosome heterochromatinization. The occurrence of CpG methylation in *P. citri* was confirmed by our group at both the molecular and cytological levels [63]. We showed that the paternally derived chromosomes were hypomethylated at CpG dinucleotides compared to maternal chromosomes in both males, where they were inactivated, and females, where they remained active. This result indicates that in mealybugs, as in mammals, parent-of-origin-specific differential DNA methylation is the molecular signal to imprint chromosomes. However, since in males paternal heterochromatic chromosomes are less extensively methylated than their maternal euchromatic counterparts, we concluded that DNA methylation in mealybugs does not induce genetic inactivation, as it occurs in vertebrates [63]. On the other hand, the lack of a direct correlation between DNA methylation and gene silencing seems to be a common feature in insects (reviewed in [64]).

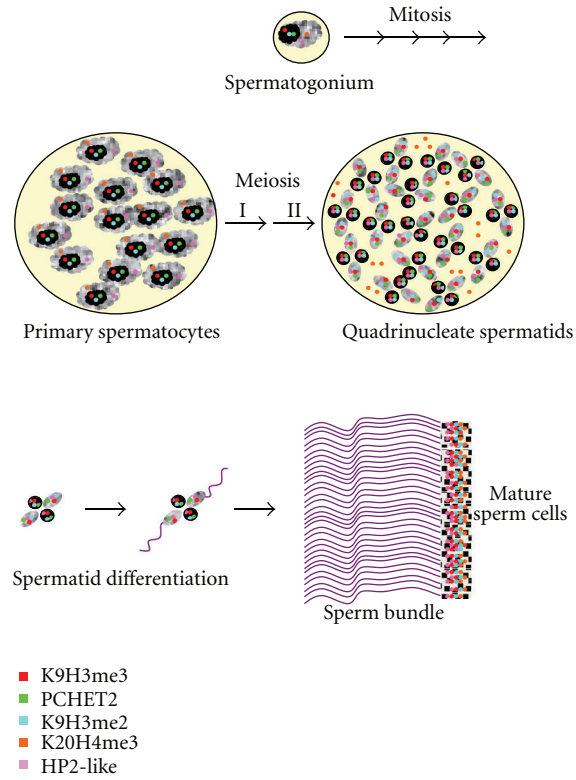


Figure 7: Epigenetic marks during *P. citri* spermatogenesis. A drawing representing the behavior of K9H3me3 (red), PCHET2 (green), K9H3me2 (light blue), K20H4me3 (orange), and of the HP2-like protein (pink), during spermatogenesis in *P. citri*. The HP1-like, PCHET2 is not detectable in sperm heads. According to Koshla et al. [66], sperm chromatin contains also a nuclease resistant fraction (NRC) that is transmitted to the progeny and that can be well considered as a component of the epigenetic machinery.

4. The Mealybugs as a Paradigm of Epigenetics

The reprogramming of the parent-of-origin-specific epigenetic marks during gametogenesis is one of the key features of genomic imprinting. In mealybugs, the chromatin remodeling events that occur during gametogenesis and lead to the facultative heterochromatinization of an entire haploid set of chromosomes in the male progeny were thoroughly scrutinized by immunocytological analysis of male and female gametogenesis (Figure 7) [42]. K9H3me3, K9H3me2, K20H4me3, PCHET2, and HP2-like could not be detected in females from meiosis to mature oocytes, whereas in males, they marked all stages from spermatogonia to spermatids, with a distribution pattern that changed according to cell type. In spermatogonia, for example, whereas K9H3me3, K9H3me2, and PCHET2 were enriched within the heterochromatin, HP2-like and K20H4me3 were found in the euchromatin [42]. However, at the spermatid stage, K9H3me3, K9H3me2, PCHET2, and HP2-like reallocated over both the euchromatin- and the heterochromatin-containing spermatids, which were produced by nonindependent assortment during inverted meiosis. The redistribution of epigenetic signals in spermatids might be related to the establishment of parental imprinting. These results were in agreement with the model proposed by Brahmachari and collaborators [65, 66], who described the reorganization of the male-specific NRC (nuclease-resistant chromatin) [67, 68] during spermatogenesis. These authors found that NRCs were acquired during maturation by sperm nuclei that contained the maternal, originally NRC-free, chromosome set [66]. Following spermiogenesis, PCHET2, the mealybug HP1-like protein was lost from mature sperm, whereas K9H3me3, K9H3me2, K20H4me3, and HP2-like were still detectable, thus ruling out the possibility that PCHET2 could play a role in the imprinting mechanism. Sperm that entered the oocyte possessed distinct K9H3me3 and K9H3me2 signals that were still found in the early pronucleus. Thus, K9H3 di- and trimethylation turned out to be the best candidates for the marks that imprint the paternal chromosomes. Buglia and Ferraro reported that the two euchromatic spermatids originating from a single meiosis were labeled with different levels of K9H3me3 and of C1A9-positive immunostaining, suggesting that the two resulting

sperms produced male or female progeny according to the amount of these epigenetic factors [69]. However, the nuclei of quadrinucleate spermatids share a common cytoplasm thus making it unlikely that an enrichment of K9H3me3 in one of the "euchromatic" spermatids could occur independently from the other "euchromatic" spermatid. Accordingly, in our scrutiny of *P. citri* spermatogenesis, we failed to observe any significant difference of labeling between the two euchromatic spermatid nuclei stemming from the same meiosis, with any of the epigenetic factors we tested [42].

Taken as a whole, all these observations suggest that the sex determination of the zygote is very likely dependent upon some unknown factor(s) that is deposited in the cytoplasm of the egg by mother. This scenario is consistent with the studies of Nelson-Rees [70], who showed that the sex-ratio widely fluctuates from female to female and is markedly influenced by the mother's age. Additionally, in insects sex ratio can be affected by different environmental factors acting on parents, like extreme temperature, starvation, and lack of resources [71–76]. In *P. citri* females, various factors, such as population density [77, 78], temperature [70, 79], and mating age [70, 78, 80], were found to influence sex allocation. Ross and collaborators tested three environmental factors (rearing temperature, food deprivation, age of mating) and showed that the effect of high temperature was rather weak, food restriction appeared to be strongly associated with reduced longevity, while older age at mating affected sex allocation, resulting in female-biased sex ratios [81]. The mechanism of this phenomenon is still unclear although PCHET2 and the histone modifications involved in the facultative heterochromatization [10, 28, 37, 40] are thought to be also involved in sex determination [82]. Females might alter the concentration of these proteins in their eggs to modulate the sex ratio of their broods. Along these lines, Buglia and collaborators observed increased concentrations of a C1A9 positive-staining protein in eggs of females that were aged prior to mating [83]. They supposed that these females would produce male-biased offspring (although the sex ratio data were not provided), whilst the opposite effect of maternal ageing prior to mating was observed in other studies [81].

We can hypothesize that the embryo cytoplasm, at blastoderm stage, determines whether the paternal chromosomes, which are marked by DNA hypomethylation [63] and K9H3 methylation [42], will undergo heterochromatinization or not, giving rise to a male or a female embryo, respectively. Given the causative role of PCHET2 in male-specific heterochromatin formation [40], the amount of PCHET2 in the developing embryo may be crucial to steer the embryo toward male or female development. As above reported, facultative heterochromatinization forms in 7th cleavage male embryos as a wave from one pole of the embryo toward the other [10], suggesting a graded distribution of PCHET2 in the embryo. Since PCHET2 could be evidenced neither in the sperm nor in the oocyte [42], its presence in the embryo should be the result of early *de novo* synthesis under the control of the above-mentioned maternal factor(s).

Khosla et al. have also suggested that a unique chromatin organization is a mechanism of genomic imprinting in coccids [65]. The nuclease resistant chromatin (NRC) [67] represents an altered organization of 10% of the paternal genome, not cytologically equivalent to heterochromatin, but perhaps containing the putative centres for facultative heterochromatin nucleation. At the cleavage stage a choice is made between the maintenance or loss of the NRC transmitted with the sperm, leading to a male or female developmental pathway, respectively [65]. The model of Koshla et al. [65] can be well reconciled with our cytological dissection of imprinting marks during spermatogenesis [42]. We can assume, as suggested by Khosla et al., that NRC regions represent chromosome inactivation centers scattered at many loci along the chromosomes. NRCs may be imprinted in the mature sperm by DNA hypomethylation and K9H3 trimethylation marks that then spread to the whole paternal genome. Then, in the cleavage embryo, some maternal factor(s) might regulate the amount of PCHET2, that gradually spreads from one embryo pole to the other. A critical amount of PCHET2 will then determine whether the paternal imprinted chromosomes will become heterochromatic, thus leading to male development, or will remain euchromatic, losing repressive histone modification and NRCs, and eventually leading to female development.

5. Perspectives

Based on the characteristics presented in this paper, the phenomenon of imprinted facultative heterochromatinization in mealybugs represents one of the most remarkable examples of epigenetics in eukaryotes.

The mealybug chromosome system offers a very acute tool with which to dissect the phenomenon of facultative heterochromatinization and the mechanisms of parental imprinting. The conservation in mealybugs of almost all the epigenetic mechanisms that act in mammals strongly supports the use of these species as a model for epigenetics. Most epigenetic mechanisms, such as histone modifications and their interplay with the HP1 proteins, show the same functional role in mealybugs as in mammals; others, namely, DNA methylation, exhibit a different involvement in epigenetics.

From the above considerations it appears that a genome-wide approach to map the distribution of epigenetic marks along coccid genome, represents a new challenge for the functional analysis of epigenomes. The epigenetic landscape of the mealybug genome might be useful (i) to determine if there are DNA sequences that act as inactivation centres, scattered along the chromosomes, as suggested by the inactivation of small fragments from irradiated chromosomes; (ii) to highlight the possible role of small RNAs in facultative heterochromatinization and imprinting; (iii) to analyze a specific functional role for the different histone modifications; (iv) to verify the presence and distribution of DNA methylation and its relationship to the histone modifications.

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Research Article

The Epigenetic Repertoire of *Daphnia magna* Includes Modified Histones

Nicole F. Robichaud, Jeanette Sassine, Margaret J. Beaton, and Vett K. Lloyd

Department of Biology, Mount Allison University, Sackville, NB, Canada E4L 1G7

Correspondence should be addressed to Vett K. Lloyd, vlloyd@mta.ca

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Daphnids are fresh water microcrustaceans, many of which follow a cyclically parthenogenetic life cycle. *Daphnia* species have been well studied in the context of ecology, toxicology, and evolution, but their epigenetics remain largely unexamined even though sex determination, the production of sexual females and males, and distinct adult morphological phenotypes, are determined epigenetically. Here, we report on the characterization of histone modifications in *Daphnia*. We show that a number of histone H3 and H4 modifications are present in *Daphnia* embryos and histone H3 dimethylated at lysine 4 (H3K4me2) is present nonuniformly in the nucleus in a cell cycle-dependent manner. In addition, this histone modification, while present in blastula and gastrula cells as well as the somatic cells of adults, is absent or reduced in oocytes and nurse cells. Thus, the epigenetic repertoire of *Daphnia* includes modified histones and as these epigenetic forces act on a genetically homogeneous clonal population *Daphnia* offers an exceptional tool to investigate the mechanism and role of epigenetics in the life cycle and development of an ecologically important species.

1. Introduction

Daphnids are freshwater crustaceans that hold the distinction of being among the relatively few genera that reproduce parthenogenetically. Under most circumstances conventional oogenesis is modified. The first meiotic division is abortive so only the mitosis-like equational division occurs producing clonal diploid eggs [1, 2]. While homologs do pair in the abortive first meiotic division [2] and many of the same meiotic genes are expressed in parthenogenetic and sexual reproduction [3], there is no cytological [2] or genetic [3, 4] evidence for recombination. As a result, other than rare mitotic recombination, conversion, or mutational events [5], the progeny produced are genetically identical [1, 2, 4]. However, while the offspring are genetically identical to each other and their mother, they are not necessarily epigenetically identical. Under stressful conditions some of these clonal diploid eggs develop as males rather than females [1, 6–8]. Additionally, in many species stressful conditions similarly trigger the restoration of conventional meiosis allowing production of haploid eggs and sperm [1–3, 6, 8]. Importantly, parthenogenetically reproducing females and sexually reproducing females are genetically identical, and both are identical to their mothers [1, 4, 5]. Moreover, parthenogenetically produced males are genetically identical to parthenogenetically produced females [1, 4, 5]. Thus, environmental signals induce epigenetic changes that control essential aspects of the life cycle—sex determination and sexual reproduction.

Epigenetic variation in daphnids has also been studied in the context of environmentally induced morphological changes, which are termed polyphenisms. In the presence of predators, *Daphnia* can produce a variety of defensive structures such as helmets, neckteeth, crests, or elongated tail spines and spikes, depending on the species [9]. As these changes occur in parthenogenetic populations in which all animals are genetically identical clones, these changes are necessarily epigenetic [9–11].

Although *Daphnia* provide an excellent system for the study of epigenetics, surprisingly, this system has not been widely exploited. This is despite the rich literature relating to their evolution, reproduction, and ecology. There are also

many genomic tools available for studying these organisms, including the genome sequence of *D. pulex* [12, 13], which has allowed the development of bioinformatic and other genomic technologies such as microarrays [14, 15], cytogenetics [16], cell culture [17], transgenics [18, 19], and RNAi gene knockdown technology [19]. *Daphnia* are ubiquitous and key members of aquatic communities, a role that has led to their extensive use in ecotoxicology, and more recently ecotoxicogenomics [15]. Because of the ecological importance of daphnids as well as their unusual development, understanding their epigenetic repertoire and its deployment in normal development and under environmental stresses is significant, yet the epigenetic resources of daphnids, which is how the environment regulates the genome, remain poorly explored.

Investigations into *Daphnia* epigenetics, to date, have focused primarily on DNA methylation. Partial sequencing of the *D. magna* genome revealed that this species has homologs of the three major vertebrate DNA methyl transferases, Dnmt1, Dnmt2, and Dnmt3A [20] and that CpG methylation does occur [21]. While the level of methylation is relatively low, it is sensitive to developmental stage, increasing modestly in adults from 0.13% of all CpG dinucleotides in 7-day-old individuals to 0.26% in 32-day-old individuals [21]. Investigation of other core epigenetic processes such as histone modification or noncoding RNA, or the role of these epigenetic mechanisms in either normal development or the well-studied predator-induced epigenetic polyphenisms, has yet to be pursued. Here, we report that *D. magna* shows both histone H3 and H4 modifications in embryonic cells. Furthermore, one of these modifications, histone H3 dimethylated at lysine 4 (H3K4me2), occurs nonuniformly in a cell-cycle-specific manner in gastrula cells and is absent from oocytes.

2. Materials and Methods

2.1. Daphnia magna Culture. *Daphnia magna* were acquired from WARD's Natural Science. They were kept at room temperature (25 ± 5°C) in 150 mL cups filled with synthetic pond water and fed with 2-3 mL of *Scenedesmus* culture (WARD's Natural Science) three to four times weekly. The algae were grown at 20°C in twenty-four hours of light in Bold's Basal Medium.

2.2. Histone Protein Analysis by Immunohybridization. 80 young embryos were rapidly dissected from the mother's brood pouch in 0.6% NaCl and 0.03% triton X-100 and stored in 1.5 mL microtubes on ice for no more than 30 min. The liquid was removed and replaced with 200 μL of 0.05 M DTT and 1X NuPAGE LDS Sample buffer (Invitrogen). The embryos and loading buffer were heated at 96°C for 5 min. and cooled to room temperature and the solution collected by centrifugation for 10 sec. 15 uL of the homogenate was electrophoresed on a 4–12% SDS-PAGE gel (Invitrogen) at 200 V for 40 min. 2.5 μL Precision Plus Protein Standards (Bio-Rad) and Magic Mark (Invitrogen) were used as molecular weight standards. Gels to be immunoblotted were transferred to a PVDF membrane (Bio-Rad) in an XCell II chamber (Invitrogen) at 30 V for 80 min. The membrane was incubated in 2% Enhanced-Chemiluminescence (ECL) blocking agent (Amersham) in 0.1% TBST (5X; 12.1 g TRIS, 40 g NaCl, pH 7.6 with HCl) for 15 min at room temperature, followed by 15 ± 5 h at 4°C. The blocking agent was removed, and 10 mL of diluted primary antibody in 2% ECL with 0.1% TBST was added to the membrane and incubated for 60 ± 2 min at room temperature. The primary antibodies (mouse monoclonal antibody to histone H3 trimethyl K27 (H3K27me3; Abcam 6002), rabbit polyclonal antibody to histone H4 dimethyl K20 (H4K20me2; Abcam 9052), rabbit monoclonal antibody to histone H3 acetyl K14 (H3K14ac; Abcam 52946), rabbit monoclonal antibody to histone H3 dimethyl K4 (H3K4me2; Abcam 32356), or rabbit polyclonal antibody to histone H3 monomethyl K9 (H3K9me; Abcam 9045)) were diluted 1 : 500. The membrane was washed with 0.1% TBST twice for 3 sec, once for 15 min, and thrice for 5 min. 10 mL of secondary antibody (1/3,000 dilution of goat polyclonal to rabbit IgG, HRP conjugated (Abcam)) was added and incubated for 60 ± 10 min. The membrane was washed twice for 3 sec, once for 15 min, and thrice for 5 min with 0.1% TBST, developed with Lumigen developing reagent (Amersham) for 5 min with minimal light exposure and imaged with a Fluor-S-Imager (Bio-Rad).

2.3. Collection and Staging of Daphnia Embryos for In Situ Immunodetection. Immunocytology was performed, with some modifications, using the procedure employed in [22] and kindly provided by Y. Shiga. For convenience, the procedure is described below. Embryos or ovaries were dissected from adults using a fine tip probe (Moria Instruments) and placed in 1.5 mL of 0.6% NaCl and 0.03% Triton X-100 in 1.5 mL microtubes. Stage 1 and 2 embryos were selected for dissection based on their size, colour, and other morphological characteristics as outlined in [23]. The ovary was collected by removing the carapace and separating the ovary from the gut with a fine tipped probe.

After collection of embryos, the NaCl Triton X-100 solution was removed and replaced with 1.5 mL of a 3 : 1 ratio of 1.33X phosphate-buffered saline (PBS) and 37% formaldehyde and 50 mM EGTA. The samples were allowed to fix for 20 minutes at room temperature. The fixative was removed by pipet, and samples were washed (for all washes 1.5 mL of the solution was added, left for 5 minutes, removed by pipet, and replaced with another 1.5 mL of solution) sequentially with 25%, 50%, 75%, and 100% methanol. The samples were then frozen in 1.5 mL of 100% methanol at −20°C.

The samples were brought to room temperature and then washed five times with 100% methanol. Samples were then washed five times with 1X phosphate-buffered saline and 0.1% polysorbate 20 (PT). Samples were then washed three times with 0.1 M Tris, 0.15 M NaCl, and 0.5% bovine serum (TNB). For mechanical lysis of the vitelline and other embryonic membranes, embryos were subjected to three freeze/thaw cycles in which the embryos, in 500 μL TNB, were frozen at −80°C for 30 min and then rapidly brought to

room temperature. Ovaries were not subjected to freeze/thaw cycles. Samples were then washed twice more in 1.5 mL TNB and left in 1.5 mL TNB for 1 hour. A 1 : 10 dilution of the primary antibodies was added to the samples in 49 μL of TNB, making a final dilution of 1 : 500, and the samples were incubated at 4°C overnight. The solution containing the primary antibody was then removed and the samples washed five times in TNB. 1 uL of the secondary antibody, goat anti-rabbit IgG FITC conjugate (Zymed), was added to 49 μL of TNB and embryos and incubated for 2 hours at 4°C in the dark. The samples were protected from light for the remainder of the experiment. The samples were washed five times with TNB and then five times with 0.1 M Tris, 0.15 M NaCl, and 0.05% polysorbate 20 (TNT). 15 μL of 10 μg/mL 4′-6-diamidino-2 phenylindole (DAPI) in TNT was added to the samples in 1.5 mL TNT and left for five minutes at room temperature. Excess DAPI solution was removed and the samples washed twice for 5 min with TNT.

To visualize the samples, excess TNT was removed and embryos or tissues were placed in a drop of Vectashield mounting medium (Vector Labs) before adding a cover slip. The embryos or tissues were viewed using an Axioscope 2 Plus (Zeiss) fluorescent microscope. Images were captured with Axiovision AC software (Zeiss).

3. Results

Histone modifications are one of the most important and conserved aspects of epigenetic gene regulation [24–27] and as such are a good target for an initial investigation into *Daphnia* epigenetics. Further, histone proteins are among the most highly conserved proteins in eukaryotes [28] so it seemed likely that commercially available antibodies raised to modified histones in other species would also work in *Daphnia*.

3.1. Confirmation of Antibody Specificity. To confirm this supposition, we performed immunohybridization of *Daphnia magna* embryos with antibodies against human histone H3 modified by trimethylation of lysine 27 (H3K27me3), dimethylation of lysine 4 (H3K4me2), monomethylation of lysine 9 (H3K9me), acetylation of lysine 14 (H3K14ac) or histone H4 modified by dimethylation of lysine 20 (H4K20me2). As expected, these antibodies all detected a predominant band at 17 kD, the expected size of histones H3 and H4 (Figure 1). Weakly hybridizing bands at approximately 15 kDa and 100 kDa were also detected, particularly when the immunoblots were overexposed. Information from the supplier indicates that the H3K27me3 antibody detects a 15 kDa band from human cells, suggesting that this band represents histone protein fragments. The 100 kDa band likely represents proteins associated with cell and body fragments not completely removed during protein preparation.

3.2. Immunocytological Analysis of Blastula and Gastrula Embryos. As the antibodies appear to detect the appropriate modified histones in *Daphnia*, we next used them to examine

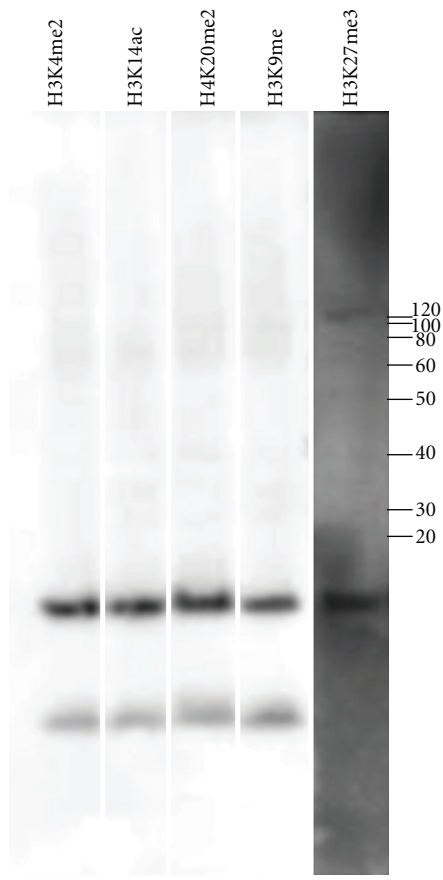

Figure 1: Immunohybridization of *Daphnia magna* embryos with antibodies specific to modified histone H3 and H4. Immunohybridization of protein extracted from *Daphnia* embryos with antibodies to histone H3 trimethyl K27 (H3K27me3), histone H4 dimethyl K20 (H4K20me2), histone H3 acetyl K14 (H3K14ac), histone H3 dimethyl K4 (H3K4me2), and histone H3 monomethyl K9 (H3K9me). These antibodies all detect a strong band at 17 kDa, the expected size for histone H3 and histone H4.

embryos for the presence and nuclear distribution of these modifications (Figure 2). To ensure that the antibodies were able to access the embryonic cells, the extraembryonic membranes were ruptured by freeze-thaw cycles, as described in Section 2, so that the normally spherical embryos show torn membranes and, occasionally, released embryonic cells.

Histone 3 trimethylation of lysine 27 (H3K27me3) and monomethylation of lysine 9 (H3K9me) are considered markers of heterochromatin [27, 29]. Dimethylation of lysine 20 of histone 4 (H4K20me2) has been shown to prevent acetylation at lysine 16 that would, in the absence of H4K20me2, promote the formation of euchromatin. Thus, the H4K20me2 modification indirectly promotes heterochromatin formation [27]. Antibodies specific to H3K27me3 and H3K9me (Figures 2(a) and 2(b)) show uniform nuclear staining, coinciding exactly with the DNA detected by DAPI staining. Similarly, H4K20me2 staining (Figure 2(c)) is uniform throughout the nucleus. This pattern was observed in multiple blastulae and gastrulae cells thus it appears invariant in these embryonic stages.

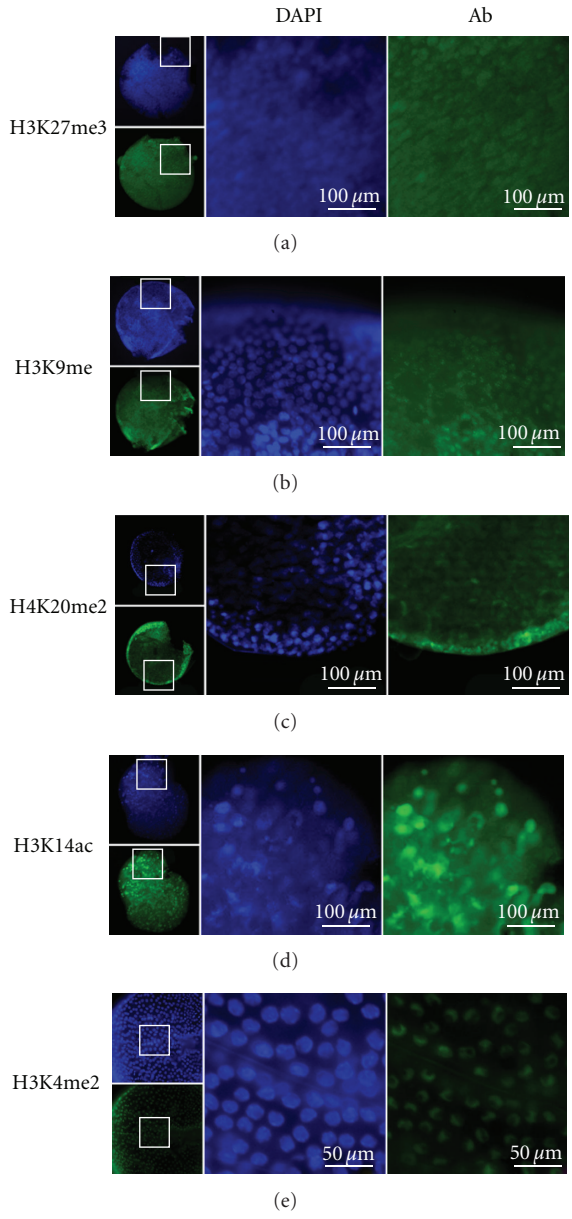

FIGURE 2: Whole mount immunocytochemistry of *Daphnia magna* embryos stained with antibodies specific to modified histone H3 and H4. The small images show the embryo from which the magnified images to the right are shown. Blue staining is with DAPI, which detects all DNA. Green staining (Ab) is for the specified histone modification. (a) H3K27me3. (b) H3K9me. (c) H4K20me2. (d) H3K14ac. (e) H3K4me2. The embryos shown in (a–d) are blastula stages, (e) is a magnified view of a gastrula embryo. The low magnification views show the torn extraembryonic membranes required to allow antibody penetration to the cells.

Acetylation of histone H3 at lysine 14 (H3K14ac) and dimethylation of lysine 4 (H3K4me2) are considered to be markers of open or euchromatic chromatin [30]. Antibodies specific to H3K14ac also showed uniform staining of the nucleus that coincides with DNA staining by DAPI (Figure 2(d)).

In contrast, staining with anti-H3K4me2 was consistently nonuniform with concentration at the nuclear periphery that did not completely coincide with DNA staining by DAPI (Figure 2(e)). The preferential staining of the nuclear periphery by H3K4me2 was not an artifact of antibody accessibility or interference from the various embryonic membranes as it was observed only with this antibody (Figure 2) and was also apparent in isolated cells released from the embryonic membranes by sonication (data not shown). The non-uniform distribution of H3K4me2 was reproducibly observed in cells from late blastulae to gastrulae embryos.

The subnuclear distribution of H3K4me2 staining also appears to be dependent on the cell cycle. In interphase nuclei, H3K4me2 staining was the strongest at the periphery of the nucleus and largely excluded from the interior. However, by prophase and metaphase, DAPI and H3K4me2 staining was largely coincident (Figure 3).

To further investigate H3K4me2 distribution in different developmental stages and cell types, antibodies specific to H3K4me2 were used to investigate ovaries from parthenogenetically reproducing females. DAPI staining shows both small cells and bigger cells with large nuclei (Figure 4). The larger cells (Figure 4 (l)) are likely polyploid lipid-containing fat cells [1]. The smaller cells are diploid germ-line cells, either the stem cells in the germarium (Figure 4 (g)) or developing oocytes and their companion nurse cells (Figure 4 (o)). Quartets of these cells remain attached as a result of incomplete cytokinesis in the two preceding divisions and so are clustered [3]. However, histological distinction between the oocyte and nurse cells is not possible until later in development [1, 3]. The staining indicates that H3K4me2 is present in both the germarium cells and somatic fat cells. However, H3K4me2 is either absent from or greatly reduced in the developing oocytes and nurse cells.

4. Discussion

While the core epigenetic mechanisms of DNA methylation and histone modification are interrelated [31], organisms vary in the extent of their reliance on each of these mechanisms [32]. For example, in mammals and plants that methylate their genomes extensively, DNA methylation is a key aspect in genomic imprinting. However, in *Drosophila*, which has a much lower level of genomic DNA methylation, genomic imprinting relies primarily on histone modifications and related chromatin-based mechanisms [32, 33]. While *Daphnia* do have DNA methyl transferases and methylate their genome [20, 21], the level of DNA methylation is low, comparable to that of *Drosophila* [21], suggesting that histone modifications may similarly play a larger role in epigenetic regulation. For this reason, we initiated an investigation of histone modifications in *Daphnia*, to our knowledge, the first such investigation.

We have demonstrated that the epigenetic repertoire of *Daphnia* includes histone modification, represented by the best-characterized methylated and acetylated modifications

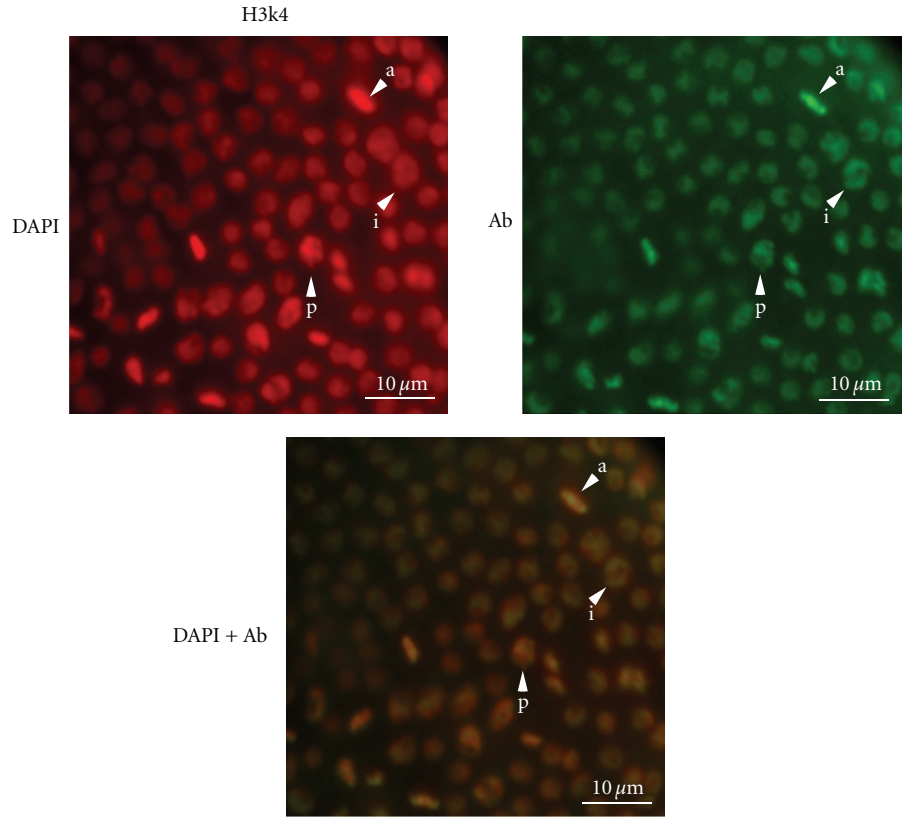

Figure 3: Localized, cell-cycle-dependent H3K4me2 staining in *Daphnia magna* gastrula nuclei. In interphase cells (i), the DAPI staining (red, left) is largely uniform whereas the H3K4me2 staining (green, right) is concentrated at the nuclear periphery producing a yellow-green circle with a red center in the merged image (lower panel). In cells undergoing prophase (p) and anaphase (a) the DAPI and H3K4me2 staining is coincident. Multiple cells in these stages are shown.

of histone H3 and histone H4. Histone modifications such as histone 3 trimethylated at lysine 27 (H3K27me3) or monomethylated at lysine 9 (H3K9me) and histone 4 dimethylated at lysine 20 are associated with heterochromatin and are present uniformly throughout the nucleus. In contrast, a modification associated with euchromatin occurs in a reproducible and distinct pattern around the inner periphery of the nucleus. Interestingly, this is the reverse of the usual organization of euchromatin and heterochromatin in the nucleus [34].

Euchromatic and heterochromatic structures influence the transcriptional status of a gene, which is conferred by a dynamic combination of different histone modifications, in conjunction with other epigenetic marks. The nature, abundance, and location within a gene of these epigenetic marks all affect the likelihood of transcription [24–27]. Thus, a single histone modification cannot unambiguously indicate the transcriptional status of a gene or the genome. Further, we are examining these modifications at the level of the nucleus rather than the gene and some of the early embryonic cells examined may not have been transcriptionally active. All of these considerations suggest that the pattern of modifications we see may not be indicative of transcriptional activity. It is, however, interesting that this pattern is the reverse of the canonical arrangement of euchromatin and heterochromatin in the nucleus. Chromosomes typically occupy distinct territories in the nucleus with heterochromatin segregated to the periphery [34]. Nuclei with the reverse organization, including the localization of H3K4me3, which like the H3K4me2 modification studied here is a marker for euchromatin, have been found in the retinal rod cells of nocturnal mammals [35, 36]. This organization has been attributed to selection for increased light transmission under low light conditions. This is unlikely to be the cause of the reversed organization of euchromatin in *Daphnia* embryos. However, as the "reversed" nuclear organization in nocturnal mice arises postnatally and only in rod cells it does demonstrate that genome architecture can be modified by natural selection. Thus, this unusual organization of the nucleus might be more common than previously thought.

This work lays the groundwork for further investigation of histone modifications associated with epigenetic events in the normal life cycle of *Daphnia*, such as the switch from parthenogenetic to sexual reproduction, including the development of males and haploid eggs, and the well described predator-induced epigenetic polyphenisms such as helmets and neckteeth. The external environment plays a role in regulating these key epigenetic events, and some of the genes involved in the signaling pathways by which the external environment influences the epigenome have

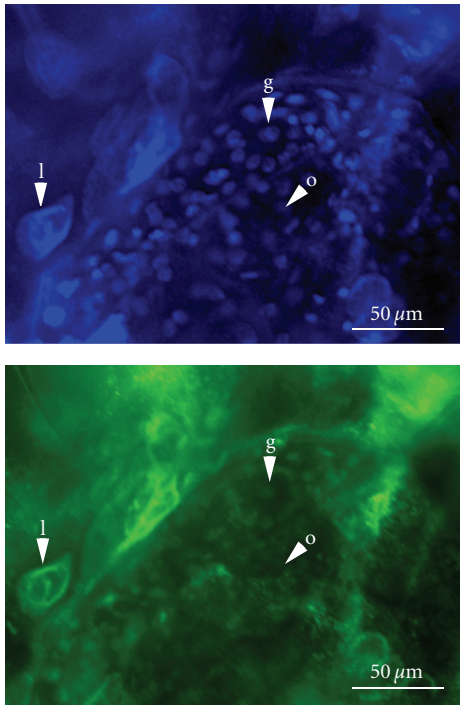

Figure 4: Whole mount immunocytochemistry of *Daphnia magna* ovaries stained with antibodies specific to H3K4me2. The top image shows DAPI staining, which detects all nuclei including the large lipid cell (l), the nuclei of the germarium (g), present in a rosette arrangement, and the nuclei of the developing oocytes and nurse cells (o). The lower image shows the same tissue stained for H3K4me2. The nuclei of the lipid cell and the cells of the germarium are detected. The nuclei of the oocytes and nurse cells are not strongly labelled with this antibody.

been identified [11]; it would be interesting to examine the epigenetic status of these genes under varying environmental conditions. Additionally, the gene knockdown technology that has been developed [18, 19] as well as conventional pharmacological inhibition of histone modifying enzymes using trichostatin A or butyrate will allow critical assessment of the role of histone modification in the interplay between the environment and genome in *Daphnia*. Finally, in organisms with conventional sexual reproduction, meiosis and gametogenesis are strictly coupled. However, in *Daphnia* oogenesis occurs with essentially mitotic nuclei. This situation would offer a unique opportunity to discriminate between chromosomal and cellular events in transgenerational epigenetic phenomena such as genomic imprinting.

Acknowledgments

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Review Article

Epigenetics in Social Insects: A New Direction for Understanding the Evolution of Castes

Susan A. Weiner[1] and Amy L. Toth[1,2]

[1] *Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, IA 50014, USA*
[2] *Department of Entomology, Iowa State University, Ames, IA 50014, USA*

Correspondence should be addressed to Susan A. Weiner, sweiner@iastate.edu and Amy L. Toth, amytoth@iastate.edu

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Epigenetic modifications to DNA, such as DNA methylation, can expand a genome's regulatory flexibility, and thus may contribute to the evolution of phenotypic plasticity. Recent work has demonstrated the importance of DNA methylation in alternative queen and worker "castes" in social insects, particularly honeybees. Social insects are an excellent system for addressing questions about epigenetics and evolution because: (1) they have dramatic caste polyphenisms that appear to be tied to differential methylation, (2) DNA methylation is widespread in various groups of social insects, and (3) there are intriguing connections between the social environment and DNA methylation in many species, from insects to mammals. In this article, we review research on honeybees, and, when available, other social insects, on DNA methylation and queen and worker caste differences. We outline a conceptual framework for the effects of methylation on caste determination in honeybees that may help guide studies of epigenetic regulation in other polyphenic taxa. Finally, we suggest future paths of study for social insect epigenetic research, including the importance of comparative studies of DNA methylation on a broader range of species, and highlight some key unanswered mechanistic questions about how DNA methylation affects gene regulation.

1. Introduction

Phenotypic plasticity is an important biological phenomenon that allows organisms with same genotype to respond adaptively to variable biotic and abiotic environments. There are several molecular mechanisms that can contribute to genomic flexibility and thus phenotypic plasticity, including transcriptional regulation, posttranscriptional modification, alternative splicing, and epigenetic modifications of DNA (reviewed in [1]). In this paper, we explore the potential role of epigenetic modifications in phenotypic plasticity in social insects in the order Hymenoptera (bees, ants, and wasps), a group of animals that exhibit many remarkable forms of morphological and behavioral plasticity [2]. Phenotypic polymorphism has arisen many times in different insect lineages [3] and not always among eusocial insects. Other well-studied examples of extreme phenotypic plasticity in insects include pea aphids with winged and wingless morphs, as well as sexual and asexual generations (reviewed in [4]), horned and hornless morphs in dung beetles [5], and phase differences in migratory locusts [6]. Studies of insects, and especially social insects, are providing intriguing new insights into the relevance of epigenetic modifications of DNA to the evolution of phenotypic plasticity [7, 8]. Eusocial insects provide some of the most dramatic examples of polyphenism found in any organism (Figure 1).

The colonies of eusocial insects can be highly complex, organized systems, sometimes containing tens of thousands or even millions of individuals [2]. In these colonies, despite the vast number of individuals, only a small percentage of individuals ever reproduce. In fact, in highly eusocial organisms such as honeybees, the workers have lost the ability to mate. The evolution of sterile workers has been a major evolutionary puzzle since Darwin [9]. One aspect that deeply concerned Darwin was that sterile female workers could be morphologically quite different from queens. Queens are generally larger, longer lived, and have large ovaries and a high reproductive output. In some species they can have

Figure 1: Examples of striking phenotypic plasticity between castes in the social insects. (a) Honey bee queen (center) and workers. (b) A winged reproductive termite *Reticulitermes flavipes* (center) and nonreproductive workers. (c) Queen leafcutter ant *Atta texana* (center) and a daughter worker (left). (d) Soldiers (with larger mandibles) and workers of the termite *Prorhinotermes inopinatus*. (e) An army ant *Eciton burcelli* soldier (center) and minor worker (bottom). (f) Major and minor workers of the leafcutter ant *Atta cephalotes*. All photos used by permission from Alex Wild.

vastly different body proportions and morphological characters compared to workers (Figure 1). How could such different phenotypes (castes) evolve if workers leave no descendants upon which natural selection can act? In some ant species, these caste systems are even more striking, with the presence of two or more types of morphologically distinct workers (e.g, specialized soldiers, major, and minor worker castes, Figure 1, [10]).

The extreme phenotypic plasticity of social insect castes has become even more compelling with the knowledge that, in most species, queen and worker caste differences are environmentally, not genetically, determined. With some notable exceptions, such as some genera of ants (e.g., [11–15]), in most social insects, there are no heritable genetic differences that dictate which individuals become queens and which become workers, nor among different morphological castes of workers [16]. Thus, the genomes of many social insects possess remarkable phenotypic flexibility, which is exquisitely sensitive to the abiotic and social environment (reviewed in [17]). Depending on the species and level of

sociality, caste differences can range from being completely behavioral and physiological (e.g., in *Polistes* paper wasps, [18]) through showing dramatically different alternate morphological phenotypes or polyphenisms (e.g., honey bees, Figure 1, [19]).

Work in rats and other mammals has uncovered that epigenetic modifications of DNA are important for mediating the effect of the early social (maternal) environment on adult phenotype (reviewed in [7, 20]). This work led to the suggestion that social modulation of the genome, and the resulting adult plasticity, may rely heavily on epigenetic effects [20]. This suggestion is made even more intriguing by the discovery that epigenetic effects are also important for caste determination in highly social honey bees [21–23] and likely in other social insect species [24]. In this paper, we summarize progress on epigenetics in social insects and compare this to work in other animals, in order to broaden the perspective on social insect studies. We also synthesize existing data into a conceptual framework of how epigenetic modifications of DNA may affect queen-worker caste phenotypes in social insects. Finally, we use this background to suggest what could be done to move the emerging field of social insect epigenetics forward.

2. Epigenetic Modifications of DNA

To facilitate our discussion of the importance of epigenetic modifications to social behavior in insects, we must first clarify what we mean by epigenetics. The term "epigenetics" has been used in a wide variety of contexts, to describe both organism-level and molecular-level phenomena [7]. Here, we refer specifically to chemical modifications to DNA that do not change the DNA sequence [7]. These modifications can be tissue specific or consistent throughout different cell types [25]. Epigenetic modifications can be made to DNA or to the histones on which DNA is stored [20]. They can even be transmitted from parents to offspring, so they can be stable over many cell divisions, though they can also be reversible (reviewed in [7]). Modifications present in the parental genome may be passed on, or new modifications may be made in the DNA of the gametes [7, 26]. This can lead to imprinting, in which paternal and maternal genes are differentially expressed [27].

A rough analogy can be made that the DNA sequence is like a written language with no spaces, capitalization, or punctuation. In other words, it contains the information to produce an organism, but that information cannot be properly decoded and understood in its raw form. Epigenetic modifications can be viewed as embellishments to the DNA language, providing punctuation that allows strings of nucleotides to be read and contain meaningful information. On a biochemical level, these modifications can help define the level at which genes are expressed (reviewed in [28]) and may also influence alternative splicing [23, 29].

Epigenetic DNA modifications can take several forms. Methyl groups can be added directly to nucleotides in a process called DNA methylation [30]. Primarily methylation occurs at the cytosines in CG dinucleotides, but methylation can occur on other cytosines or even other nucleotides [31]. In addition, modifications can be made to the histones around which DNA is packaged [20]. These modifications include methylation, acetylation, and ubiquitination [32]. All these different modifications have the potential to affect transcription via changes in chromatin structure and/or gene splicing patterns [20, 23, 32]. Most of the current literature, particularly in social insects, has focused on DNA methylation, so this paper will also focus on DNA methylation. However, histone acetylation is strongly negatively correlated with DNA methylation, and the two may be maintained in a dynamic equilibrium [20]; thus, it is important to keep in mind that other types of epigenetic modifications may have equally important effects on gene regulation.

DNA methylation appears to be an ancestral trait in eukaryotes but may serve different purposes in different taxa [33]. In plants and vertebrates, DNA methylation is important for the suppression of transposable elements [33]. Transposable elements are DNA sequences that can move themselves from one location to another in the genome, either by copying themselves or by cutting out of one region and reattaching elsewhere. In vertebrates, regions-containing transposable elements are heavily methylated, which both suppresses their expression and inactivates them over time by increasing the rate of mutation [34]. These elements tend to be more common in plants and vertebrates, although invertebrates are more subject to the effects of transposons than mammals, suggesting that one of the benefits of methylation is as a defense against transposons [35]. Gene body methylation is also common in plants and animals (but less so in fungi) [33]. In invertebrate animals, in particular, most methylation occurs within gene bodies [33]. Methylation can also occur at promoters or other noncoding regions, particularly in vertebrates and plants. When promoter regions are methylated, the expression of the gene or region is generally silenced [33].

Although DNA methylation has been associated with silencing of gene expression in vertebrates, more recent studies in insects suggest that gene body methylation is highest in genes with intermediate expression and in genes that are ubiquitously expressed in different tissues [22, 33, 36]. Differential methylation of a gene between different tissue types, social roles, or life stages may have important effects on gene expression [20, 21, 23, 33, 37]. In vertebrates, socially mediated methylation is known in promoter regions [20], but in insects, all evidence thus far suggests that social effects on methylation, and indeed nearly all methylation, occurs within gene bodies [33]. Methylation within genes may regulate splicing by an as-of-yet poorly understood mechanism [23, 38, 39].

The enzymatic addition of methyl groups to nucleotides involves several DNA methyltransferases (DNMTs). Most organisms with a fully functional DNA methylation system have at least one copy of each of DNMT1, DNMT2, and DNMT3. DNMT1 maintains methyl tags, while DNMT3 is involved in *de novo* methylation. DNMT2 is not considered a true DNA methyltransferase and may be involved in methylation of tRNAs [40, 41]. Despite the many important functions of DNA methylation, some organisms do without a complete set of methylation enzymes in their genomes

and have little or no methylation in their DNA [33]. For example, *Drosophila* does not possess DNMT1 or DNMT3 orthologs (reviewed in [33]). Therefore, *Drosophila* has very little methylation in its genome (reviewed in [41]) although a low level is present in embryonic stages [42]. Because of early studies with *Drosophila*, it was initially thought that DNA methylation was not important in insects (reviewed in [41]). However, recent work has revealed evidence of DNA methylation in several insects, including all Hymenoptera and some Orthoptera (crickets), Hemiptera (aphids), and Lepidoptera (moths) ([24, 33, 43], reviewed in [44]). DNA methylation is inferred to occur in all eusocial insects thus far examined [24, 45, 46]. Other insects with phenotypic polymorphism such as aphids have also been demonstrated to possess moderate levels of genome-wide methylation [47, 48].

There are many open questions relating to our understanding of how DNA methylation affects phenotype, and the social insects are a promising new model with which to better understand these questions. (1) Are epigenetic modifications of DNA a key mechanism in the evolution of extreme phenotypic plasticity [8]? (2) Did epigenetic effects facilitate the evolution of division of labor and eusociality? (3) What is the raison d'etre of epigenetic DNA modifications, and can the study of this theme in social insects help shed light on this question?

3. Connections between Epigenetics and Sociality

Because of their potential to be passed between generations, epigenetic changes to DNA have been of great interest as mediators of intragenomic conflict. Observations have long suggested that genes from maternal and paternal genomes (matrigenes and patrigenes) may have opposing effects on offspring phenotypes, such as the amount of resources offspring take from their mothers [27]. Paternally imprinted genes tend to cause offspring to take more resources to maximize their own fitness, while maternally imprinted genes tend to decrease the amount of resources taken to allow the mother to spread her investment over more offspring.

In a haplodiploid system such as the eusocial Hymenoptera (ants, bees, and wasps), it has been suggested matrigenes and patrigenes will be in further conflict over the treatment of social partners and offspring to which they are differentially related [26]. The haplodiploid genetic system of hymenopteran insects, in which females are diploid and males are haploid, results in "supersister" relationships in which sisters with the same father are on average 75% related [49]. Queller [26] predicted that genes promoting reproductive cooperation among closely related (e.g., supersister) females founding a nest together (such as in paper wasps in the genus *Polistes*) would be paternally imprinted (because patrigenes will be 100% shared whereas matrigenes only 50% shared). These and many other predictions related to imprinting in social insects still await experimental verification. Some of these questions could potentially be addressed by looking specifically at germline methylation. However, even in mammalian systems, in which imprinting has been best studied, imprinting through methylation is relatively uncommon and its mechanisms are still poorly understood [50].

Research on mammals has found that DNA methylation can be very important in mediating the effects of early life nutrition and social circumstances on phenotype [7, 20, 37, 51]. For example, rat pups that are cared for by more attentive mothers (mothers that perform more grooming and arched-back nursing behaviors) are less reactive to stress later in life [37]. This change is mediated by enhanced methylation of the exon 1_7 promoter of the glucocorticoid receptor in pups cared for by less attentive mothers [37]. Feeding adults methionine increased methylation of this exon and caused adults that were cared for by attentive mothers to have behavioral stress responses typical of rats receiving poor maternal care, indicating that methylation is changeable even in adult life and that methylation levels are directly linked to behavioral differences [51]. This work, in addition to other studies in mammals (reviewed in [52]), suggests that DNA methylation can be a key mechanistic link between the genome and the maternal and social environment [20].

Maternal effects (or similar effects mediated by workers) are very important in caste determination in social insects [26, 53, 54]. It is well known that brood-caregiver interactions (whether between mothers and offspring or workers and alloparental brood) are essential to caste differences [53, 54]. This can occur via differential feeding or nourishment [55], pheromonal signaling [56] or even vibrational cues [57]. Thus, there are fascinating (and heretofore unexplored) parallels between the potential effects of the maternal environment in mammals and brood care effects in social insects. A rough analogy between mammalian maternal effects and social/nutritional effects on caste determination in social insects suggests great potential for the role of DNA methylation in insect social organization [45].

4. Evidence for DNA Methylation in Social Insects

Evidence to date suggests important and widespread roles for DNA methylation in the Hymenoptera. The honey bee genome revealed that honeybees possess a complete set of DNA methyltransferases (two copies of DNMT1, and one each of DNMT2 and DNMT3) and DNA methylation has been experimentally verified in several studies [41, 45, 46]. Subsequently, a full complement of DNMTs was discovered in the solitary parasitoid jewel wasps, *Nasonia vitripennis* and two closely related species [58], as well as in 7 recently sequenced ant genomes ([59–64], reviewed in [44]) and in the paper wasp *Polistes dominulus* (A. L. Toth, unpublished data).

While the honey bee and *Nasonia* possess multiple copies of DNMT1 (three in *Nasonia* and two in honey bees), all sequenced ant genomes show evidence for only one DNMT1 (reviewed in [44]). This suggests the number of DNMT1 genes is evolutionarily labile within the Hymenoptera; however, further studies on additional solitary and social taxa are needed to understand this pattern of apparent expansion and contraction of DNMT1 genes. Based on rough estimates using methylation-sensitive restriction enzyme assays,

relatively high levels of methylation (similar to or higher than that in the honeybee) have been estimated in the paper wasp *Polistes dominulus*, the carpenter ant *Camponotus festinatus*, the advanced eusocial wasp *Polybia sericea,* and the yellowjacket *Vespula pennsylvanicus* [24], as well as the harvester ant *Pogonomyrmex barbatus* [61]. Somewhat lower levels have been estimated in several other social Hymenoptera, including several advanced eusocial species as well as a small set of more primitively eusocial species [24]. Subsequent studies also experimentally confirmed the presence of DNA methylation in the genomes of the fire ant *Solenopsis invicta* [64] as well as the jumping ant *Harpegnathos saltator* and the carpenter ant *Camponotus floridanus* [59]. The latter study suggested lower DNA methylation levels may be associated with the more primitively eusocial lifestyle of *H. saltator* compared to *C. floridanus* [59].

Some insects have little to no DNA methylation (e.g., the flour beetle *Tribolium castaneum,* the flies *Anopheles gambiae,* and *Drosophila melanogaster*, [22], reviewed in [44]). Recently, however, it has come to light that many other invertebrates possess a full complement of DNA methylation enzymes and/or show genome wide levels of DNA methylation that are comparable to those of the Hymenoptera (*Daphnia* water flea: [65], stick insect: [66], crickets: [67], cabbage moth: [43], silkworm: [68], aphids: [47, 48, 69, 70], and human body louse: [71], also reviewed in [44]). This suggests that, while methylation may be important for eusociality, it is by no means unique to social taxa among insects. This indicates that DNA methylation, while not essential to all insects, may play distinct and important roles in certain insect groups. We do not yet know of the presence, nor the extent of divergence of methylation systems in many lineages of insects; thus there is a great deal still to be learned about what factors drive the maintenance or loss of DNA methylation machinery in insects.

5. DNA Methylation and Caste Determination

After the discovery of a functional DNA methylation system with the sequencing of the honey bee genome [45], there has been a flurry of research to better understand the significance of DNA methylation in honeybees and, in particular, how methylation affects caste determination. Kucharski and colleagues [21] inhibited the expression *of dnmt3*, the *de novo* DNA methyltransferase, in worker larvae, which typically have elevated *dnmt3* expression compared to queen larvae [72]. They demonstrated that *dnmt3* knockdown caused demethylation of a biomarker gene, *dynactin p62*. Typically, *dynactin p62* is more highly methylated in worker honeybees than in queens, and queen larvae show higher expression of *dynactin p62*, though its role in caste determination is not known [21]. After *dmnt3* knockdown, emerging adults showed queen-like traits, both phenotypically (larger size, larger ovaries, and queen-like morphological traits) and in their methylation patterns. These data strongly suggested DNA methylation plays a direct causal role in honey bee caste determination, and this striking finding led to a series of studies, both experimental and computational, aimed at characterizing the "methylome" or complete set of methylated sites, in the honey bee genome.

In order to estimate DNA methylation levels in sequenced genomes, researchers have used bioinformatic approaches, focused on the CpG dinucleotide content of genes [22, 36]. Methylation primarily occurs on the cytosines of CpG dinucleotides. Methylated cytosines are more prone to mutation, and, therefore, regions that are consistently highly methylated will, over time, become CpG depleted [22]. The fruit fly *Drosophila melanogaster*, the mosquito *Anopheles gambiae*, and the flour beetle *Tribolium castaneum* (all of which have little to no DNA methylation) have a unimodal distribution of CpG richness [22]. Honeybees, like several other organisms with substantial DNA methylation, have a bimodal distribution of CpG richness in their genes, indicating that some genes are highly methylated (leading to CpG depletion) and some genes are nonmethylated or weakly methylated (allowing for the maintenance of CpG rich DNA) [22]. The solitary parasitoid wasp *Nasonia vitripennis* also shows a bimodal distribution of CpG richness, which is more pronounced in introns [61]. However, more recent evidence suggests the classic bimodal pattern may not always be present in insect species with functional methylation systems. In two ants, *Pogonomyrmex barbatus* and *Linepithema humile,* despite the presence of a full complement of DNMTs and experimental evidence of DNA methylation, there is no evidence of bimodality in CpG content in exons nor in introns [61, 62].

The aforementioned data on CpG composition in honey bees were subsequently used to examine connections between DNA methylation and gene expression. Lists of "predicted methylated genes" in the honey bee genome were compared to global gene expression data (using microarrays). These analyses found that genes predicted to be most heavily methylated in honeybees were ubiquitously expressed "housekeeping genes" involved in basic biological processes such as cell communication, development, cell adhesion, and signal transduction [22, 36].

However, because CpG content measurements are based on mutational changes, they only reflect methylation patterns of genes that are methylated in the germ line, as somatic mutations will not be passed on to the next generation nor accumulate over time [22]. Such a limitation could potentially be more serious in the honeybee. Since workers rarely reproduce, genes that are methylated in workers but not in queens or males would not be expected to show substantial CpG depletion. Thus, this method may not pick up key differences in methylation between castes, nor in genes that are methylated in specific tissues, but not in the germline. Nonetheless, to date there is good agreement between CpG predictions of methylation status and the actual presence of DNA methylation [36], supporting the use of this metric as a proxy for methylation status.

Experimental approaches have uncovered evidence of differential methylation of particular genes in queens and workers [21, 23]. Foret and colleagues [36] confirmed their bioinformatic assessment of methylation levels of several genes from CpG content estimates with bisulfite sequencing. Bisulfite sequencing involves treating DNA with bisulfite,

which converts unmethylated cytosines into uracils, but leaves methylated cytosines. By treating DNA with bisulfite and then comparing the sequences to untreated DNA, methylated cytosines can be identified. This method has been used to demonstrate differential methylation in several genes, including *dynactin p62* [21, 72] and *hexamerin 110* [73]. Differential methylation of *dynactin* has been demonstrated to correlate with queen-like and worker-like traits, even in intercastes when rearing changes are made after the critical period [72]. However, to date, there have been no demonstrated causal roles for any known differentially methylated gene, including *dynactin p62* and *hexamerin 110*. These are clearly areas that are ripe for future study.

In honeybees, evidence to date is unclear on how differential methylation is relevant to caste-specific differential gene expression; relatively few differentially methylated genes have actually turned out to be differentially expressed between castes [21–23, 44]. However, new evidence from both honey bees [23] and mammalian cells [39] suggests differential methylation may be important for alternative splicing. Based on studies in human lymphoma cell lines, Shukla and colleagues [39] proposed a potential mechanism linking gene body methylation with splicing. Their data suggest that CTCF, a DNA-binding protein that promotes exon inclusion during transcription, is inhibited by gene body methylation. In this way, DNA methylation may affect the frequency of transcription of certain exons.

In honeybees, there is also evidence for a connection between DNA methylation and alternative splicing. GB18602 is a gene that has two splice variants, one that is found in both queens and workers and one that is significantly upregulated in queens [23]. GB18602 is also differentially methylated in the brains of queens and workers, particularly around the areas of alternate splicing, suggesting that the differential methylation is relevant to the splicing [23]. Using bisulfite sequencing on a genomic scale, Lyko et al. [23] identified hundreds of putative differentially methylated genes encoding highly conserved proteins involved in core cell functions. In the brains of adult queens and workers, 550 differentially methylated genes were found, including genes involved in metabolism, RNA synthesis, nucleic acid binding, and signal transduction [23].

6. Conceptual Framework

In the paragraphs that follow, we have synthesized existing information from honey bees into a conceptual framework to describe the potential role of DNA methylation in caste determination in social insects. First, we suggest that DNA methylation in social insects can be divided into two types: consistent and differential (Table 1). Both types of methylation are primarily found in gene bodies and particularly exons [33].

Consistent methylation describes sites that are equally likely to be methylated across different castes and tissues. We predict that these genes will tend to have deeply conserved methylation patterns that are shared across a wide variety of insect taxa, for example, pea aphids and honey bees [47]. The functions, as well as the sequences of consistently methylated genes, appear to be especially well conserved over hundreds of millions of years of insect evolution [47]. Evidence from honey bees suggests consistently methylated genes are consitutively expressed across tissues and castes and are involved in core cell functions [22]. Genes that are consistently methylated in the germline should be accompanied by decreased CpG content due to mutation of methylated cytosines over time [22]. (Note that low CpG content may potentially identify both genes that are truly consistently methylated, as well as genes that are differentially methylated but more highly methylated in the germ line of queens).

Table 1: Features of consistent and differential methylation in social insects.

Consistent methylation	Differential methylation
Sites consistently methylated	Methylation varies across tissues, castes, and individuals
Depleted CpG content [22, 23]	Less depleted CpG content [22, 23]
Primarily found in exons [33]	Primarily found in exons [33]
Consistent expression levels/splicing patterns across tissues and castes [22]	Variable expression levels/splicing patterns across tissues and castes [23]
Well-conserved across insect taxa [47]	Not yet known whether patterns conserved or divergent across taxa

Differential methylation describes sites that are more likely to be methylated in certain tissues or castes. Differentially methylated genes are predicted to be more variable in their expression and/or splicing patterns in space, time, and across individuals [23]; however, at this time there is limited empirical data on how differential methylation actually affects gene regulation in social insects. Areas with higher methylation in workers and in nongermline tissue are less likely to accumulate CpG-depleting mutations over time [22, 23]. Evidence to date suggests that differentially methylated genes in honey bees tend to have higher CpG content than genes that are consistently methylated, though they still show some evidence of moderate CpG depletion [23].

Differential methylation has been demonstrated to be involved in caste determination in honeybees [21], although the exact mechanism by which differential methylation is translated into differential gene regulation is not yet clear. Caste in honeybees is also known to be controlled by environmental factors, especially larval nutrition, which have downstream effects on hormonal signaling (e.g., juvenile hormone), gene expression, and developmental fate [55]. A recent study has also demonstrated the importance of a dietary factor, the peptide royalactin in royal jelly, that may stimulate growth factor signaling pathways, leading to queen development [74]. The effect of nutrition on methylation in mammals has been well documented, particularly in transgenerational metabolic syndromes (reviewed in [75]); thus it is intriguing to postulate a similar role in social insects. Evidence to date suggests the effects of diet on caste phenotype can be mediated by methylation of particular genes [21]. This differential methylation could potentially affect both

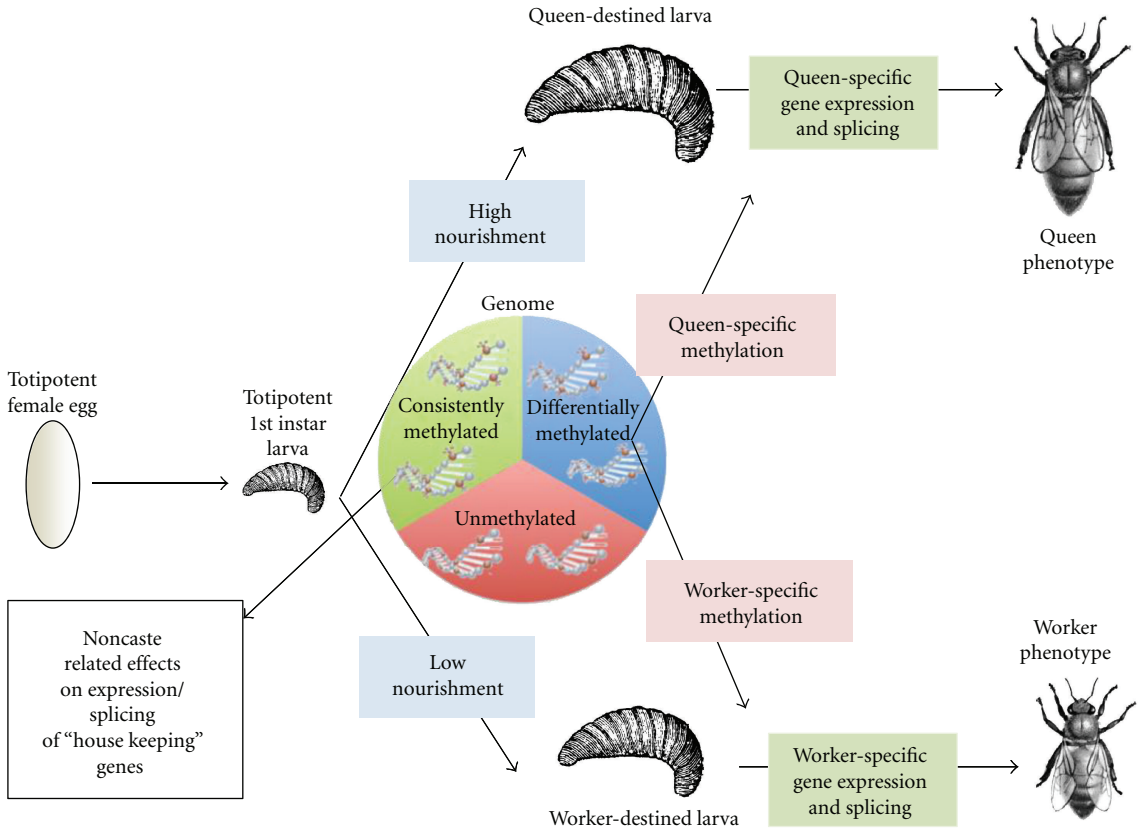

Figure 2: Schematic diagram describing the role of DNA methylation in caste determination in honey bees. Each female egg begins in a totipotent state, which lasts through early larval instars, that can potentially develop into either a queen or worker. Differential nourishment, in the form of royal jelly in the case of queens and lower-quality/quantity food in the case of workers, differentially affects the genomes of queen-and worker-destined larvae. The genome can be roughly divided into unmethylated DNA, consistently methylated DNA, and differentially methylated DNA. Differential methylation can potentially affect the downstream levels of expression and splicing patterns of many genes related to growth, metabolism, and development, leading to alternative queen and worker phenotypes.

expression and splicing, both of which can contribute to the expression of alternative phenotypes through the activation of different gene networks.

In our conceptual framework (Figure 2), we propose that dietary differences lead to differential methylation. This, in turn, leads to alternative splicing and possibly caste-biased expression, which leads to caste-biased phenotypes, such as restricted ovarian development in workers or larger body size and longer lifespan in queens (Figure 2). Numerous studies have already begun to identify specific genes and pathways associated with queen and worker caste determination in honey bees (reviewed in [17]). These include significant changes in gene expression of storage proteins [76], mitochondrial enzymes [77], lipid metabolism enzymes [78], insulin pathway genes [79], heat shock proteins [78], and growth factors [74]. It remains to be seen whether differential methylation directly affects the expression and/or splicing patterns of these genes, or whether they are downstream effectors of other differentially methylated genes.

The purpose of consistent methylation is less well understood. Methylation in honeybees occurs primarily in exons, and methylated cytosines have a higher mutation rate, which should incur a cost to maintaining high levels of methylation. The presence of consistent methylation that is conserved over millions of years of insect evolution [47] suggests that consistent methylation is serving some important purpose, or it would be selected against; indeed DNA methylation has been lost in some insects [33]. However, despite the higher mutation rates of methylated genes, many methylated genes are especially highly conserved on the protein level, suggesting strong selection against sequence divergence in these genes [23]. One possibility is that methylation of certain classes of genes may repress potentially damaging alternative transcription patterns; this may be especially important in "housekeeping" genes that are ubiquitously expressed across many tissue types [44]. Nonetheless, it is also possible that consistent methylation could be a nonadaptive side effect of the evolutionary maintenance of DNA methylation systems for differential methylation.

7. Where Do We Go from Here?

There is still a great deal still to be learned going forward in the study of DNA methylation in social insects. Important

groundwork has been laid in *Apis mellifera*, but we do not yet know whether DNA methylation is relevant to caste differences in other social species. We suggest it will be particularly illuminating to take a comparative perspective on the study of DNA methylation and castes in Hymenoptera, as this group represents at least 11 different origins of sociality and has species with various different levels of sociality, from facultatively social to advanced eusocial, and even some lineages in which sociality has been lost or obligate social parasitism has evolved. In each of these cases, comparisons to what is currently known about honey bees could provide many useful and interesting answers to a long list of open questions relating to epigenetics and the evolution of sociality. Below, we provide a few provocative examples.

7.1. DNA Methylation and Caste Determination

7.1.1. Is DNA Methylation Important in Social Organization during the Early Stages of Social Evolution, or Is It more of a Feature of Highly Derived Social Systems Such as Honey Bees? Data thus far suggest some primitively social lineages, such as the paper wasps *Polistes dominulus* have even higher DNA methylation levels than the advanced eusocial honey bees [24]. In primitively eusocial species, queen and worker castes are phenotypically very similar, and adults can switch between castes, but each individual actually retains *greater* phenotypic plasticity in its behavior and physiology throughout its lifetime than in an advanced eusocial species. Thus, it is possible that DNA methylation could be as important or even more important in mediating phenotypic plasticity during the early stages of eusocial evolution.

7.1.2. Does Having a Functional DNA Methylation System in Place Predispose or Allow a Lineage to Evolve a Broader Range of Phenotypic Plasticity? Eusocial Hymenoptera, and their nonsocial kin within the aculeate (stinging Hymenoptera) lineage, evolved from parasitoid ancestors. We know that members of at least one parasitoid Hymenopteran lineage, the jewel wasps in the genus *Nasonia*, do possess a fully functional methylation system suggesting such a system existed in the solitary ancestors of social Hymenoptera. This suggests that the solitary ancestors of social Hymenoptera already possessed a fully functional DNA methylation system. Could the existence of a DNA methylation system have provided a baseline level of genomic plasticity that allowed for or facilitated the evolution of different castes? Regev et al. [80] suggested that within invertebrates, higher DNA methylation was associated with higher rates of cell turnover, and perhaps developmental complexity and/or flexibility. Gaining a better understanding of the association between developmental plasticity and DNA methylation could begin to provide some hints about the adaptive advantages conferred by evolutionary maintenance of DNA methylation machinery.

7.1.3. What Happens to DNA Methylation Systems When Sociality Is Lost, or When the Queen or Worker Caste Is Lost, during Evolution? If DNA methylation is maintaining phenotypic plasticity in eusocial species, we may expect relaxed selection or evolutionary changes in DNA methylation patterns and DNMT enzymes in species in which sociality is lost. For example, It would be informative to examine DNA methylation systems in species where caste polyphenism is lost or reduced, e.g. in halictid (sweat) bees in which there have been reversions to solitary behavior [81], during the evolution of queenless or workerless social parasites (as found in several bee, ant, and wasp lineages) [82], or in cases where morphological caste differences have been secondarily reduced as in the swarm founding wasps [83]. If caste flexibility is lost, is selection for the maintenance of DNA methylation systems also relaxed?

7.1.4. Does DNA Methylation Play a Role in Caste Differentiation in Multiple, Independent Origins of Sociality, and If so, Are the Same Genes and/or Pathways Methylated in Each Origin, or Are These Largely Lineage Specific? Functional DNA methylation systems are now inferred to be present in numerous species of social bees, ants, and wasps [24, 59–64]. Based on gene expression studies in a wide variety of social Hymenoptera (reviewed in [17]), it appears that many of the same genes and pathways, especially those involved in metabolism, nutrient signaling, and hormone signaling, are involved in caste determination across a wide variety of species as well. If caste-related expression differences are convergent, and methylation is involved in caste differences in multiple lineages, are differential methylation patterns associated with caste differences also convergent across social insect taxa?

7.1.5. What Role Does Methylation Play in Nonhymenopteran Eusocial Systems? Thus far, there is no published work on DNA methylation in termites or other nonhymenopteran social arthropods with castes such as aphids, thrips, or snapping shrimp [84]. Nonetheless, there are intriguing commonalities in the mechanistic underpinnings of queen and worker caste determination in Hymenoptera and solider caste differentiation in termites, including the involvement of juvenile hormone and storage proteins such as hexamerins [85]. Since termite workers are derived from juvenile stages, and in many species, can mature into neotenic reproductives or soldiers, the path of caste determination is very different (reviewed in [86]). In addition, hymenopteran workers are all female, while termite workers are both male and female (reviewed in [86]). Comparing the effects of DNA methylation on reproductive and solider caste determination in termites to effects in Hymenoptera could be extremely informative.

7.2. Mechanistic Understanding of DNA Methylation. In order to more fully understand the effects of methylation on caste determination, we need to better understand the effects of differential methylation on gene expression and splicing. There is growing knowledge on the precise locations within social insect genomes that are generally methylated relative to the beginning and end of transcription [33]. With more studies that directly compare the locations of methylated sites to splicing sites, we can better understand how alternative splicing may be regulated by DNA methylation. In addition, it would be valuable to know whether there are differences between consistent methylation and differential methylation in

how and where genes are methylated. For example, are consistently methylated genes methylated more frequently in certain regions of genes, and how does this affect expression and splicing [33]?

Another avenue that could help us better understand the effect of DNA methylation on caste determination is understanding the dynamics of methylation patterns during development and during adulthood. How changeable are methylation patterns within an individual? Methylation changes may even be important for shorter-term plasticity, specifically, learning in adult worker honeybees [87]. In addition, we know that it is possible to reverse the effects of maternal care on methylation in adult mice [51]; what about caste-related methylation differences? Do methylation patterns change when workers reproduce under queenless conditions? If these patterns are changeable in adults, perhaps this stems from behavioral flexibility in solitary ancestors. Do solitary species that have laying and nonlaying periods undergo shifts in methylation? Such comparisons could provide new insight into the mechanistic regulation and evolution of castes.

In conclusion, the study of epigenetic modifications in social insects has already provided useful and intriguing information about the mechanisms of caste determination in honeybees, as well as a better appreciation of the complexities of gene regulation. There is still a great deal of work to be done in this area related to mechanisms, evolution, and imprinting. Further research could provide valuable insights into not only the mechanisms, but also the evolutionary origins of eusociality.

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Review Article

Transgenic Epigenetics: Using Transgenic Organisms to Examine Epigenetic Phenomena

Lori A. McEachern[1,2]

[1] *Department of Biology, Dalhousie University, Halifax, NS, Canada B3H 4R2*
[2] *Department of Physiology, Dalhousie University, Halifax, NS, Canada B3H 4R2*

Correspondence should be addressed to Lori A. McEachern, lori.mceachern@dal.ca

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Non-model organisms are generally more difficult and/or time consuming to work with than model organisms. In addition, epigenetic analysis of model organisms is facilitated by well-established protocols, and commercially-available reagents and kits that may not be available for, or previously tested on, non-model organisms. Given the evolutionary conservation and widespread nature of many epigenetic mechanisms, a powerful method to analyze epigenetic phenomena from non-model organisms would be to use transgenic model organisms containing an epigenetic region of interest from the non-model. Interestingly, while transgenic *Drosophila* and mice have provided significant insight into the molecular mechanisms and evolutionary conservation of the epigenetic processes that target epigenetic control regions in other model organisms, this method has so far been under-exploited for non-model organism epigenetic analysis. This paper details several experiments that have examined the epigenetic processes of genomic imprinting and paramutation, by transferring an epigenetic control region from one model organism to another. These cross-species experiments demonstrate that valuable insight into both the molecular mechanisms and evolutionary conservation of epigenetic processes may be obtained via transgenic experiments, which can then be used to guide further investigations and experiments in the species of interest.

1. Introduction

Transgenic model organisms have been widely used to study a variety of epigenetic processes and mechanisms. The majority of these studies have examined epigenetic control regions (i.e., DNA sequences targeted by epigenetic modifications, also referred to herein as epigenetic sequences) that have been relocated to a novel chromosomal position in the same model organism, an approach that can provide valuable information regarding the minimum sequences required at the endogenous locus, as well as the mechanisms and proteins that contribute to epigenetic expression or repression [1–6]. An alternative, but less used, type of transgenic epigenetic study involves transferring an epigenetic control region from one species into another. This cross-species approach can provide valuable insight into the molecular mechanisms that act on an epigenetic sequence of interest, which may be difficult to study at the endogenous locus, and can be facilitated in transgenic studies by including easy-to-monitor reporter genes adjacent to the epigenetic sequence in the transgenic construct. In addition, this method holds tremendous potential in the study of the evolution of epigenetic mechanisms, allowing for the rapid determination of whether an epigenetic process is based on widespread, evolutionary conserved mechanisms that are found in a wide range or eukaryotes, or whether it is a species-specific unique process.

Despite the great potential of this technique, it has thus far been vastly underutilized and has not yet been employed in the study of non-model organism epigenetics. Non-model organisms are traditionally difficult to work with in a laboratory environment for a wide range of reasons, including size, life cycle, viability, breeding ability, and a lack of well-established propagation- and housing-methods. In addition, non-model organisms generally lack genetic and epigenetic tools and protocols that are well developed, widely tested, and accepted within the scientific community. By

transferring an epigenetic sequence of interest from a non-model organism to an amenable model organism for which a plethora of tools are available, such as *Drosophila* or mice, new information regarding how the original sequence works may be obtained. For example, this method can be used to identify the minimum sequence required for epigenetic effect on gene expression, the identity of DNA regulatory elements contained within the sequence, the presence or absence of methylation at the sequence, and whether the sequence stimulates the formation of a compact heterochromatin domain. Furthermore, analysis of proteins and protein complexes bound to the sequence, histone modifications acquired by the sequence, the effect of small interfering RNA (siRNA) or short hairpin RNA (shRNA) knockdowns, and the effect of DNA methylation- or histone modification-inhibitors may be more quickly and easily examined in a transgenic model organism than in the original non-model organism. Finally, the transgenic approach may be especially useful to quickly and thoroughly examine the effect of a wide range of mutant strains or genetic knockouts on the epigenetic sequence of interest, as well as the inheritance pattern of the epigenetic state across several generations.

This cross-species transgenic approach is predicated on the assumption that epigenetic processes and proteins are evolutionary conserved, and that an epigenetic process can be studied in a transgenic environment. These assumptions will be examined here by detailing several cross-specie transgenic epigenetic experiments that studied the processes of genomic imprinting and paramutation, by transferring epigenetic control sequences from one model organism to another.

2. Conserved Epigenetic Mechanisms

Epigenetic effect on gene expression is accomplished by a variety of molecular mechanisms that lead to gene expression or repression, including histone modifications, changes in higher-order chromatin structure, DNA methylation, RNA interference (RNAi), and noncoding RNAs. These mechanisms have been observed in a wide range of organisms, from yeast to plants and mammals, suggesting that they are both widespread and evolutionary conserved [7–13].

Histone modifications are at the very core of epigenetic gene regulation, and many other epigenetic processes ultimately contribute to the epigenetic status of a locus by directing or targeting modifications of histone proteins. DNA is packaged within the nucleus by its association with nucleosomes, protein structures that consist of two copies of four different histone proteins (H2A, H2B, H3, and H4). This complex of DNA and protein is termed chromatin; densely packed "inactive" chromatin is termed heterochromatin, while loosely packed chromatin is termed euchromatin. Chemical modifications of amino acids in the histone proteins, such as methylation, acetylation, phosphorylation, sumoylation, ubiquitination, and ribosylation, can lead to the formation of heterochromatin or euchromatin, depending on the nature and position of the modification. The inclusion of variant histones, and the availability of histone chaperones, can also contribute to changes in chromatin structure [14–16].

Further changes in higher-order chromatin structure may be facilitated by DNA-binding proteins that mediate the formation of chromatin loops or other complex chromatin structures and thereby modify the access of regulatory proteins, chromatin remodelling proteins, and histone modification enzymes, to their target sequences or sites. These DNA-binding proteins and higher-order chromatin structures may also contribute to epigenetic gene expression by localizing the target sequences to a particular region within the nucleus [17]. Maintenance of silent or active chromatin states often also involves the well-characterized Polycomb group (PcG) and trithorax group (trxG) proteins, which regulate the expression of many developmental genes and exhibit extensive evolutionary conservation in eukaryotes, with homologues identified in fungi, plants, and animals [18]. These proteins form large multimeric complexes that maintain transcriptional repression and activation, primarily by directing histone modifications and chromatin remodelling [18]. Epigenetic processes mediated by PcG and other chromatin proteins have also been observed to involve noncoding RNAs, small RNAs, and the RNAi pathway, demonstrating the interconnectedness of these epigenetic mechanisms [19–21]. Importantly, a number of studies have demonstrated the functional conservation of this family of proteins and other chromatin modifiers, by showing that *Drosophila* PcG proteins can function as repressors in mammalian cells [22], and mammalian homologues can rescue *Drosophila* mutant phenotypes [23–26].

DNA methylation is the process through which a methyl group is added to nucleotides in the DNA sequence. The most frequent target of DNA methylation in animals is cytosine bases present in CpG dinucleotides [27], although non-CpG methylation also occurs [28, 29], and is quite common in plants and some insect species [30–33]. In most organisms that exhibit DNA methylation, *de novo* methyltransferases establish DNA methylation, while maintenance methyltransferases replicate pre-existing methylation patterns as the DNA is replicated. DNA methylation at promoter sequences is frequently associated with repression of gene expression; however, methylation-requiring enhancers, repressors, and protein-binding sequences are also important in epigenetic gene regulation. Evidence suggests that DNA methylation and histone modifications frequently exhibit epigenetic "cross-talk", with DNA methylation guiding histone modifi- cations, and histone modifications similarly influencing DNA methylation [34, 35]. These two epigenetic processes therefore often function in a mutually reinforcing epigenetic loop that ensures maintenance of a repressive chromatin state.

RNAi pathways involve the processing of large coding or noncoding RNAs into small RNAs. These small RNAs can modify gene expression post-transcriptionally, by degrading an mRNA transcript or inhibiting its translation, or transcriptionally, by mediating chromatin modifications that promote the formation of heterochromatin and thereby inhibit transcription [8]. The molecular mechanisms underlying RNAi-directed heterochromatin formation have been

most thoroughly studied in yeast, where transcripts from heterochromatic regions of the genome were found to be processed into siRNAs, which then recruited histone methylation that contributed to heterochromatin formation [36]. Noncoding RNA transcripts may also orchestrate changes in chromatin structure directly, rather than through an RNAi pathway, by mediating protein recruitment, histone modifications, and DNA methylation at a target site [8, 37, 38]. Both transcriptional and post-transcriptional RNAi-mediated silencing has been observed in a wide range of eukaryotic organisms, and key components of the RNAi machinery are conserved in plants, yeast, and animals [9]. The diverse range of RNAi-mediated pathways and processes that have been reported throughout the eukaryotic kingdom are therefore likely based on an evolutionarily conserved silencing process that was present in ancient eukaryotes.

3. Epigenetic Inheritance

Epigenetic changes to DNA sequences must be stably transmitted through mitosis, to ensure that the appropriate set of genes are expressed or repressed during growth and development, and cellular replacement and repair. Loss of the "correct" epigenetic state of a gene can lead to aberrant gene expression and the development of many types of cancer and other diseases [39, 40]. In addition to being mitotically heritable, epigenetic states can also be meiotically heritable, via mechanisms that result in a silent epigenetic state being inherited from one generation into the next. Genomic imprinting and paramutation are two epigenetic processes that exhibit this phenomenon of *trans*-generational epigenetic silencing.

Genomic imprinting is a process in which an allele is marked based on the sex of the parent transmitting it. This epigenetic mark can lead to transcriptional repression of fully functional alleles based strictly on whether they were inherited through the male or female germline. Imprinting has been observed in a wide range of eukaryotic organisms, including plants [41, 42], insects [43, 44], *C. elegans* [45], zebrafish [46], and mammals [47, 48]. In the process of imprinting, an epigenetic mark is differentially established in the male and female germlines, the maternal and paternal epigenetic states are maintained during the development of the organism, and finally the epigenetic states are erased in the gametes so that the organism transmits the "correct" epigenetic state to its offspring, according to whether it is male or female.

Paramutation is another *trans*-generational epigenetic silencing process, in which alleles of the same gene exhibit different epigenetic states. However, in paramutation, the epigenetic status of an allele is not dependent on its parent of origin, but it is instead influenced by the epigenetic status of an allele present in *trans*. In the process of paramutation, the epigenetic state and expression level of one allele changes after it is combined with another allele in a heterozygous organism. The allele's new epigenetic state is meiotically stable, and so it is inherited and maintained in the next generation [49].

Cross-species transgenic organisms have provided tremendous insight into the evolutionary conservation of the epigenetic silencing mechanisms underlying imprinting. Similarly, I have recently used transgenic *Drosophila* to examine the epigenetic mechanisms underlying maize paramutation. In order to assess whether this approach can successfully be used to examine epigenetic processes from other species, such as non-model organisms, this paper will summarize and examine the mechanistic and evolutionary insights gained from cross-species transgenic experiments studying genomic imprinting and paramutation.

4. Imprinting at the Mammalian *H19/Igf2* Locus

In mammals, imprinted genes are often found in clusters that contain two or more imprinted genes, a shared imprint control region (ICR) or regions, and several gene-specific regulatory elements, all of which work together to establish and/or maintain the appropriate imprinted expression of the genes in the cluster. One of the best characterized examples of mammalian imprinting is that of *Insulin-like growth factor 2* (*Igf2*) and *H19*. *Igf2* is expressed from the paternal allele only and is located approximately 90 kb from the noncoding *H19* transcript, which is expressed from the maternal allele only ([50, 51], Figure 1). A shared ICR that is required for imprinting of both genes is located approximately 2 kb upstream of the *H19* transcription start site [52]. In addition to the ICR, several tissue-specific enhancers 10–120 Kb downstream of the *H19* gene [53–56], several differentially methylated regions (DMRs) near *Igf2* [57–60], and a central A6-A4 DNAse hypersensitive region [61–63], are also important in establishing the correct expression profiles of these two imprinted genes.

Imprinting of *H19* and *Igf2* require CCCTC-binding factor (CTCF), an enhancer-blocking insulator protein that is conserved from *Drosophila* to humans [64] and is similarly required for imprinting in *Drosophila* [65]. The *H19*/*Igf2* ICR contains several binding sites for CTCF which can only bind when these sites are unmethylated [66, 67]. Further, the ICR exhibits differential methylation in male and female gametes, with methylation detected in sperm but not oocytes [68]. Thus, CTCF is able to bind to the maternally inherited unmethylated ICR, but not the paternally inherited methylated ICR. The binding of CTCF to the maternal ICR blocks the downstream enhancers from activating *Igf2*, and instead the enhancers mediate expression of *H19*. Conversely, in the absence of CTCF on the methylated ICR of the paternal allele, the downstream enhancers activate expression of *Igf2* ([66, 67], Figure 1). Differential methylation of the ICR in the gametes therefore mediates differential binding of CTCF, and results in *H19* expression from the maternally inherited chromosome only and *Igf2* expression from the paternally inherited chromosome only.

Several differentially methylated regions (DMRs) also exist in the *Igf2* gene region. In mice, DMR0 is hypermethylated on the maternal allele in the placenta and encompasses the promoter of a placental-specific transcript [57]. DMR1

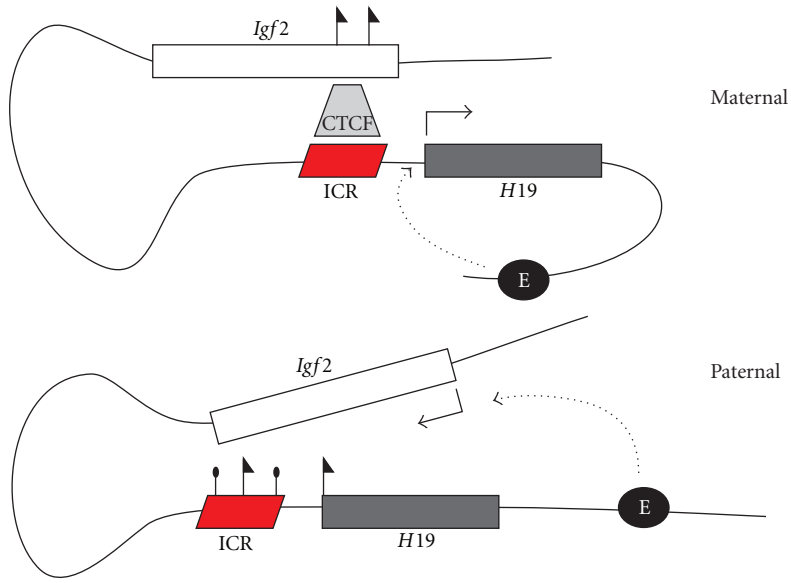

Figure 1: Imprinting at the *Igf2* and *H19* locus. On the maternally inherited allele, CTCF binds to the unmethylated ICR (red parallelogram) and the downstream enhancers (black circle) drive expression of *H19*. On the paternally inherited allele, the ICR is methylated (black lollipops), which prevents binding of the insulator protein CTCF, and enables the downstream enhancers to stimulate expression of *Igf2*. The maternal *Igf2* gene exhibits repressive histone marks (black flags), while these marks are found on the paternal ICR and *H19* gene.

is a methylation-sensitive mesodermal repressor that is hypermethylated on the paternal allele and is required in the unmethylated state to mediate repression of the maternal *Igf2* allele in mesoderm tissues [58, 60]. Conversely, DMR2 is a methylation-dependent *Igf2* enhancer that is hypermethylated on the paternal allele and is important for stimulating high levels of paternal *Igf2* expression [59].

The process of *Igf2*/*H19* imprinting also involves the formation of higher-order chromatin structures. On the paternal chromosome, chromosome conformation capture (3C) has demonstrated interactions between the ICR and DMR2, as well as interactions between the *Igf2* promoter and downstream enhancers [69–71]. Conversely, the maternal chromosome exhibits chromatin interactions between the ICR and the *Igf2* promoter region, including DMR1, and between the downstream enhancers and the *H19* promoter [69–72]. In addition to binding at the ICR, CTCF binding is also detected at *Igf2* at DMR1 and the two major *Igf2* promoters, P2 and P3 [69, 72]. Disruption of CTCF binding to the ICR also eliminates CTCF binding at DMR1, suggesting that the long range interactions between the ICR and *Igf2* gene region recruit CTCF to *Igf2*. CTCF binding at the maternal DMRs appears to protect these regions from acquiring the paternal-specific methylation pattern [69].

Histone modifications are also important in determining the correct patterns of *H19* and *Igf2* expression. The maternal *Igf2* allele is enriched for several repressive marks, including methylation at Lysine 9 of Histone H3 (H3K9), methylation at Lysine 27 of Histone H3 (H3K27), and the heterochromatic histone variant macroH2A1 [72–74]. H3K27 methylation is mediated by the highly conserved Polycomb repressive complex 2 (PRC2), which contains Suz12, a protein that can directly interact with CTCF [72]. Both H3K27 and Suz12 are required to maintain maternal *Igf2* repression [72], suggesting that the CTCF-mediated maternal chromatin loop represses *Igf2* by recruiting PRC2 to catalyze H3K27 methylation and maintain *Igf2* repression [72]. On the paternal chromosome, repressive histone modifications are found at the ICR and *H19* gene [73, 74]. Similarly, and consistent with their expression profiles, the paternal *Igf2* gene region is enriched for activating histone marks, such as histone acetylation and Histone H3 lysine 4 (H3K4) methylation, while these marks are predominant at the ICR and *H19* gene region of the maternal chromosome [73, 74].

CTCF is a master regulator of *H19* and *Igf2* imprinting, and elimination of CTCF binding to the maternal ICR causes the chromosome to adopt both the paternal pattern of histone modifications [73] and chromatin interactions [69]. Overall, the process of *Igf2* and *H19* imprinting is complex and requires an interplay between many underlying epigenetic mechanisms, including DNA methylation, histone modifications, higher-order chromatin structures, and chromatin binding proteins. Despite this complexity, cross-species transgenic experiments have provided many insights into the evolutionary conservation of genomic imprinting.

5. *H19/Igf2* Transgenic Experiments

5.1. Human and Mouse → Drosophila. Epigenetic effects on gene expression, such as position effect variegation, telomeric position effect, *trans*-inactivation, and transvection, have been extensively studied in *Drosophila melanogaster* [75]. Given the evolutionary conservation of many epigenetic proteins and core epigenetic silencing mechanisms, transgenic *Drosophila* have also proven an invaluable tool for analysing epigenetic control sequences from other species. Early cross-species transgenic experiments examining *H19*/*Igf2* genomic

imprinting in *Drosophila* provided insight into both the nature of the imprint control region and the evolutionary conservation of the mechanisms underlying genomic imprinting. In fact, a distinct silencer element contained within the mouse *H19* ICR was discovered in transgenic *Drosophila* [76] prior to its identification at the endogenous mouse locus [77]. In this experiment, a 3.8 kb fragment of the *H19* upstream region, including most of the ICR, was found to silence both *lacZ* and mini-*white* reporter genes in transgenic *Drosophila.* Transgenic deletion constructs were able to further delineate the silencer element to a 1.2 kb region that includes approximately 900 bp of the 2 kb UTR. Importantly, subsequent experiments showed that targeted deletion of only this 1.2 kb silencer element in mice, while leaving the remainder of the UTR and surrounding region intact, caused a loss of *H19* silencing following paternal transmission but did not affect paternal *Igf2* expression, differential methylation of the UTR, or expression of *H19* and *Igf2* following maternal transmission [77]. Thus the mouse *H19* silencer that was discovered in transgenic *Drosophila* appears to be evolutionarily conserved in its function, acting as an epigenetic silencer both at its native locus and in the distantly related transgenic flies.

Transgenic *Drosophila* experiments also identified a similar 1.5 kb silencer element at the 3′ end of the human *H19* ICR [78]. This region silenced a mini-*white* reporter gene in transgenic *Drosophila*, while additional regions from the human ICR did not. The silencing activity of this specific fragment was confirmed in transient transfection assays using a human embryonic kidney cell line [78]. Interestingly, despite the lack of sequence similarity between the human and mouse ICRs, and the failure of the human ICR to imprint in mice (described in the next section), both ICRs appear to contain an evolutionarily conserved silencer element that functions in transgenic *Drosophila*.

Further examination of the mouse *H19/Igf2* ICR in transgenic *Drosophila* provided additional evidence that the epigenetic mechanisms underlying genomic imprinting are conserved [79]. This study used transgenic *Drosophila* containing the larger 3.8 kb *H19* upstream region, which includes the full ICR, and found that the ICR is transcribed in the sense and antisense direction, from both the maternal and paternal alleles, both at the endogenous mouse locus and in transgenic *Drosophila* [79]. The ability to rapidly test many mutant strains and easily manipulate the transgenic *Drosophila* system proved extremely useful in this study and provided further insight into the possible function of the ICR transcription. Mutations in several RNAi genes, including *piwi*, *aubergine*, *dicer-2*, *r2d2*, and *spindle-E*, failed to relieve reporter gene silencing in *Drosophila* [79]. Furthermore, despite the bidirectional transcription of the ICR, no siRNAs from the ICR could be detected in transgenic *Drosophila*. In fact, artificially producing *H19* ICR siRNAs by expressing a fragment of the ICR as an inverted repeat resulted in a significant reduction in the ICR transcripts and a loss of reporter gene silencing. Thus the *H19* ICR transcripts appear to induce gene silencing in an RNAi-independent manner [79]. At the endogenous mouse locus, the ICR transcripts may be involved in forming a repressive chromatin structure that contributes to paternal *H19* repression. Similar cases of a noncoding RNA transcript mediating the formation of a repressive chromatin structure have been observed at the imprinted *Cdkn1c-Kcnq1* domain [80], pericentric heterochromatin [81], and a ribosomal gene cluster [82]. Potentially, the repressive effect of the *H19* ICR transcripts on the maternal allele may be prevented or blocked by CTCF binding to this region, or the transcripts may serve a different functional role that has not yet been elucidated [79].

Similar transgenic experiments have shown that the central A6-A4 region also functions as a silencer in *Drosophila*. The placement of this region adjacent to mini-*white* and *lacZ* reporter genes in transgenic *Drosophila* resulted in overall silencing of both reporter genes and occasional eye pigment variegation, which is indicative of the formation of a repressive chromatin structure [83]. Silencing from the A6-A4 region in *Drosophila* may be consistent with the observation that this region includes a tissue-specific repressor in mice [62]. Again, transgenic *Drosophila* mutational analysis provided insight into the potential mechanism of repression. Two Polycomb group genes, *Enhancer of Zeste (E(z))* and *Posterior Sex Combs (Psc),* were found to be important for reporter gene silencing and were observed to bind to the transgene integration site [83]. E(z) and Psc are highly conserved proteins that are involved in chromatin remodelling and the formation of repressive chromatin states [18]. The repressor activity of the A6-A4 region in mice may therefore require the mouse homologues of these proteins to mediate the formation of a condensed chromatin domain, although this has not yet been confirmed endogenously.

Overall, these transgenic results suggest that genomic imprinting in mammals may use evolutionary conserved silencing mechanisms. Silencers contained within the *H19/Igf2* mammalian imprint control regions are recognized and targeted for silencing in *Drosophila,* affecting the expression of nearby reporter genes. Bidirectional transcription of the ICR is also conserved between mice and *Drosophila*, and the transgenic *Drosophila* system was able to provide significant inscight into the potential role of these transcripts in mouse *H19/Igf2* imprinting. The silencing mediated by the upstream A6-A4 conserved region in *Drosophila* further suggests that the mechanisms governing epigenetic modifications at other sites important for imprinted expression are also conserved between mammals and flies, indicating tremendous evolutionary conservation of the mechanisms underlying this complex epigenetic process.

5.2. Human → Mocuse. The general mechanism of imprinting at the *Igf2*/*H19* locus shares many similarities between mice and humans, including the chromosomal arrangement of the two genes and the position of the ICR, the pattern of methylation and gene expression, and the binding of CTCF to the ICR. However, with the exception of the CTCF binding sites, the sequences of the mouse and human ICRs are significantly different [66, 67, 84], suggesting that some aspects of the mechanisms underlying imprinting might have diverged in the mammalian lineage.

Transgenic experiments confirm that there may be divergence in some of the mechanisms governing imprinted

expression of *H19* between mice and humans. Mice containing a 100 kb human *H19* transgene failed to imprint the human *H19* gene [85], despite containing significantly more flanking sequence than a 15.7 kb mouse transgene that successfully imprinted in mice when present in a single transgene copy [2]. Interestingly, in all lines with more than a single copy of the 100 kb human *H19* transgene, methylation of the ICR was detected in sperm but not oocytes, with the level of methylation increasing as the number of transgene copies increased. Paternal methylation of the ICR began to decrease early in embryonic development and was undetectable in the somatic tissues of transgenic mice. *H19* was expressed equally after both paternal and maternal inheritance in all multicopy lines. However, a single copy line showed a complete absence of both methylation and *H19* expression [85].

The requirement for multiple transgene copies to establish methylation at the ICR in the paternal germline may suggest that imprint signals in the human transgene are only weakly recognized in the mouse, and thus multiple copies are necessary to accumulate a strong enough signal for transgene methylation. Since methylation at the ICR of the human transgene is lost in the embryo, it is possible that the imprint is not established correctly or completely in the paternal germline, or is not recognized and maintained by the mouse machinery in the early embryo. While the presence of multiple transgene copies may trigger methylation that is unrelated to the ICR and imprinting machinery, as has been documented for other tandem repeats of transgenes [86, 87], this would not explain why the observed methylation was only acquired in the paternal germline, consistent with its epigenetic status in mice.

It would be intriguing to know whether the human ICR exhibits silencing activity in mice, despite failing to imprint or acquire methylation at the ICR region. Potentially, the human ICR and *H19* gene may acquire repressive histone modifications that lead to *H19* repression following both maternal and paternal inheritance. If the human ICR functions as a silencer in mice, this would be consistent with the lack of *H19* expression in the single-copy line, and with the transgenic *Drosophila* experiments described previously. In the case of the multi-copy lines, the silencing ability of the ICR may decrease as the copy number increases, resulting in equivalent *H19* expression following both maternal and paternal inheritance.

6. Transgenic Insights: Genomic Imprinting

The transgenic experiments described here indicate that several core epigenetic mechanisms underlying mammalian imprinting are highly conserved. This is perhaps not surprising given that plants, mammals, and insects utilize many of the same mechanisms to establish and maintain imprinted expression. These mechanisms include DNA methylation, histone modifications, changes in higher-order chromatin structure, and noncoding RNA and RNA interference, all of which are frequently interrelated and mutually reinforcing. Histone modifications have been observed to play an essential role in plant, insect, and mammalian imprinting and can result in parent of origin-specific higher-order chromatin structures or modifications that contribute to the imprinting of genes, gene clusters, or chromosomes [72, 88–91]. A homologous Polycomb complex participates in both plant and mammalian imprinting, further emphasizing their relatedness [72, 92, 93].

The fact that the human and mouse *H19/Igf2* imprint control regions function as silencers but do not confer imprinting of marker genes in *Drosophila* may indicate that a silenced epigenetic state is the default at these imprinted loci. In support of this, a human imprinting centre from the Prader-Willi/Angelman syndrome region was also found to function as a silencer in *Drosophila* [94]. In these experiments, a 740 bp sequence from the imprint centre was found to be sufficient for silencing of reporter genes, while other nonspecific DNA fragments exerted no effect [94].

The apparent divergence of *H19/Igf2* imprinting between mice and humans, despite the conservation of the underlying silencing mechanisms between mammals and *Drosophila*, may again indicate that the silent epigenetic state is the default. While silencing may use core widespread epigenetic mechanisms, such as histone modifications, DNA methylation, and higher-order chromatin modifications, imprinting may be a more divergent species-specific modification of these processes in the gametes. That is, the parent-of-origin patterns of gene expression observed in genomic imprinting may be accomplished by simply using conserved core epigenetic mechanisms differently in the maternal and paternal germ-line. However, it is important to note that the potential divergence between the human and mouse *H19* ICRs is not indicative that all imprinting processes at all imprinted loci have diverged between the two lineages. For example, transgenic mice containing sequence from the human Prader-Willi/Angelman syndrome domain successfully imprint the transgene [95–97], and subsequent experiments have found several *cis*-acting elements and protein binding complexes that function at the transgenic locus [98]. Similarly, a differentially methylated region near two paternally expressed human genes, *HYMAI* and *PLAG1*, was concluded to be an imprint control region following transgenic mouse experiments in which it successfully acquired differential methylation and conferred imprinting of an *eGFP* reporter gene [99]. It is therefore possible that including additional distant sequence, or modifying the sequence contained within the *H19* transgene (e.g., by decreasing the distance between regulatory elements), could result in successful imprinting of the human *H19/Igf2* imprint control region in mice.

7. Paramutation at the Maize *B1* Locus

The maize *b1* locus provides one of the best characterized examples of paramutation. The *b1* gene encodes a transcription factor that regulates expression of genes required for the synthesis of purple anthocyanin pigments [100]. Two alleles at the *b1* locus participate in paramutation: the highly transcribed *B-I* allele, and the weakly transcribed *B′* allele. Paramutation occurs when a *B-I* allele is combined with a *B′* allele in heterozygous plants, and results in epigenetic

silencing of the normally highly expressed *B-I* allele ([101, 102], Figure 2).

Paramutation at the *b1* locus requires a control region that is located 100 kb upstream of the *b1* transcription start site and consists of a 6 kb sequence containing seven tandem repeats of an 853 bp sequence [103]. Despite containing identical DNA sequences, the *B′* tandem repeats exhibit a closed chromatin structure, repressive histone modifications, and higher levels of DNA methylation, whereas the *B-I* tandem repeats have an open chromatin structure and histone H3 acetylation, an activating histone modification [103, 104].

Higher-order chromatin structure may also play a role in determining the epigenetic status of the repeats and the transcriptional status of *B-I* and *B′* [105]. Chromosome conformation capture (3C) has demonstrated that the high-expressing *B-I* allele exhibits a higher frequency of chromatin interactions than *B′*, involving the transcription start site, the tandem repeats, and several additional upstream regulatory regions, suggesting the formation of a complex multi-loop structure that facilitates *b1* expression. In contrast, the weakly expressed *B′* allele exhibits less frequent interactions involving only the transcription start site and the tandem repeats, suggesting the formation of a less stable single-loop structure [105].

Paramutation at the *b1* locus requires several proteins, including *mediator of paramutation 1*, or *mop1* [106], which encodes an RNA dependent RNA polymerase [107], *mediator of paramutation 2* (*mop2*, also known as *rmr7*), which encodes the second largest subunit of both RNA polymerases IV and V [108], and *required to maintain repression 6* (*rmr6*) [109], which encodes the largest subunit of RNA polymerase IV [110]. In *Arabidopsis*, these RNA polymerases participate in the production of siRNAs and noncoding RNAs, transcriptional gene silencing, silencing of transposons and repetitive DNA, RNA-directed DNA methylation, and heterochromatin formation [111–116]. In addition, a protein termed CXC domain *b1*-repeat binding protein, or CBBP, has been shown to bind to the *b1* tandem repeats and appears to be involved in establishing, rather than maintaining, the silenced epigenetic state [117].

The maize *b1* tandem repeats are transcribed from both strands, with a similar level of transcription in *B-I* and *B′* plants, as well as in plants with a neutral allele that contains only a single copy of the repeat [107]. 24-nt siRNAs from the tandem repeats have been detected in plants with *B-I*, *B′*, and the single-repeat allele, but are reduced in the presence of a *mop1* or *mop2* mutation [108, 118]. It is thus likely that the bidirectional transcription of the repeats produces double stranded RNA (dsRNA) and that MOP1 and MOP2 are required to produce significant levels of siRNAs from the dsRNA molecules.

The current model for paramutation at the *b1* locus is that RNA-mediated communication between the *B-I* and *B′* alleles establishes the chromatin states of the control regions, which thereby determines the level of *b1* transcription ([118, 119], Figure 2). The open chromatin structure of the *B-I* tandem repeats, and the multi-chromatin loops that are formed at this allele, may promote *b1* transcription, whereas the closed chromatin structure of the *B′* tandem repeats, and single chromatin loop, may inhibit or prevent *b1* transcription. Importantly, however, the presence of siRNAs in the nonparamutating single-repeat allele suggests that the siRNAs alone are not sufficient to induce paramutation. The number of tandem repeats is also important and may mediate or stabilize pairing-interactions between alleles, potentially via increased accumulation of proteins or chromatin modifications. In addition to RNA-mediated communication, interactions between the DNA sequences, proteins bound to the DNA, or the formation of higher-order protein complexes may also play a role in paramutation by bringing the two alleles together physically, or localizing them to a particular nuclear compartment where silencing and a heritable chromatin state can be established by the siRNAs [119].

Given that the highly expressed *B-I* allele contains seven tandem repeats that are transcribed and produce siRNAs, there is necessarily an additional mechanism that normally prevents silencing at this allele. The active chromatin structure of the repeats, or specific proteins that bind to the active chromatin structure, may inhibit the formation of the silenced epigenetic state, or the allele may be localized to a different nuclear environment that inhibits silencing [118, 119]. Alternatively, pre-existing repressive modifications at the *B*' allele may make it susceptible to further siRNA-directed modifications [104]. This could be similar to the mechanism at the *Arabidopsis FWA* locus, where siRNAs direct DNA methylation at the tandem repeats of silenced alleles with pre-existing methylation, but not at active epialleles [120].

8. Transgenic Experiments

8.1. Maize → Drosophila. In order to analyze the conservation of epigenetic mechanisms underlying maize *b1* paramutation, I generated transgenic *Drosophila* carrying the seven maize *b1* tandem repeats adjacent to the *Drosophila white* reporter gene [121]. In this transgenic system, the *b1* repeats are located between two Flipase Recombinase Target (FRT) sequences. This experimental design allows for the removal of the repeats by crossing to a source of Flipase recombinase (FLP), which mediates site-specific removal of FRT-flanked sequences, thereby enabling analysis of reporter gene expression from transgenes with and without the *b1* repeats, at identical chromosomal positions. A similar type of construct could theoretically be used to examine the effect of any epigenetic sequence of interest, from any organism, on reporter genes in *Drosophila* (Figure 3).

In all eleven transgenic *Drosophila* lines examined, the maize *b1* repeats functioned as a silencer, with a visible increase in *white* expression observed following repeat removal [121]. Silencing strength increased as the number of tandem repeats increased; however, silencing was also observed with a single copy of the repeat sequence, indicating that the observed silencing of *white* is not due to nonspecific silencing of a tandem repeat array, and further suggesting that each 853 bp tandem repeat contains evolutionarily conserved silencing sequences that are recognized and targeted

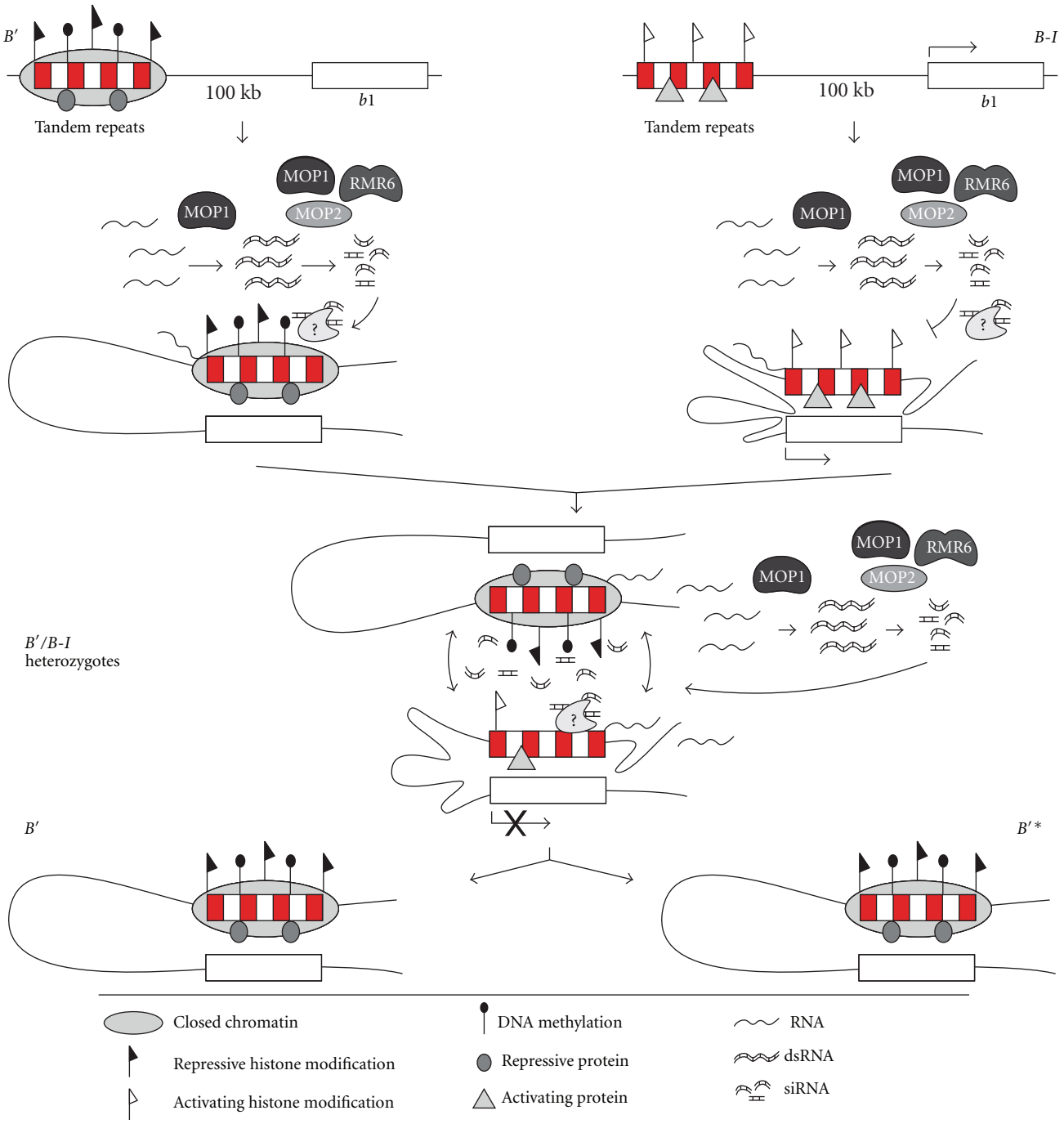

Figure 2: Paramutation at the maize *b1* locus. The two alleles that participate in paramutation at the *b1* locus are identical in sequence and contain an identical control region consisting of seven tandem repeats (red and white boxes). However, the *B-I* allele is highly transcribed while the *B′* allele is not. The two alleles exhibit epigenetic differences in chromatin structure, histone modifications, and DNA methylation and may be associated with distinct proteins that maintain these epigenetic states. The tandem repeats are bidirectionally transcribed in both *B-I* and *B′* plants, producing repeat RNA that then forms dsRNA and is processed into siRNAs. The proteins MOP1, RMR6, and MOP2 are important for the production and amplification of the dsRNA and siRNAs. The siRNAs are hypothesized to direct chromatin modifications at the tandem repeats via mechanisms and proteins that are currently unknown, but this process is blocked at the *B-I* allele, potentially by the active chromatin state, bound proteins, or nuclear environment. Paramutation occurs in heterozygous plants, when the highly transcribed *B-I* allele is "paramutated", or converted, to the silenced *B′* state. siRNAs produced from the tandem repeats are hypothesized to mediate *trans*-interactions or communication between the alleles, as well as direct the establishment of a closed chromatin structure at the *B-I* tandem repeats. The conversion of *B-I* to a silenced epigenetic state is meiotically stable, and in the next generation all progeny will inherit a silenced *B′* allele. The newly paramutated allele is termed *B′**.

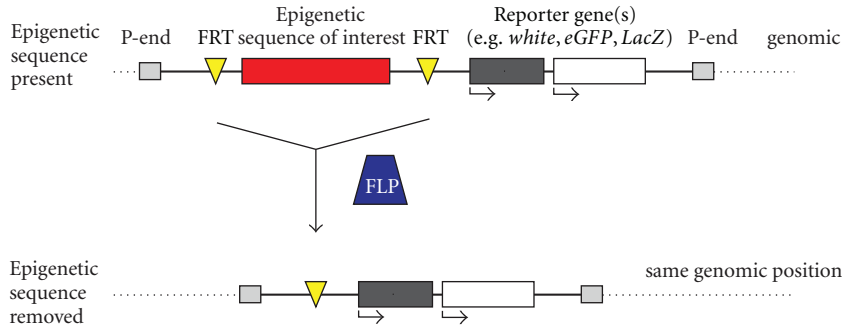

Figure 3: An FRT-FLP mediated approach that can be used to analyze any epigenetic sequence of interest from any organism, in *Drosophila*. The epigenetic sequence of interest is placed between two FRT sites, with one of more reporter genes positioned adjacent to the sequence of interest, but outside of the FRT sites. Following incorporation into the genome, the transgenic line can be crossed to a source of FLP recombinase, which excises FRT-flanked DNA sequences and thereby mediates removal of the test epigenetic sequence. This approach produces transgenic *Drosophila* with reporter genes at the same genomic position, but with or without the epigenetic sequence of interest present, thereby controlling for nonspecific genomic and position effects on reporter gene expression.

for silencing in transgenic *Drosophila*. Importantly, this is in contrast to other experiments in which no effect on *white* expression was observed for *Drosophila* containing tandem repeats from mosquito subtelomeric heterochromatin [122], and no change in *white* expression was observed following FLP-mediated removal of adjacent repetitive sequence from the *Drosophila 1360* transposable element [123]. Thus, the maize repeats appear to specifically recruit epigenetic silencing in transgenic *Drosophila*.

Evidence of *trans*-silencing was also observed in transgenic *Drosophila*, as several transgenes exhibited reduced expression when homozygous. *Trans*-silencing was only observed when transgenes at the same genomic position were combined, suggesting that it requires direct pairing of the homologous transgenes. Further, the *b1* control region produces bidirectional transcripts in the transgenic *Drosophila* system [121], as in the endogenous maize system. Interestingly, despite the role of the *b1* tandem repeats in both mediating paramutation and acting as an enhancer that drives high expression of the *B-I* allele [103], *cis* activation of the *white* transgene by the tandem repeats was not observed in *Drosophila* [121]. This may indicate that the silent epigenetic conformation of the *b1* tandem repeats is the default epigenetic state. This agrees with the observation that the highly expressed *B-I* allele will spontaneously convert to the silenced *B′* state at a frequency of 1–10% [101, 102], whereas the reverse, conversion of the silenced *B′* state to the active *B-I* state, has never been observed [101, 119]. In maize, it is likely the case that the mechanism used to ensure high levels of transcription of the *B-I* allele occasionally fails, causing the allele to adopt the default silenced epigenetic state [118]. Similarly, while the default silenced epigenetic state is readily adopted in transgenic *Drosophila*, the mechanisms required for the tandem repeats to act as an enhancer are likely absent, unstable, or fail. Alternatively, this may be explained by the fact that the *b1* tandem repeats are only ~500 bp from the *white* reporter gene in transgenic *Drosophila*, whereas in maize, the *b1* gene is ~100 kb from the tandem repeats. This greater distance may be required for the formation of the higher order chromatin loops, which involve additional upstream sequences, and may facilitate the formation of the active epigenetic state [105]. As paramutation necessarily requires both active and silent epialleles, conservation of the full paramutation process could not be assessed in the transgenic *Drosophila* system.

9. Transgenic Insights: Paramutation

The transgenic *Drosophila* results suggest that paramutation is likely based on conserved epigenetic silencing mechanisms, as silencing was observed in all transgenic lines containing the *b1* tandem repeat sequence [121]. Similarly, it has recently been proposed that paramutation employs widespread RNA-based mechanisms that direct epigenetic modifications at target sites, and is simply an extreme manifestation, or aberration, of these processes [124, 125]. The transgenic results agree with these assertions, as evidence of silencing, *trans*-interactions, and bidirectional transcription, are all observed when the *b1* control region is moved to a new species. Further, the extensive evolutionary distance between maize, an angiosperm plant, and *Drosophila,* a dipteran insect, provides support for the hypothesis that core epigenetic mechanisms are conserved throughout the eukaryotic kingdom, and seemingly unique epigenetic phenomena function by exploiting these core mechanisms.

10. Conclusions

Transgenic organisms have proven to be an extremely valuable tool for studying a wide range of epigenetic processes. While the traditional transgenic method of moving an epigenetic control region to a novel chromosomal position in the same organism has been widely used, an alternate, but underutilized, method involves using transgenic organisms to examine epigenetically-regulated regions from a different species. The examples described here clearly demonstrate that significant insight into both the molecular mechanisms and the evolutionary conservation of these mechanisms may be obtained by examining epigenetic control regions from

one species in another. As non-model organisms are traditionally difficult to work with in a laboratory environment, this approach should prove especially useful in future non-model organism epigenetic studies.

Drosophila, in particular, have long been a valuable resource for a wide range of genetic and epigenetic studies. The conservation of core epigenetic mechanisms enhances the utility of *Drosophila* in transgenic epigenetic studies, as the transgenic system can be used to advance the understanding of the molecular mechanisms and proteins that function at the endogenous locus. Transgenic *Drosophila* also provide a unique opportunity to study the molecular mechanisms of epigenetic processes from other species in more detail. A tremendous number of *Drosophila* mutant strains are readily available and can be tested with relative speed and ease, compared to mutational testing in other species.

While an epigenetic control region does not always successfully confer an epigenetic process in full when transferred to a novel organism, it frequently causes an observable epigenetic effect on adjacent marker genes. For example, imprint control regions from mice and humans cause gene silencing, but not imprinting, in *Drosophila*, and a paramutation control region from maize causes silencing and pairing-sensitive *trans*-silencing, but not paramutation, in *Drosophila*. Despite not fully recreating the endogenous epigenetic effect, these experiments nonetheless allow for dissection of the underlying conserved epigenetic processes from the species-specific modulations of these processes. Indeed, such experiments allowed for the discovery of a silencer at the mouse *H19*/*Igf2* imprint control region in transgenic *Drosophila*, prior to its discovery in mice. Even the apparent failure of a human imprint control region to function in transgenic mice is of interest, as it indicates that additional sequences may be required for successful imprinting, or that species-specific modification of a finely tuned epigenetic mechanism may have led to rapid evolution of imprinting control between the two mammalian species.

It is important to note that this methodology is not without its caveats. It is possible that an epigenetic control region may recruit different proteins or modifications in a transgenic organism than at the endogenous locus, or it may fail to recruit any epigenetic modifications in the transgenic environment, despite conferring epigenetic effects in the original organism. Species-specific unique proteins may function at the endogenous locus, preventing these interactions from being detected in the transgenic organism, or similarly, species-specific proteins may be recruited to the transgenic locus, leading to false conclusions regarding the processes involved in the original species. Importantly, the proteins or modifications recruited to the transgenic se- quence may be influenced by the larger genomic environ- ment, including the proximity of nearby heterochromatin domains and the identity of neighbouring regulatory se- quences and genes, necessitating examination of several transgenic lines with different transgene insertion sites. It is therefore best, whenever possible, to use these transgenic experiments to provide insight and guide new studies in the original species that could lead toward confirmation of the results.

In all, the transgenic experiments described here suggest that seemingly unique epigenetic processes, such as genomic imprinting and paramutation, function via exploitation of conserved epigenetic mechanisms. Given the silencing observed from imprinting and paramutation control regions in transgenic *Drosophila*, it appears likely that the silencing mechanisms underlying these processes are core mechanisms that are highly conserved from one species to another, while the unique patterns of gene expression observed at the endogenous loci are due to species-specific modulations of these mechanisms. As many epigenetic proteins and processes are highly conserved, and transgenic model organisms have proven useful in analyzing epigenetic control regions from other species, this approach holds great promise for future non-model organism epigenetic studies.

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Review Article

Aphids: A Model for Polyphenism and Epigenetics

Dayalan G. Srinivasan[1] and Jennifer A. Brisson[2]

[1] *Department of Biological Sciences, Rowan University, Glassboro, NJ 08028, USA*
[2] *School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE 68588, USA*

Correspondence should be addressed to Dayalan G. Srinivasan, srinivasan@rowan.edu

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Environmental conditions can alter the form, function, and behavior of organisms over short and long timescales, and even over generations. Aphid females respond to specific environmental cues by transmitting signals that have the effect of altering the development of their offspring. These epigenetic phenomena have positioned aphids as a model for the study of phenotypic plasticity. The molecular basis for this epigenetic inheritance in aphids and how this type of inheritance system could have evolved are still unanswered questions. With the availability of the pea aphid genome sequence, new genomics technologies, and ongoing genomics projects in aphids, these questions can now be addressed. Here, we review epigenetic phenomena in aphids and recent progress toward elucidating the molecular basis of epigenetics in aphids. The discovery of a functional DNA methylation system, functional small RNA system, and expanded set of chromatin modifying genes provides a platform for analyzing these pathways in the context of aphid plasticity. With these tools and further research, aphids are an emerging model system for studying the molecular epigenetics of polyphenisms.

1. Introduction

While the genome has been portrayed as a "blueprint" instructing the development of an adult organism, the articulation of genotype into phenotype is a more complex phenomenon. Context-dependent development and environment-dependent phenotypic variation have been observed for decades [1]. Like the changes in gene expression that intrinsically occur in development, environment can affect gene expression and alter developmental trajectories [2]. If these developmental responses to the environment, and plasticity itself, can increase fitness and are heritable, then morphology, physiology, behavior, or life history strategies can evolve elements of adaptive phenotypic plasticity [1, 3]. This can result in the production of continuous or discrete phenotypic variation (polyphenism). The possibility for nongenetic heritable effects of environment on development raises doubts about the "blueprint" view of the genome [4].

Waddington originally defined "epigenetics" as the study of phenomena that act to produce phenotype from genotype all within in a framework of evolutionary biology [5–7]. Waddington's view of epigenetics now largely encompasses the fields of developmental biology and evolutionary developmental biology, which describe, in part, how patterns of gene expression change during ontogeny and through evolution [8]. The modern field of epigenetics examines how patterns of gene expression, instructed by extrinsic biotic or abiotic factors, can be passed to offspring through means other than the inheritance of DNA sequence. Examples of inherited epigenetic phenomena include stable cell fate specification during pluripotent stem cell divisions, dosage compensation and X chromosome inactivation, imprinting, and position effect variegation in *Drosophila* [9]. Models for seemingly disparate phenomena have converged on common mechanisms for establishing heritable gene expression patterns: changes in chromatin architecture due to the effects of DNA methylation, small RNAs, and chromatin modifying enzymes [10, 11].

2. Predictive Adaptive Developmental Plasticity through the Aphid Life Cycle

Aphids, soft-bodied insects that feed on the phloem sap of plants, have long been a model for studying the causes

and consequences of phenotypic plasticity. They exhibit both a wing polyphenism (consisting of winged and unwinged females) and a reproductive polyphenism (consisting of asexual and sexual individuals). The production of alternative morphs by genetically identical individuals by definition involves epigenetic mechanisms. Here, we describe these two polyphenisms within the context of the aphid life cycle. We then discuss the environmental cues that trigger the polyphenisms. Finally, we discuss what is known about epigenetic mechanisms in the pea aphid.

The life cycle of a model aphid species, the pea aphid *Acyrthosiphon pisum*, begins as a "foundress"—a female aphid that hatches in the spring from an overwintering egg. The foundress produces, via live birth (viviparity), a population of female unwinged aphids through asexual reproduction (apomictic parthenogenesis) that continues to reproduce asexually over several generations. This population is genetically identical, aside from spontaneous mutations [12], and lacks males during the spring and summer months. Environmental factors such as high aphid density, host plant quality, and predation can induce unwinged females to produce winged offspring. Winged asexual females disperse and colonize new host plants, founding new colonies via parthenogenesis. The parthenogenetic production of winged and unwinged female aphids continues during the spring and summer.

In fall, a change from asexual to sexual reproductive modes occurs. Asexual females sense the changing photoperiod and temperature and respond by parthenogenetically producing sexual females and males. Males are produced genetically by the loss of one X chromosome during parthenogenetic oocyte division and can be winged or unwinged. Since only sperm containing an X chromosome are viable, sexual females lay only female eggs on the host plant. The egg must "overwinter" for three to four months at cold temperatures in order to complete development and hatch as a foundress in the spring [13, 14]. Other aphid species switch host plants, produce winged sexual females or produce males earlier than pea aphids. These adaptations (sexual versus asexual, winged versus unwinged) have evolved in response to environmental changes that are predictable (seasons) and unpredictable but common (population density, host plant quality, and predation).

3. Experimental Evidence for Epigenetic Phenomena in Aphids

The wing and reproductive polyphenisms are examples of how the maternal environment affects the development of the offspring as a "predictive adaptive response" [15]. Several groups have described the triggering environmental cues and aphid responses. Though the cues differ for the reproductive and wing polyphenisms, the developmental response for both is separated by at least one generation from the triggering cue. Additionally, the resulting morphs are discrete forms and not simply continuous differences along a phenotypic gradient. This binary phenotypic output from an inductive signal gives the aphid experimental system an advantage for studying the epigenetic contribution to phenotypic plasticity.

3.1. Induction of Winged Aphids. Winged offspring can be induced by tactile stimulation of unwinged asexual aphids, either by interactions with other aphids, interactions with nonpredator insects, or experimental stimulation [16–18]. Unwinged mothers produce both unwinged and winged offspring; winged aphid mothers rarely produce winged offspring [16]. Other factors, such as the age of the mothers and temperature, can also modulate the degree of wing induction [18, 19]. The production of winged offspring can also be induced by the presence of aphid predators [20–22]. However, this effect may be driven by increased aphid walking, and thus increased inter-aphid interactions, in response to predator presence [23]. The environmental changes listed above are unpredictable but generally common, and aphids facultatively express the wing phenotype to limit predation and competition for resources.

In some aphids, wing induction occurs prenatally [24] while other species can be induced postnatally [16]. In prenatal determination, the environmental cue perceived by the mother must be transmitted to its embryos *in utero*, and the daughter embryos respond to this maternal signal. The precise nature of this maternal signal or its response is not known, though some studies implicate the juvenile hormone (JH) pathway (but see [16]). However, wing development itself does not occur until the second to third larval stage and is accompanied by the development of wing musculature, increased sclerotization of the cuticle, changes in eyes, antennal sensory rhinaria, and reproductive output [25, 26]. Thus, several days and presumably several rounds of cell division separate induction and resulting developmental response.

3.2. Induction of Sexual Aphids. The production of sexual morphs and the resulting overwintering egg coincides with predictable, seasonal changes in photoperiod and temperature. Sexual aphid morphs are observed in temperate zones during the fall and winter but not in the spring or summer, and aphids were the first animals shown to respond to changes in photoperiod [27]. Later studies defined the lengths of light and dark phases necessary for the induction of sexual aphids (reviewed in [28]). An embryo that developed under experimentally controlled long-day "summer" conditions (16 hours of light, 8 hours of darkness), and shifted to short day "fall/winter" conditions (12 hours light, 12 hours darkness) upon birth, can produce sexuals-producing mothers that consequently give birth to sexual offspring [28]. Experimental manipulations of temperature can modulate the degree of sexual morph production [29], and high temperatures can override the effect of short days on sexual induction [29, 30]. Based on the timing of sexual offspring birth, determination of embryos destined to become sexual morphs is thought to occur after embryonic germ cell cluster formation and migration, roughly corresponding to stage 17 of asexual embryo development [13, 31]. Induction of sexual-producing aphids and their

sexual offspring requires at least 10 consecutive days of fall/winter conditions [32], which appears to prevent aphids born prior to the vernal equinox from undergoing sexual induction. Some strains of aphids also produce sexual aphids followed by asexual females, possibly to hedge bets against a harsh winter and the lack of host plants [33, 34].

Similar to winged aphid induction, the induction of sexual aphids is a complex process that involves multiple tissues and extends over several days of development. Though external morphological differences between asexual and sexual females are few, the difference in internal morphology is striking. Aphid ovaries consist of 12–16 ovarioles, each of which contains germ cells housed in an anterior germarium. In sexual ovaries, germaria are connected to oocytes [31, 35]. The sexual haploid oocyte will fill with yolk contributed by nurse cells in the germarium, grow in size, and pass through the uterus to undergo fertilization. However, in asexual ovaries, the germarium is connected to a posterior string of successively older asexual embryos progressing through development, from one-celled embryos to fully developed embryos ready for parturition.

Both aphid polyphenisms are examples of the maternal epigenetic determination of offspring phenotype. The maternal inducing signal, received by the offspring as embryos, is translated into an expansive suite of developmental changes well after birth. Over 90 years ago, Ewing [36] reviewed several studies on wing induction and postulated a transgenerational "physiological inheritance" that is "not dependent on the germplasm (or at least the chromosomes) but which modifies the expression of somatic characters." Sutherland [37] also hypothesized a nongenetic "intrinsic factor" that delayed production of winged offspring from mothers born early from winged grandmothers. The transgenerational response to changing environmental conditions in aphids in some cases may involve juvenile hormone (JH). Application of JH or JH analogs to aphid mothers can prevent sexual induction under fall/winter conditions [38, 39]. Neurosecretory cells within the mother's brain likely perceive light and dark and transduce the photoperiod signal to the progeny directly or indirectly through JH [40, 41]. Thus, in the reproductive polyphenism, this "physiological inheritance" may be due to maternal hormonal signals that establish heritable epigenetic information that sets gene expression patterns in the developing embryo. Below, we discuss how genomics technologies and bioinformatics have invigorated investigation of the molecular basis of this epigenetic phenomenon.

4. The Aphid Genome: A Model for Plasticity

The genome of the pea aphid *A. pisum* is distinctive among insect and even animal genomes for several reasons [42]. With its large size (~517 Mbp) and large number of predicted genes (~35,000 genes, many well-supported by homology, EST, or RNA-seq data), the pea aphid possesses one of the largest gene repertoires among animals, rivaling that of *Daphnia pulex*, another polyphenic arthropod [43]. Repetitive elements (REs) account for a large fraction of the assembled genome (38%) [42, 44]. The large number of genes is due to a large number of gene duplications: 2,459 gene families of various functions have undergone duplication, with many families containing more than 5 paralogs. Indeed, paralogs account for nearly half of the total aphid genes, similar to that of *Daphnia* [43]. Notable among these are duplications of genes involved in DNA methylation, small RNA pathway, and chromatin modifications and remodeling (discussed in detail below). Furthermore, the aphid genome has the lowest G/C content among sequenced insects at 29.6%. The pea aphid community now has an impressive set of genomic data and tools: a draft genome sequence, expressed sequence tags (EST), full-length cDNA sequences, microarrays, and RNAi [42, 45–60].

This genome information can be leveraged toward understanding the basis of aphid plasticity and the role of epigenetics in that plasticity. For example, aphid-specific gene duplications may have facilitated the evolution of developmental plasticity, as greater phenotypic space can be explored through the differential expression of diverged paralogs in response to environmental variation. Indeed, reports of differential paralogous gene expression between different aphid morphs lend support to this hypothesis [51, 61–65]. The molecular basis for the differential expression of aphid paralogs is thus far unknown. We speculate that, in a manner similar to other arthropods [66], environmentally sensitive expression of maternal hormones helps establish heritable patterns of chromatin architecture in the embryo that affect gene expression patterns during development. This could involve DNA methylation, which can regulate gene expression in arthropods [67, 68], small RNAs, and chromatin modifications. Below, we discuss recent results lending support to a functional epigenetic system in aphids that may underlie polyphenic aphid development.

5. DNA Methylation

Several epigenetic processes rely on DNA methylation, which involves the addition of a methyl group ($-CH_3$) to the 5-carbon of cytosine in genomic DNA to form 5-methylcytosine. Methylation modifications are most commonly found on cytosines at CG dinucleotides, resulting in a symmetrical double-stranded pattern. They are less commonly found in a CHG or CHH context, where H = A, G, or T [69]. These methyl groups act as a "memory" at particular genes and function during the normal growth and differentiation of many organisms [70, 71]. DNA methylation can negatively affect transcription by either physically interfering with the binding of proteins that activate transcription, or recruiting other proteins that affect chromatin structure (see Chromatin Remodeling section). They also silence the activity of transposons and inactive genes [72].

In aphids, DNA methylation was originally observed at the E4 esterase gene in insecticide-resistant green peach aphids, *Myzus persicae* [73–75]. Contrary to the generally understood role of DNA methylation in negatively regulating transcription, the E4 esterase gene was only expressed when it

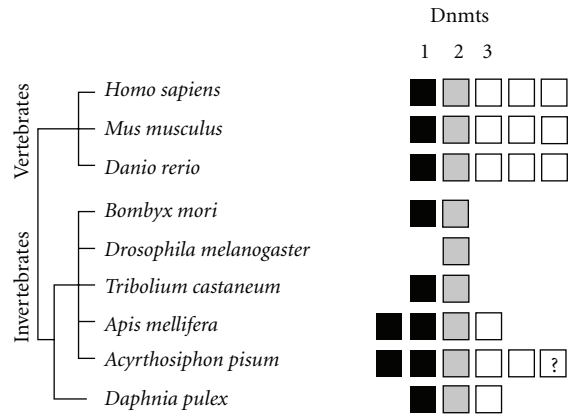

Figure 1: Vertebrates and invertebrates vary in Dnmt subfamily enzyme copy number. The number of boxes in each color (black, grey, white) indicates the number of paralogs of each type of Dnmt.

was methylated [76]. At the time, few studies had investigated the functional consequences of the observed low levels of methylation in insects [67]; thus, few conclusions could be made about the role of methylation in vertebrates versus invertebrates. Mandrioli and Borsatti [77] reported the presence of DNA methylation in the heterochromatic regions of pea aphid DNA, although they did not identify specific regions that were methylated.

DNA methyltransferases (Dnmts) are the enzymes that add methyl groups to nucleotides in DNA, using S-adenosyl methionine as the methyl donor. Animals use three classes of Dnmts [69, 78]. Dnmt1 acts as a maintenance methyltransferase, attaching methyl tags to newly synthesized DNA strands; Dnmt3 typically methylates DNA *de novo*; Dntmt2, an RNA cytosine methyltransferase, is no longer considered a true DNA methyltransferase [79, 80]. However, current evidence suggests that all three active Dnmts (Dnmt1, Dnmt3a, and Dnmt3b) may be involved in the maintenance of DNA methylation [81]. Considerable variation across taxa exists as to the presence or absence of each category of Dnmt [82]. For example, the honey bee (*Apis mellifera*) has two copies of Dnmt1, one of Dnmt2 and one of Dnmt3 [83], while *C. elegans* has lost all Dnmts and seems to lack DNA methylation [70, 84] (Figure 1). Clearly, some organisms develop and reproduce successfully without methylation enzymes and thus without methylated DNA.

The previous reports of methylated aphid DNA indicated the presence of Dnmts in the aphid genome. However, given variation among taxa in Dnmt occurrence, it was not obvious *a priori* that an aphid genome would contain all of the DNA methylation enzymes. By searching the pea aphid genome sequence [42], Walsh et al. [85] found two copies of Dnmt1, a Dnmt2 a Dnmt3, and a gene distantly related to the other Dnmts that they called Dnmt3X. Dnmt3X lacks key amino acids thought to be necessary for Dnmt function. It may, therefore, be a pseudogene. Additional proteins involved in DNA methylation are present in the pea aphid genome: the methylated-CpG binding proteins MECP2 (one copy) and NP95 (three copies), and Dnmt1 associated protein that associates with Dnmt1 to recruit histone deacetylases [85, 86]. Walsh et al. [85] also quantified overall methylcytosine levels, finding that 0.69% (±0.25%) of all of the cytosines were methylated. This low percentage closely matches the low methylation levels observed in other insect genomes [82]. Further, twelve pea aphid genes are methylated in their coding regions, but not in their introns [85]. Three of those genes are juvenile hormone (JH) associated genes, chosen for analysis because JH has previously been shown to be involved in phenotypic plasticity in aphids [28]. Further investigation of the gene for JH binding protein revealed one methylated site that had a marginally significant higher level of methylation in winged relative to wingless asexual females [85]. Overall, these data indicated that the pea aphid has a functional DNA methylation system.

6. Aphid Genome Methylation Patterns

With the pea aphid genome sequence, patterns of DNA methylation could be investigated using an indirect measure that utilizes the observed versus expected levels of CpG methylation ($CpG_{O/E}$). This method is based on the fact that methylated cytosines are hypermutable, resulting in a loss of CpGs in methylated regions. Regions of DNA with low $CpG_{O/E}$ are inferred to have been historically methylated and thus are considered areas of dense methylation [87].

Walsh et al. [85] used this method to examine the coding regions of all predicted genes in the pea aphid genome. The resulting histogram of gene frequency by $CpG_{O/E}$ exhibited a clear bimodal distribution, indicating two gene classes: genes with and without a history of DNA methylation. This same pattern was observed in another polyphenic species, the honey bee, whereas it was not observed in nonpolyphenic species like the red flour beetle (*Tribolium castaneum*), *Anopheles gambiae,* and *Drosophila melanogaster* [88]. These data began to approach the intriguing question of whether methylation levels associate with aphid alternative phenotypes, but to take this question a step further required gene expression data.

Brisson et al. [89] used a pea aphid microarray to identify significantly differentially expressed (DE) genes between fourth instars and adults, males and females, and wing morphs within each sex (wing morphology in asexual females is polyphenic, while in males it is genetically determined). Using these data, Hunt et al. [90] asked whether gene methylation density associated with patterns of DE genes among the different phenotypic groups. Overall, genes with condition-specific expression (i.e., genes with DE among categories) showed higher $CpG_{O/E}$ levels than genes that were more ubiquitously expressed. They concluded that morph-biased genes have sparse levels of methylation while non-morph-biased genes have dense levels of methylation. In a similar study, Elango et al. [88] showed that genes with DE between honey bee queens and workers had higher $CpG_{O/E}$ levels. These studies, along with others [68, 91], suggest that ubiquitously expressed genes in insects are the most likely to be densely methylated.

What are the gene categories with low and high $CpG_{O/E}$ values? The highly methylated class includes gene ontology (GO) terms associated with general organismic functions such as metabolic processes. In contrast, genes with sparser methylation encompass a wider variety of functions such as signal transduction, cognition, and behavior [90]. Given the putative role for methylation in alternative morphologies, these patterns are counterintuitive since morph-biased genes would be presumed to be the most highly methylated. One way to reconcile this contradiction is to modify the hypothesis: if morph-biased genes have sparser CpG methylation, their CpG sites are available for the action of *de novo* methylation. These genes could then acquire differential methylation states, and thus different expression states, on a generation-by-generation basis, induced by relevant environmental circumstances. In support of this, RNAi of the Dnmt3 *de novo* methyltransferase in honey bees led to changes in reproductive morph specification [92].

These previous studies relied on indirect measures of methylation specifically focused on methylation at CG dinucleotides. A catalog of all base positions in the genome that exhibit methylation, known as a "methylome," would allow for global comparative analyses of DNA methylation. This has been achieved in other organisms (e.g., [91, 93, 94]), and indeed a pea aphid methylome is currently being pursued (O. Edwards, D. Tagu, J. A. B., S. Jaubert-Possamai, unpublished data). With the methylome, it will be possible to answer the following questions: Are there differences in CG, CHG, or CHH methylation patterns between winged and wingless or sexual and asexual females? If so, what specific genes exhibit methylation differences between morphs? Does methylation associate with alternative splicing? Does methylation have a role in regulating the abundant paralogs in the pea aphid genome? Does methylation correlate with expression levels?

7. Chromatin Modification and Remodeling Pathway

The production of a cell fate relies on stable gene expression patterns specified by intrinsic and/or external factors during development. Current models propose that DNA methylation and chromatin architecture set stable, yet modifiable, patterns of gene expression. An array of different DNA-bound proteins, largely consisting of histones, acts in concert to create higher-order structures that alter chromatin shape from local to global scales. Histones H2A, H2B, H3, and H4 form an octamer on which DNA is wrapped, forming a structure known as a nucleosome, that can make DNA locally inaccessible to DNA-binding factors. Histone tails extend from the core octamer and are available for modification such as acetylation, ADP ribosylation, methylation, phosphorylation, SUMOylation, and ubiquitylation. These modifications affect local chromatin function by adjusting its accessibility and attractiveness to regulatory complexes [95]. Variant histones can replace core octamer subunits, endowing the local chromatin environment with unique structural and functional properties [96]. Nucleosomes themselves can be repositioned to allow local access to DNA by nucleosome remodeling complexes [97]. This large array of activities is thought collectively to establish a "code" of chromatin characteristics, which reflects the functional and structural state of the underlying chromosomal DNA. Histone modifications, nucleosome remodeling, DNA methylation, and even small RNA pathways may be functionally linked and interdependent in a context-dependent manner [98–100].

Increasing evidence shows that a simple model of "open" and "closed" chromatin is insufficient to explain functional and structural differences among different regions of the genome. Instead, chromatin structure can be viewed as a composite of structural and functional domains with unique combinations of histone post-translational modifications, DNA methylation patterns, variant histone members, nucleosome position and chromosome territory within the nucleus [101, 102]. Chromatin structure is maintained across mitotic divisions, although theoretical and experimental evidences have not yet converged on a mechanism for that transmission.

A survey of aphid chromatin genes is the first step in understanding how heritable chromatin structure may be associated with aphid polyphenisms. The current draft of the aphid genome indicates expansions of antagonistic chromatin modifying and remodeling pathways [61]. Histones and histone variants are conserved in the aphid genome at numbers similar to *Drosophila melanogaster*, though histone variants such as *Cenp-A* and protamines appear absent [61]. The major chromatin remodeling complexes (SWI/SNF, CHD1, ISWI, and NURD) are represented in the aphid genome. The most striking observation is that expansions of gene families involved in histone acetylation are mirrored by expansions of genes involved in histone deacetylation. A similar situation is seen for genes involved in histone methylation and histone demethylation [61, 103]. Since the effect of acetylation and methylation on chromatin state and gene expression is context-dependent, these multiple antagonistic activities could contribute towards a complex regulation of chromatin state in aphids.

Evidence thus far for morph-associated chromatin architecture in aphids is in its early stages. The holocentric structure of aphid chromosomes (which presumably have diffuse kinetochores) could have effects on higher-order chromatin structure. Stainings of pea aphid chromatin detected several histone modifications, such as methylation of histone H3 on lysine 4 and lysine 9 [61, 77, 104]. In particular, largely overlapping differential histone methylation of these residues was observed in specific regions of chromatin [61]. Duplications of antagonistic histone modifying genes could be interpreted as a "need" for a balance of chromatin modifying activities. Alternatively, these duplications could be merely coincident with the general level of gene duplication in the aphid genome and may not have biological relevance for any specific trait. Chromatin immunoprecipitation (ChIP), expression analysis, and evolutionary analysis of these genes should help distinguish between these hypotheses. Additionally, next-generation sequencing technologies can be used to survey morph-specific chromatin modifications [105].

8. Small RNA Pathway

Work over the last 15 years has implicated small noncoding RNAs as a layer of epigenetic control. Small RNAs direct the transcriptional or post-transcriptional repression of gene activity in a gene-specific manner. Classes of small noncoding RNAs include endogenous microRNAs (miRNAs); exogenous and endogenous short interfering RNAs (siRNAs and esiRNAs); and Piwi protein-associated small RNAs (piRNAs) [106–108]. This dizzying array of small RNAs is generated by transcription either of endogenous miRNA- and siRNA-encoding genes, or of repetitive elements, transposons and noncoding regions [109]. These classes differ in their biogenesis, processing, function, and partner proteins [110]. Here, we discuss progress in studying aphid miRNA and piRNA pathways.

The miRNA and siRNA pathways provide animals and plants a means of attenuating the activities of viruses and selfish genetic elements [111]. Additionally, miRNAs post-transcriptionally regulate the expression of many endogenous genes [112, 113]. Primary miRNA transcripts in the form of a stem-loop are processed by the Drosha/Pasha complex [114] into pre-miRNAs which are exported from the nucleus via Exportin 5 [115–117]. The Dicer1/Loquacious complex then pares the pre-miRNA down to a 21-nucleotide miRNA duplex [118–120]. Mature miRNAs or endogenous siRNAs are then loaded onto a RNA-induced silencing complex (RISC), which contains an Argonaute (Ago) family member protein, of which there are five in *Drosophila* (Ago1–3, Piwi and Aubergine) [121]. In *Drosophila*, Ago1-containing RISCs bind miRNAs while Ago2 RISCs contain siRNAs [122]. One strand of the duplex is retained in this complex as the single-strand miRNA or "guide" siRNA [123, 124]. RISC facilitates annealing of the single-strand miRNA to 3′ UTRs of target mRNAs to either block protein translation and promote target mRNA degradation [109], or, if the miRNA is nearly fully complementary to the target, direct cleavage of the target mRNA by RISC, similar to a siRNA (Figure 2).

In *Drosophila*, *Ago3* and the germline-specific *Piwi* and *Aubergine* Argonaute subfamily members associate with Piwi-associated piRNAs [125–127]. This class of small RNAs arises from "piRNA clusters" in heterochromatin in a manner distinct from siRNAs [107]. Piwi and Aubergine proteins exhibit "Slicer" activity when bound to piRNAs and cleave their piRNA's cognate RNA [128, 129]. Tudor domain proteins and arginine methylation of Piwi/Aubergine by the PRMT5 methyltransferase modulate Piwi/Aubergine association with piRNAs [130–133]. In addition, Piwi subfamily members may regulate the translation of germline transcripts [134, 135] and affect chromatin architecture to promote silencing [69, 136–138].

Analysis of the aphid genome sequence has revealed that the miRNA pathway has expanded in aphids [139]. *Drosophila* contains two Dicer genes, *Dicer1* and *Dicer2*, while mammals and *C. elegans* possess only one *Dicer* [140]. Jaubert-Possamai et al. [139] showed that the aphid genome, however, contains single copies of the *Dicer2* and *Ago2* siRNA pathway components and duplicates of *Pasha*, *Dicer1*, *Loquacious* and *Ago1* miRNA pathway genes relative to *Drosophila* (Figure 2). Aphid *Ago1b* and two of the four *Pasha* paralogs have undergone rapid evolution since duplication. The aphid *dcr-1a* and *dcr-1b* genes are lineage-specific duplications distinguished by a 44-amino acid insertion in the first RNAse III domain of DCR-1B. The aphid-specific duplication of Loquacious, a partner protein of Dicer1 that binds to precursor miRNAs and esiRNAs, complements the *Dicer1* duplication. These potential binding partners could form an array of complexes that regulate gene expression.

The identification of miRNAs encoded by the aphid genome firmly establishes the presence of active small RNA pathways in aphids [141]. Legeai et al. [141] used homology, deep sequencing, and predictive methods to converge on 149 pea aphid miRNAs, of which 55 are conserved among insects and 94 are thus far aphid-specific. Seventeen miRNAs showed differential expression among asexual, sexuals-producing asexual, and sexual females. Polyphenic locusts [142] and honeybees [143] also express small RNAs in morph-specific patterns. Aphid miRNAs can now be tested for their roles in aphid plasticity. As of yet, no aphid esiRNAs or piRNAs have been identified, but these small RNAs could be identified by prediction or by empirical methods.

The piRNA pathway also expanded in aphids. Within aphids, the *Piwi/Aubergine* subfamily has undergone extensive gene duplications, with eight *Piwi* paralogs and three *Ago3* paralogs found in the genome ([144], and Figure 2). Parallel to the expansion of aphid *Piwi/Aubergine* members, the aphid genome contains three PRMT5 methyltransferase paralogs (compared to one in *Drosophila*) [61], at least three Tudor-domain containing proteins and two copies of the HEN1 2′-OH RNA methyltransferase (D. G. Srinivasan, unpublished results). Similarly, in *C. elegans*, 27 Argonaute family proteins have been identified—some without Slicer activity—that act in different aspects of the small RNA pathway [145]. This may be the case in aphids as aphid *Piwi* paralogs do show differential expression between different aphid morphs [144]. The high number of repetitive and mobile genetic elements in the aphid genome mirrors the expansion of the Argonaute protein family in aphids [42, 44, 146]. This suggests morph-specific regulation of transposons and mRNAs in a Piwi-dependent manner.

9. Current Hypotheses and Comparisons with Other Arthropod and Nonarthropod Systems

Most of what is known about the patterns and processes associated with DNA methylation come from studies in noninsect systems, primarily in mammals and plants. From these studies, a view emerged that methylation levels are high in CpG contexts, with transposons, other repeats, promoters, and gene bodies exhibiting methylation [78, 93, 147]. Promoter methylation is associated with a downregulation of transcription. More recent studies have shown that gene body methylation is ancestral to eukaryotes, but other methylation patterns, such as methylation of transposons, are taxon specific [148, 149].

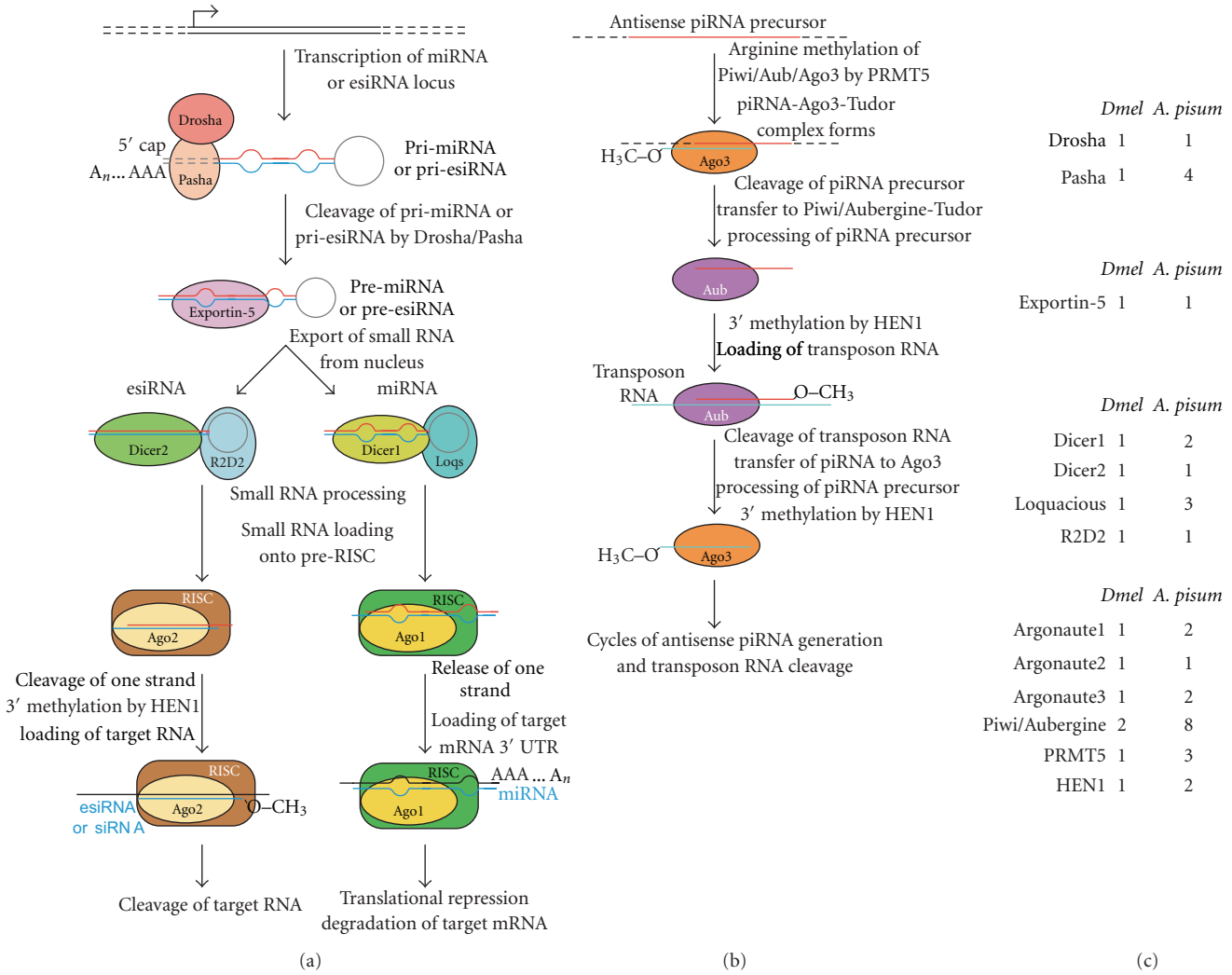

Figure 2: Small RNA pathways are conserved between *Drosophila melanogaster* and *A. pisum*. (a) The esiRNA and siRNA pathway is initiated typically with nearly perfectly complementary dsRNA produced endogenously or introduced exogenously, respectively. miRNAs are endogenously transcribed and processed by a parallel pathway in *Drosophila*, arises from imperfectly complementary dsRNAs, and repress translation of endogenous genes. (b) piRNAs are generated from piRNA clusters in the genome and are processed by a different set of Argonaute family proteins to repress transposon activity. (c) Comparison of small RNA pathway gene copy number between *D. melanogaster* and *A. pisum* reveals aphid-specific duplications. *Dmel*: *D. melanogaster*.

Methylation in insects has traditionally been understudied due to the finding that *Drosophila melanogaster*, the most well-developed insect model, has almost no detectable DNA methylation [150]. It was therefore assumed that DNA methylation does not play an integral role in insect biology as it does in mammals and plants. Recent efforts have changed this impression. Whole-genome bisulfite sequencing of *Apis mellifera* [151] and *Bombyx mori* [91] has shown that insect genomes are, indeed, methylated. However, these studies have also shown that there are key differences between insect methylomes and vertebrate or plant methylomes. First, less than one percent of cytosines in insects is methylated compared with 20–80% in plants and mammals. Second, as mentioned above, insects exhibit variable numbers of each of the Dnmt enzymes. Third, methylation in insects is highest in gene bodies. And finally, transposable elements and other repetitive elements do not appear to be methylated at high levels. One pattern is shared among insects, plants, and mammals: genes with the highest and lowest expression levels show the least gene body methylation, while those with moderate levels of expression are the most highly methylated [68, 88, 148].

One intriguing idea that insect methylation studies have raised is the possibility that gene body methylation controls alternative splicing of transcripts. In fact, methylation in *A. mellifera* is enriched near alternatively spliced exons, and alternative transcripts of at least one gene are expressed in workers versus queens [68, 151]. Thus, methylation could control alternative splicing, with alternative transcripts being deployed to achieve alternative phenotypes. In general, because of their smaller genomes, accessibility as study organisms, and gene body methylation, insects may emerge

as valuable systems for understanding the causes and consequences of DNA methylation [82].

How can DNA methylation be coupled to other epigenetic pathways in aphids? The measurement of relative methylation and accompanying chromatin states is a clear first step to test the connections between aphid gene duplications, gene expression, and chromatin structure. The interplay between chromatin modifications and DNA methylation may converge on differential expression and/or splicing of morph-specific genes. Additionally, small RNAs are expressed in morph-specific expression patterns in polyphenic locusts [142] and honeybees [143], and loss of *piwi* in *Drosophila* is associated with the loss of heterochromatic histone modifications and of HP1 association with chromatin in somatic cells [138]. Interestingly, the piRNA pathway in *Drosophila* has been associated with the suppression of phenotypic variation through the Hsp90 pathway [152] and with *de novo* DNA methylation of an imprinted locus in mice [153]. Additionally, *Drosophila* piRNAs can be epigenetically transmitted from mother to egg and affect the suppression of transposons in the next generation [154]. Identification, characterization, and correlation of small RNAs, DNA methylation, and chromatin structure to polyphenic aphid traits will help resolve the epigenetics underlying aphid life cycles.

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Review Article

Peromyscus as a Mammalian Epigenetic Model

Kimberly R. Shorter, Janet P. Crossland, Denessia Webb, Gabor Szalai, Michael R. Felder, and Paul B. Vrana

Peromyscus Genetic Stock Center and Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA

Correspondence should be addressed to Paul B. Vrana, pbvrana@gmail.com

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Deer mice (*Peromyscus*) offer an opportunity for studying the effects of natural genetic/epigenetic variation with several advantages over other mammalian models. These advantages include the ability to study natural genetic variation and behaviors not present in other models. Moreover, their life histories in diverse habitats are well studied. *Peromyscus* resources include genome sequencing in progress, a nascent genetic map, and >90,000 ESTs. Here we review epigenetic studies and relevant areas of research involving *Peromyscus* models. These include differences in epigenetic control between species and substance effects on behavior. We also present new data on the epigenetic effects of diet on coat-color using a *Peromyscus* model of agouti overexpression. We suggest that in terms of tying natural genetic variants with environmental effects in producing specific epigenetic effects, *Peromyscus* models have a great potential.

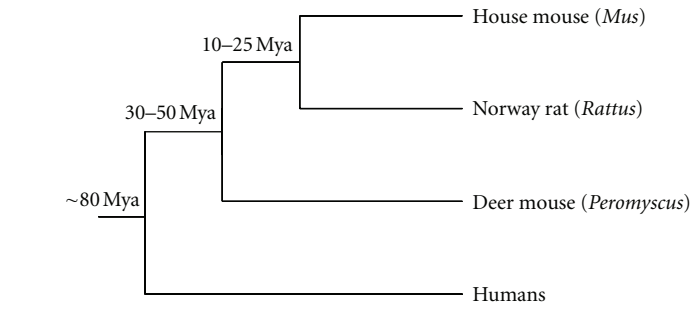
1. Introduction

1.1. Importance of Epigenetics. Understanding epigenetic effects and their associated gene-environment causes is important in that they are thought to play a large role in human disease susceptibility and etiology. Epigenetic effects are also important in agriculture, evolution, and likely in understanding ecological interactions. Gene-environment interactions are central to the concept of epigenetics, which may be defined as heritable phenotypic changes not mediated by changes in DNA sequence. Research within the last decade has revealed that many classes of genes are subject to epigenetic regulation. Such regulation likely explains much of the lineage/tissue-specific gene expression observed in mammals [1]. For example, several stem cell regulatory loci are regulated in this fashion [2, 3]. Moreover, epigenetic responses to environment, including brief exposures, appear to regulate gene expression involved in many biological processes [4–7].

These environmental response mechanisms inducing epigenetic change are largely unknown. Environmental sensitivity is illustrated by the epigenetic abnormalities seen in cultured mammalian embryos [8–10] and influences of maternal diet and behavior on offspring epigenetic marks such as DNA methylation and histone modifications [11–13]. Therefore, epigenetic effects might be predicted to vary across organisms with diverse life histories and reproductive strategies.

1.2. Caveats of Mammalian Systems. Surprisingly, there is no widely used mammalian system for studying epigenetic effects in wild-type genomes. Model systems such as rats, dogs, cows, and sheep do not represent natural populations and have been altered by domestication and other human selection [14]. The most widely used biomedical mammalian model systems are the common inbred strains of laboratory mouse (*Mus*). The common inbred strain genomes differ from wild type in two respects in addition to conscious human selection. First, the complete homozygosity of these strains is not natural. The full scope of changes induced or selected for by inbreeding is not yet known; one that seems highly likely is the presence of highly elongated telomeres in these strains [15] and attenuated behaviors [16].

The final (and perhaps least appreciated) difference of common inbred strain genomes from wild type are the combinations of alleles [17–19] and corresponding patterns of



(a)

(b)

Figure 1: (a) Phylogenetic placement of *Peromyscus* and approximate divergence times from laboratory mice, rats, and humans. (b) Map showing locales where PGSC stocks' founders were caught.

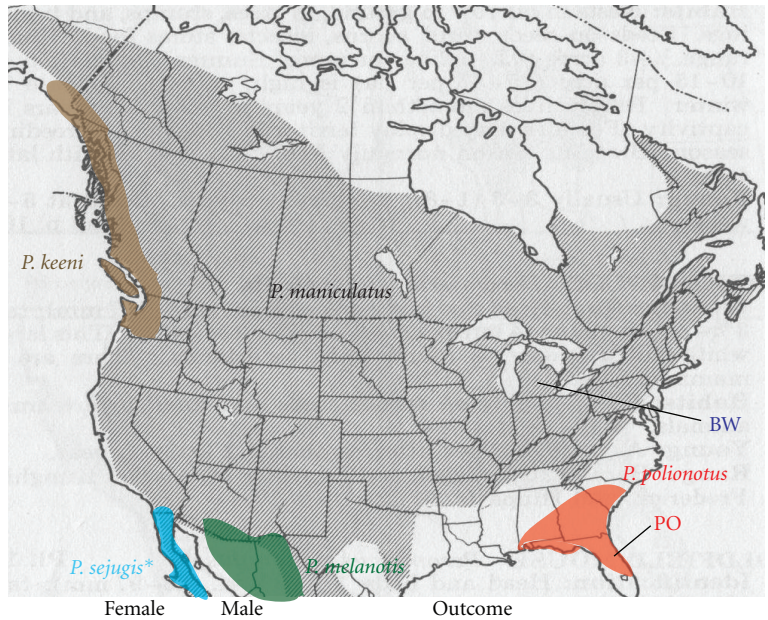
variation. That is, the genome-wide combination of alleles (whether homo- or heterozygous) found in these strains does not exist in nature. Moreover, recent studies show that the genetic diversity found in the inbred strains is limited [20]. That is, the genetic architecture of model systems does not resemble humans [21]. An obvious solution that has been proposed is to incorporate more wild-derived/non traditional systems [16, 20].

1.3. Introduction to Peromyscus and the PGSC. The rodent genus *Peromyscus*, colloquially termed deer- or field-mice, is the largest and most wide-spread group of indigenous North American mammals [22]; the group's 55+ species are found in every terrestrial ecosystem. Despite superficial resemblances, these animals represent a relatively old divergence (30 to 50 MYA) from both *Mus* and rats (*Rattus*) within the muroid rodents [23] (Figure 1(a)). Most of these species are easy to capture and breed well in captivity, facilitating study of natural variants.

The major stocks maintained by the *Peromyscus* Genetic Stock Center (PGSC; http://stkctr.biol.sc.edu/) are wild-derived. That is, a number of founder animals were caught at a specific locale over a short time period, and their random-bred descendants are considered a single stock. Among these are three of the few species of mammals which have shown to be monogamous and to exhibit pair bonding (*P. californicus*, *P. polionotus*, and *P. eremicus*). Figure 1(b) depicts the origins of these major stocks. The additional natural variants and mutants housed by the PGSC have typically been bred onto the *P. maniculatus bairdii* (BW; http://stkctr.biol.sc.edu/wild-stock/p_manicu_bw.html) stock genetic background.

The *Peromyscus maniculatus* species complex is particularly wide-spread and variable across North America (Figure 2). Viable and fertile interspecific hybrids are possible between many populations and species within this group (e.g., *P. maniculatus* females × *P. polionotus* males). Due to these factors, the majority of resource development has occurred within this group. These resources include a recently completed genetic map of *P. maniculatus* (BW stock)/*P. polionotus* (PO stock; http://stkctr.biol.sc.edu/wild-stock/p_polion_po.html), ~90,000 ESTs to date (additional transcriptome data of other organs will follow), and completed sequencing of both the BW and PO genomes. Assembly of these two genomes is in progress. Genome sequencing of two additional species, *P. leucopus* (also quite widespread in North America, and exceptionally long-lived [22, 24–26]) and *P. californicus* (arguably the best known mammalian monogamy model [27–29]) will follow.

Further, major advances have been made in reproductive manipulation of *P. maniculatus* [30]. We have greatly increased the number of oocytes/embryos recovered after induced ovulation. Second, we have also optimized conditions for culturing embryos. These advances (1) allow for easier study of early developmental stages, (2) allow a greater



Female	Male	Outcome
BW	PO	Growth retardation (viable), X skewing
PO	BW	Overgrowth, defects, LOI, X skewing

FIGURE 2: *Peromyscus maniculatus* species complex, captive stock origins, and cross results. Ranges are indicated by color, except *P. maniculatus*, which is shaded. **P. sejugis* range includes adjacent *P. maniculatus* populations which exhibit greater affinities to this species [31, 32]. Ranges of *P. keeni*, *P. maniculatus*, *P. melanotis,* and *P. sejugis* extend beyond map. LOI: Loss of (genomic) imprinting; X skewing: skewing of X chromosome during inactivation in somatic tissues. Studies from the 1930s–1950s period suggest asymmetries in crosses between other populations/species (i.e., besides PO and BW).

chance for success in cryopreservation, and (3) allow embryo manipulation (e.g., transgenics, chimera production).

Here we review epigenetic studies and relevant areas of research involving *Peromyscus* models as well as presenting new data on the epigenetic effects of diet on coat-color using a *Peromyscus* model of agouti overexpression.

2. Incompatibility between *P. polionotus* and *P. maniculatus* Epigenetic Regulation

2.1. Epigenetics in Mammalian Reproductive Isolation. An emerging theme in mammalian development is the involvement of epigenetic control of key regulatory loci [1, 2, 33–36]. The epigenetic modifications at these loci are of the same type as those observed at imprinted loci, retroelements (i.e., to prevent their transcription), the inactive X-chromosome, and in heterochromatin [37–39]. Therefore, changes in epigenetic regulation could both alter development and contribute to reproductive isolation.

Reproductive isolation is thought to be driven by sets of interacting loci in which derived allele combinations are deleterious [40]. One approach to studying such variants is to utilize interspecific hybrids, which exhibit dysgenic or maladaptive phenotypes [41]. A number of studies have employed such hybrids to map and identify the causative loci [42–45]. However, the few studies in mammals largely involve hybrid sterility [46] and thus offer little information on genes involved in developmental isolating mechanisms. Despite the lack of mapping studies, epigenetic mechanisms have been implicated in mammalian reproductive isolation in several cases, including (a) Gibbon (*Nomascus*) karyotypic evolution [47], (b) hybrid sterility between the house mouse species *Mus musculus—M. domesticus* [48], (c) retroelement activation in both Wallaby (*Macropus*) [49], and (d) *Mus musculus—M. caroli* hybrids [50].

The *Peromyscus maniculatus* species complex of North America offers great potential for such genetic studies [14]. Among the many variable characteristics in this group are the heterochromatic state of some genomic regions [51, 52]. This heterochromatin variation itself indicates epigenetic variation. Interspecific crosses within this group exhibit great variation in offspring viability. The best characterized of these are the asymmetries in crosses between *P. maniculatus* (particularly *P.m. bairdii*, the prairie deer mouse; BW stock) and *P. polionotus* (PO stock) [53–56], whose range is significantly more limited (Figure 2). One potential explanation of such asymmetries involves genes subject to the epigenetic phenomenon of genomic imprinting, which is the differential expression of the two parental alleles of a given locus.

2.2. Genomic Imprinting. Demonstration of the epigenetic nonequivalence of mammalian maternal versus paternal genomes [57–59] led to the discovery of imprinted loci. Imprinted genes exhibit biased allelic expression dependent on parental origin. That is, some loci are silenced during oogenesis and others during spermatogenesis. Differential allelic DNA methylation of cytosine residues is thought to

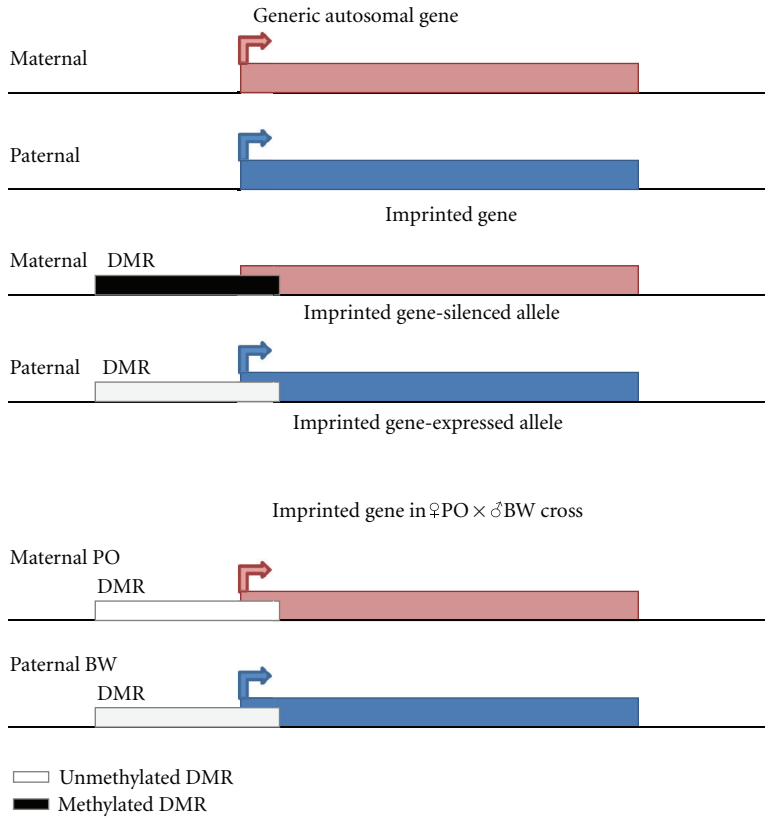


Figure 3: Diagram of effects of hybridization on genomic imprinting. A generic autosomal gene expressed from both parental alleles is shown on top. An imprinted gene expressed from the paternal allele with a methylated DMR on the silenced maternal allele is shown in the middle. The same (normally imprinted) gene losing imprinting and DMR methylation in the ♀PO × ♂BW offspring is shown at the bottom.

be the primary epigenetic mark responsible for genomic imprinting [60–62]. These discrete differentially methylated regions (DMRs) arise in gametogenesis, where the responsible epigenetic marks must be reset [63–65]. DNA methylation at these DMRs survives the global genomic demethylation during embryogenesis [66–68] and may have long-range effects on gene expression [69].

2.3. Loss of Imprinting in Peromyscus Hybrids. *P. maniculatus* females × *P. polionotus* males (♀bw × ♂po, so denoted to indicate the growth retardation outcome of the cross) produce growth-retarded, but viable and fertile offspring [55, 70, 71]. The ♀bw × ♂po hybrids display few alterations in imprinted gene allelic usage or expression levels [72, 73]. For example, the *Igf2r* gene shows slight reactivation of the normally silent paternal allele in ♀bw × ♂po extraembryonic tissues. The product of this gene negatively regulates the Insulin-like Growth Factor 2 (*Igf2*) protein. The growth-retarded hybrids also exhibit lower levels of the imprinted *Igf2* transcript in embryonic and placental tissues at some time points [73, 74]. However, normal *Igf2* paternal expression is maintained.

In contrast, *P. polionotus* females × *P. maniculatus* males (♀PO × ♂BW) produce overgrown but dysmorphic conceptuses. Most ♀PO × ♂BW offspring are dead by mid-gestation; those surviving to later time points display multiple defects [73]. A portion (~10%) of ♀PO × ♂BW conceptuses consist of only extraembryonic tissues, indicating major shifts in cell-fate. Roughly a third of pregnancies have one or more live embryos at this age. Most of these embryos have visible defects that suggest nonviability (e.g., hemorrhaging) [73]. The rare ♀PO × ♂BW litters that reach parturition typically result in maternal death due to inability to pass the hybrid offspring through the birth canal [75].

Our research has shown that many loci lose imprinted status and associated DMR DNA methylation in the ♀PO × ♂BW hybrids [72, 73, 76, 77] (Figure 3). While the extent of ♀PO × ♂BW DNA methylation loss is not known, restriction digests suggest it is not genome-wide. Excluding a *Peromyscus*-specific prolactin-related placental lactogen, which displays paternal expression [76], we have tested the expression of over twenty known imprinted genes in the hybrids [77]; the majority exhibit hybrid perturbations. In the case of *H19* and *Igf2*, two tightly linked loci are differentially affected. *H19* loses imprinting (and exhibits higher expression levels), while neither *Igf2* allelic expression nor levels have been affected in the ♀PO × ♂BW hybrids examined [72, 73]. Also pure strain PO and BW embryos exhibit significantly different expression levels of some imprinted genes (*Igf2*, *Grb10)* [73].

Two imprinted loci contribute to the ♀PO × ♂BW overgrowth: *Mexl* (maternal effect X-linked) and *Peal* (paternal

effect autosomal locus) [78, 79]. The *Mexl-Peal* interactions do not account for the loss of genomic imprinting or the developmental defects. Rather, these effects are due to the *Meil* (maternal effect on imprinting locus) locus where the effect is dependent on maternal genotype [80]. Females homozygous for the PO *Meil* allele produce the severe dysgenesis in their offspring when mated to BW males. The imprinted genes perturbed in the ♀PO × ♂BW cross do not match the patterns displayed by targeted mutations of any of the DNA methyltransferase encoding (*Dnmt*) loci [80], though those also produce maternal effects [81–84].

2.4. Hybrid X Inactivation. Both hybrid types display skewed X-chromosome inactivation in somatic tissues [78]. That is, the PO allele is preferentially silenced. This difference is mediated by the X-chromosome inactivation center. Surprisingly, imprinted X-inactivation, in which the paternally-inherited X is silenced, is maintained in the extraembryonic tissues of both hybrid types. Note that paternal X inactivation is believed to be the default and ancestral state in mammals [85, 86].

Thus it is clear that epigenetic control of individual loci as well as genome-wide epigenetic control differs between *P. maniculatus* and *P. polionotus*. We suggest that this may be the case between other species within the *P. maniculatus* species complex [14].

2.5. Use of Peromyscus in Other Genomic Imprinting/X Chromosome Studies. The frequent polymorphisms between the two species has facilitated the discovery of novel imprinted loci. A screen in the lab of SM Tilghman used a differential display approach on PO, BW, and reciprocal hybrid placental tissues which led to the discovery of imprinting of *Dlk1*, *Gatm,* and a *Peromyscus*-specific placental lactogen encoding gene. [76, 87, 88]. However, many of the putative newly discovered imprinted loci were never vetted.

The phylogenetic placement of *Peromyscus* (more divergent from lab rats and mice, Figure 1(a)) renders them useful for evolutionary studies. Several studies have shown absence of genomic imprinting at specific loci (*Rasgrf1*, *Sfmbt2*) in *Peromyscus* along with absence of putative regulatory elements, thereby strengthening the mechanistic hypotheses [89, 90].

A recent study utilized animals of the PGSC *P. melanophrys* (XZ) stock to investigate reports of anomalous sex chromosomes in this species [91]. Using *P. maniculatus* chromosome paints, they identified a region common to both the X and Y chromosomes, which has translocated to an autosome. This region has some characteristics of the inactive X chromosome (e.g., late-replication) but lacks others such as trimethyl-H3K27 modification [92].

3. Effects of a High-Methyl Donor Diet on the *Peromyscus* Wide-Band Agouti Phenotype

3.1. The Agouti A^{vy} Allele and Epigenetics. The best studied example of dietary effects on mammalian epigenetics concerns the viable yellow allele of the agouti locus (A^{vy}) in laboratory mice [11, 93]. The A^{vy} allele displays variable misexpression due to the insertion of an intracisternal A particle (IAP) retroviral-like element 5′ of the agouti promoter. Overexpression of agouti results in obesity and cancer susceptibility as well as a yellow coat-color [94, 95]. The latter phenotype differs from that of wild-type mice, whose individual hairs exhibit bands of yellow and brown (as do those of many mammals).

Maternal diets supplemented with additional methyl-donor pathway components (all taken as human dietary supplements) result in A^{vy} offspring with wild-type coloration and adiposity [11, 93]. This rescue occurs in spite of the fact that these animals are genetically identical to unrescued animals. These effects are due to the selective DNA methylation (and hence silencing) of the IAP element promoter. A maternal diet with a greater amount of supplementation resulted in a greater reduction of the abnormal phenotypes.

A nearly identical phenomenon has been documented with a lab mouse variant of the Axin gene. An IAP insertion into an Axin intron resulted in the fused allele ($Axin^{Fu}$) [96]. The IAP element results in a truncated protein, which interferes with the WT product's role in axial patterning. Thus $Axin^{Fu}$ animals have a variable degree of tail-kinking.

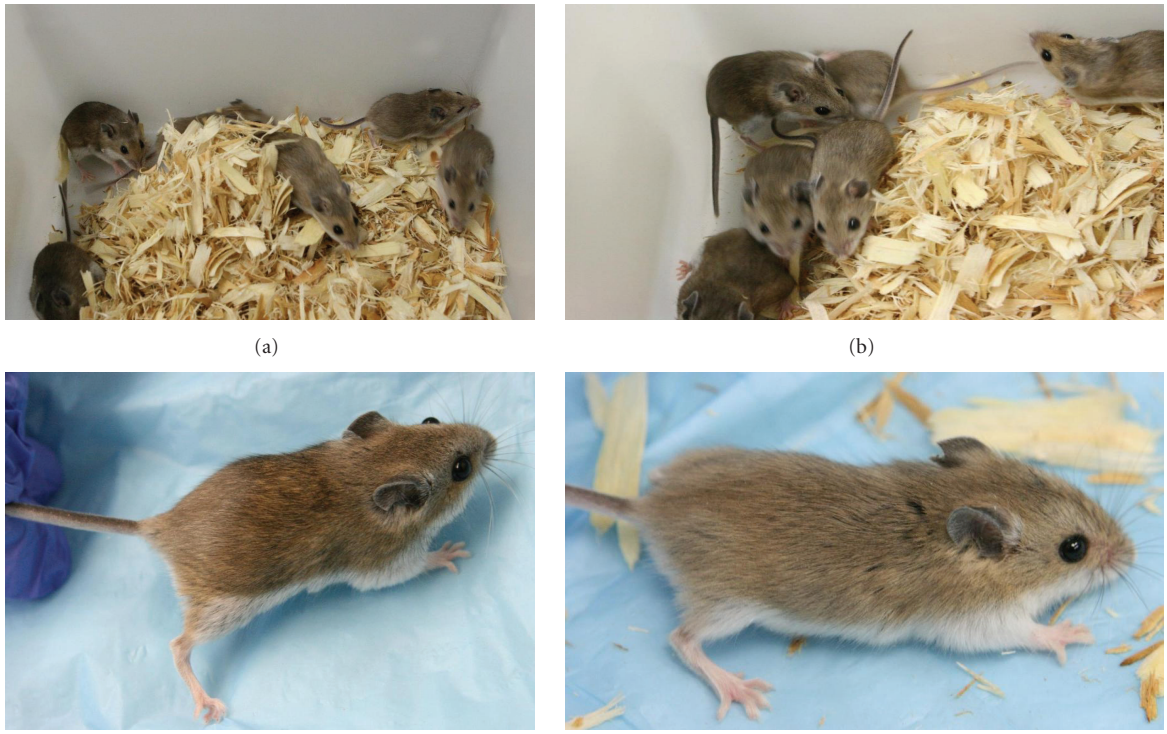
A high methyl-donor maternal diet identical to that used in the A^{vy} studies (which of the two diets is not specified) results in lower incidence and less severe tail-kinking. The rescued $Axin^{Fu}$ offspring also exhibits greater methylation of the IAP retroelement. Further, the tail appears to be more labile than the liver in terms of DNA methylation at this allele. These findings have particular import if such gestational dietary modification promotes methylation at loci other than these unusual IAP alleles.

Table 1

	8604	7517
Betaine	0	5
Choline	2.53	7.97
Folic acid	0.0027	0.0043
Vitamin B12	0.051	0.53

Comparison of standard (8604) and Methyl-Donor (7517) diet components (g/Kg of chow).

3.2. Effects of Diet on the Peromyscus A^{Nb} Allele. To test the hypothesis that such a diet may not only affect IAP elements, we utilized the same high methyl-donor chow used in the agouti A^{vy} and $Axin^{Fu}$ studies (Table 1). We employed a naturally occurring *Peromyscus* allele, which overexpresses agouti, termed wide-band agouti (A^{Nb}; http://stkctr.biol.sc.edu/mutant-stock/wide_band.html) [97–99]. We mated standard BW females to homozygous A^{Nb} males and analyzed the resulting offspring either fed a normal diet (Harlan 8604 Teklad Rodent Diet; http://www.harlan.com/) or the methyl-donor-enriched diet Harlan Teklad TD.07517 Methyl Diet; the latter is the "MS" diet used in prior methyl-donor diet studies [11, 93]. A comparison between this diet and the standard chow is shown in Table 1. Offspring were fed the same diet postweaning, until sacrificed at ∼six months of age



(a) (b) (c) (d)

Figure 4: (a, b) Heterozygous wide-band Agouti (A^{Nb}) litters exposed to methyl-donor diet; note variation. (c) Individual from litter shown in (a, b). (d) Age-matched heterozygous A^{Nb} animal exposed only to standard rodent chow. All animals shown within 4 days post weaning (24–28 days postnatal). All animals used in laboratory studies presented were bred or derived from stocks kept at the *Peromyscus* Genetic Stock Center. Most species (including all those discussed below) may be kept in standard mouse cages. *Peromyscus* are kept on a 16 : 8 light/dark cycle (rather than a 12 : 12 cycle) to facilitate breeding. All experiments presented were approved by the University of South Carolina Institutional Animal Care and Use Committee (IACUC).

(when coat color is mature; note that these animals live >4 years). After being euthanized, the animals were skinned, and tufts of hair were analyzed by light microscopy.

Whereas A^{Nb} heterozygous animals are uniformly light in coloration (Figure 4(d)), we observed large variability in the animals whose mothers were fed the methyl-donor diet as soon as weaning (Figures 4(a) and 4(c)). Thus the maternal diet alone can affect the status of a non-IAP-regulated agouti locus.

Analysis of the coats of other animals at six months of age (maintained on the diet) confirmed this variation in animals exposed to the methyl-donor-enriched diet. Some animals had a yellow hair band of only 2-3 mm, whereas this band extended to 5-6 mm in other animals. This length corresponds to the overall appearance of the coat (i.e., the longer the band, the lighter the coat, Figure 5). Future studies will examine the DNA methylation status of the agouti gene and other loci in these animals as well as potential behavioral effects.

4. Toxicology and Epigenetics

4.1. Peromyscus as a Toxicology Model. Due to their ubiquity, *Peromyscus* are found in most North American contaminated (e.g., due to mining or manufacturing) sites, even where other animals are absent [100–102]. Comparison of PGSC animals exposed to these compounds is a fruitful way to study the physiological consequences of xenobiotic exposure. One unexplored research avenue is whether animals at sites contaminated with heavy metals exhibit epigenetic changes, as cadmium and nickel (among others) have been shown to induce such change [103–106].

Stock Center animals have been employed for studies involving PCBs [107–112], 4,4′-DDE [113], Aroclor 1254 [114, 115], 2,4,6-trinitrotoluene [116], ammonium perchlorate [117], and RDX [118–120]. One of the PGSC stocks has a natural deletion of the alcohol dehydrogenase (ADH) gene [121], which has proven useful for delimiting the relative roles of ADH and microsomal oxidases in ethanol metabolism [122] and the metabolic basis of ethanol-induced hepatic and pancreatic injury [123]. Ethanol and its metabolites have also been associated with changes in epigenetic marks [124, 125].

4.2. BPA Peromyscus Studies. Bisphenol A (BPA) is a chemical used in the production of poly-carbonate plastic and epoxy resins. BPA is commonly used in products including food and beverage containers, baby bottles and dental composites; it is present in 93% of human urine samples in the United States and is a known endocrine disruptor [126].

(a) (b)

Figure 5: Pelts and hair clumps from selected heterozygous wide-band Agouti (A^{Nb}) raised on the methyl-donor diet. (a) Pelts from animals exhibiting differential coat-colors. (b) Microscopy of dorsal hair clumps from same animals (and same order) as in (a). Note the longer yellow band in the rightmost sample.

Dolinoy and colleagues found that prenatal exposure to BPA through maternal dietary supplementation (50 mg/kg) produced significantly decreased methylation of nine sites of the A^{vy} locus, as well as at the CDK5 activator-binding protein locus [127]. Coat color distribution was shifted towards the yellow coat color phenotype.

A 2011 study demonstrated behavioral disruptions in BW animals by bisphenol A (BPA). BPA altered certain behaviors in male offspring of mothers administered BPA during pregnancy. Specifically, these males had decreased spatial navigational ability and exploratory behavior, traits necessary for finding a mate. Females also preferred non-BPA exposed males, despite the lack of detectable physical effects on the BPA-exposed males [128]. This study, therefore, has broad implications for the effects of these compounds on mammals.

5. Additional Areas of *Peromyscus* Epigenetic Study

There are several additional areas of research where *Peromyscus* models appear to have potential.

As noted, *P. leucopus* is a model for ageing, as they live >8 years, ~3-4 times longer than other rodents of comparable size. That longevity is associated with increased vascular resistance to high glucose-induced oxidative stress and inflammatory gene expression [25] and a relatively slower rate of loss of DNA methylation [26].

P. maniculatus has a propensity to perform repetitive movements, for example, jumping, whirling, and back flipping [129]. Such behaviors are not only representative of a number of human disorders [130] but also an issue in captive animal welfare. Thus the PGSC BW stock of *P. maniculatus* has become recognized as a model for stereotypy [131]. Attenuation of stereotypy was seen after environmental enrichment [132], suggesting a potential epigenetic effect.

Further, BW populations can be grouped into high and low stereotypic behavior groups, with high and low doses of fluoxetine reducing the phenotype in both groups [133]. The two stereotypy levels found in the BW population make them a model for basic research on brain function during repetitive motion and also provide a model for gene-environment epimutation analysis.

6. Conclusions

The interplay between environment and genotype that results in specific epigenetic changes appears complex. *Peromyscus* offers the opportunity to study natural genetic variants in both laboratory and natural settings and the ability to examine mechanistic and evolutionary aspects of changes in epigenetic control. We suggest that in terms of natural genetic variation and associated epigenetic effects, *Peromyscus* models have a potential not yet realized with any mammalian system. We encourage anyone interested in the possibility of using these animals in their research program to contact the PGSC (http://stkctr.biol.sc.edu/).

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Review Article

Epigenetic Mechanisms Underlying Developmental Plasticity in Horned Beetles

Sophie Valena and Armin P. Moczek

Department of Biology, Indiana University, 915 E Third Street, Myers Hall 150, Bloomington, IN 47405-7107, USA

Correspondence should be addressed to Armin P. Moczek, armin@indiana.edu

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All developmental plasticity arises through epigenetic mechanisms. In this paper we focus on the nature, origins, and consequences of these mechanisms with a focus on horned beetles, an emerging model system in evolutionary developmental genetics. Specifically, we introduce the biological significance of developmental plasticity and summarize the most important facets of horned beetle biology. We then compare and contrast the epigenetic regulation of plasticity in horned beetles to that of other organisms and discuss how epigenetic mechanisms have facilitated innovation and diversification within and among taxa. We close by highlighting opportunities for future studies on the epigenetic regulation of plastic development in these and other organisms.

1. Introduction

Organismal form and function emerge during ontogeny through complex interactions between gene products, environmental conditions, and ontogenetic processes [1, 2]. The causes, nature, and consequences of these interactions are the central foci of epigenetics [3]. Broadly, epigenetics seeks to understand how phenotypes emerge through developmental processes, and how that emergence is altered to enable evolutionary modification, radiation, and innovation. Epigenetic mechanisms can operate at any level of biological organization above the sequence level, from the differential methylation of genes to the somatic selection of synaptic connections and the integration of tissue types during organogenesis. Here, we take this inclusive definition of epigenetics and apply it to the phenomenon of developmental plasticity, defined as a genotype's or individual's ability to respond to changes in environmental conditions through changes in its phenotypes [4]. All developmental plasticity is, by definition, epigenetic in origin, as the genotype of the responding individual remains unaltered in the process. It is the nature, origins, and consequences of the underlying epigenetic mechanisms that we focus on in this review. We do so with specific reference to horned beetles, an emerging model system in evo-devo in general and the evolutionary developmental genetics of plasticity in particular.

We begin our review with a general introduction to the concept of developmental plasticity. We then introduce our focal organisms, horned beetles, summarize the most relevant forms of plasticity that have evolved in these remarkable organisms, review what is known about the underlying epigenetic mechanisms, and highlight future research directions. Lastly, we discuss how studies in *Onthophagus* species could provide meaningful insight into three major foci in evo-devo research: the development and evolution of shape, the process of evolution via genetic accommodation, and the origin of novel traits. We begin, however, with a brief introduction of the significance of plasticity in development and evolution.

2. The Biology of Developmental Plasticity

Developmental plasticity refers to an individual's ability to respond to environmental changes by adjusting aspects of its phenotype, often in an adaptive manner. In each case a single genotype is able, through the agency of environment-sensitive development, to give rise to vastly different phenotypes. Developmental plasticity is perhaps most obvious

in the expression of alternative morphs or polyphenisms, as in the seasonal morphs of butterflies, winged or wingless adult aphids, aquatic or terrestrial salamanders, or the different castes of social insects (reviewed in [2]). However, developmental plasticity is also inherent in more modest, often continuous changes in response to environmental conditions, such as tanning (in response to sun exposure), muscle buildup (in response to workouts) or immunity (following an infection resulting in an immune response). Lastly, developmental plasticity is a necessary prerequisite for developmental canalization, or the production of an *in*variant phenotype in the face of environmental fluctuation. Here, plastic compensatory adjustments on some level of biological organization enable the homeostatic maintenance of developmental outputs at others, such as the maintenance of blood sugar levels in the face of fluctuating nutrition and activity, or the maintenance of scaling relationships despite nutrition-dependent variation of overall body size in most organisms. Developmental plasticity is thus a ubiquitous feature of organismal development, applicable to all levels of biological organization, and rich in underlying mechanisms.

Developmental plasticity not only enables coordinated and integrated responses in development but also has great potential to affect evolutionary processes and outcomes (reviewed in [4, 5]). Developmental plasticity enables organisms to adaptively adjust their phenotype to changing environmental conditions. On one side, developmental plasticity may thus impede genetic divergences that might otherwise evolve between populations subject to disparate environmental conditions. On the other, plasticity buffers populations against local extinctions, thus increasing the opportunity for the evolution of local adaptations and diversification.

Similarly, developmental plasticity may both impede and facilitate evolutionary diversification by providing additional targets for selection to operate on, by offering modules for the regulation of development that can be reused across developmental contexts, and by creating novel trait interactions. In each case, developmental plasticity may result in pleiotropic constraints on adaptive evolution, but also has the potential to shift the evolutionary trajectories available to lineages into phenotypic space that otherwise would remain unexplored [5].

The role of developmental plasticity in evolution is perhaps most important when we consider the consequences of organisms encountering novel environments, for instance during the natural colonization of a new habitat or the anthropogenic alteration of ecosystems due to global climate change, habitat degradation, and the invasion of alien species [5]. Here, developmental plasticity enables the production of functional, integrated phenotypes, despite development occurring in previously unencountered, or greatly altered, conditions. Moreover, such novel conditions may result in the formation of novel traits or trait variants previously unexpressed, alongside the release of previously cryptic, conditionally neutral genetic variation. Developmental plasticity thus has the potential to determine which phenotypic and genetic variants become visible to selection in a novel environment, thus delineating the nature and magnitude of possible evolutionary responses. Consistent with a long-assumed role of developmental plasticity in evolution (reviewed in [2]), a growing number of artificial selection experiments on a broad range of organisms (*Drosophila*: [6]; but see [7], Arabidopsis [8], fungi [9], and Lepidoptera [10]) have now demonstrated unequivocally that developmental systems confronted with challenging or novel environments can indeed expose novel phenotypic and genetic variants that, in turn, provide ample substrate for rapid, selective evolution of novel phenotypes. Similarly, studies on natural populations are providing growing evidence that ancestral patterns of plasticity have enabled and guided more refined evolutionary responses in derived populations (e.g., [11]).

Developmental plasticity thus plays a central role in the production and evolution of phenotypic variation. Further understanding of the nature of this role likely requires a thorough understanding of the epigenetic mechanisms that enable plastic responses to environmental variation. As outlined in the following sections horned beetles have begun to provide diverse opportunities to investigate the mechanisms underlying the epigenetic regulation of developmental plasticity and to probe their significance in the developmental origin and evolutionary diversification of form and behavior. We begin with a brief introduction of the biology of these organisms.

3. The Biology of Horned Beetles

Beetles are holometabolous insects and constitute the most diverse insect order on the planet. Horned beetles comprise a polyphyletic group of diverse beetle families marked by the development of horns or horn-like structures in at least some species (reviewed in [12, 13]). Horn evolution has reached its extremes, both in terms of exaggeration and diversity, in two subfamilies within the Scarabaeidae, the Dynastinae (i.e., rhinoceros beetles), and the Scarabaeinae, or true dung beetles (Figure 1). In both subfamilies, thousands of species express horns and have diversified with respect to location, shape, and number of horns expressed. In extreme cases, horn expression more than doubles body length and may account for approximately 30% of body mass.

Despite the remarkable morphological diversity that exists among horned beetle species, horns are used invariably for very similar purposes: as weapons in aggressive encounters with conspecifics (reviewed in [12]). In the vast majority of species, horn expression is restricted to, or greatly exaggerated in, males, and absent or rudimentary in females. In these cases horns are used by males as weapons in male combat over access to females (e.g., [14]). In all species studied to date, body size has emerged as the most significant determinant of fighting success. In a subset of species, horns are expressed by both sexes. Here, males and females use horns as weapons in defense of mates and nesting opportunities, respectively (e.g., [15]). Lastly, in a very small number of species, horn expression is exaggerated in females and greatly reduced in males. Such reversed sexual dimorphisms are rare and the ecological conditions that have facilitated their evolution are largely unknown [16, 17].

Figure 1: Examples of the exuberance and diversity of horn phenotypes across genera. top to bottom: Scarabaeinae: *Phanaeus imperator, Onthophagus watanabei;* Dynastinae: *Eupatorus gracilicornis, Trypoxylus (Allomyrina) dichotoma, Golofa claviger.*

We know most about the biology of horned beetles through studies on one particular genus in the Scarabaeinae: *Onthophagus.* Adults of the *Onthophagus* genus colonize dung pads of a variety of dung types, consume the liquid portions and bury the more fibrous fraction in subterranean tunnels as food provisions for offspring in the form of brood balls. Brood balls typically contain a single egg and constitute the sole amount of food available to a developing larva. Variation in the quantity or quality of parental provisions or abiotic factors such as soil moisture can greatly affect the amount of food that is effectively available to sustain larval development, which in turn results in substantial variation in larval mass at pupation and final adult body size, as detailed below.

Also similar to many other horned beetles species, *Onthophagus* frequently have to contend with high levels of male-male competition for females and female-female competition over breeding resources such as dung and tunneling space [18]. This unique combination of developmental conditions (marked by partly unpredictable larval resources) and ecological conditions (marked by intense intraspecific competition) has facilitated the evolution of a remarkable degree of plasticity in development, physiology, and behavior in *Onthophagus* beetles, as overviewed in the next section.

4. Developmental Plasticity in Onthophagus

4.1. Plasticity in Timing of Life History Transitions. Larval *Onthophagus* develop in a partly unpredictable resource environment, as their feeding conditions depend on the quantity and quality of dung provisioned for them by their parents and the physical properties of the nesting site. Unlike the highly mobile larval stages of many other holometabolous insects, larval *Onthophagus* cannot change their location or add to the resources made available to them. *Onthophagus* larvae meet these unpredictable conditions with a striking degree of plasticity in the timing of life history transitions, specifically by molting to the pupal stage at a range of larval body sizes far greater than what has been observed for other insects (reviewed in [19]). For instance, *Onthophagus taurus* larvae will routinely feed for 15 days during the third and final larval instar under *ad libitum* conditions, but are capable of completing metamorphosis if food deprived after just 5 days of feeding. The resulting larvae pupate at a fraction of the body mass of larvae fed *ad libitum* and eclose as tiny adults. Such striking flexibility in the dynamics of larval development and the body mass at pupation allows *Onthophagus* larvae to respond to unpredictable variation in larval feeding conditions while ensuring eclosion to a viable adult capable of reproducing. As a consequence of this phenomenon, natural populations of adult *Onthophagus* commonly display a remarkable amount of intraspecific variation in male and female body sizes.

4.2. Morphological Plasticity. Recall that in the vast majority of species horn expression is restricted to males, which use horns in male combat over access to females or nesting sites. Recall also that body size is the most important determinant of fighting success, yet ecological conditions generate males

Figure 2: (a) Examples of male polyphenism in *O. taurus* (top) and *O. nigriventris* (bottom). Large males are shown on the left and small males on the right. Note that females (not shown) are entirely hornless in both species. (b) Rare reversed sexual dimorphism in *O. sagittarius*. Males also lost ancestral male dimorphism.

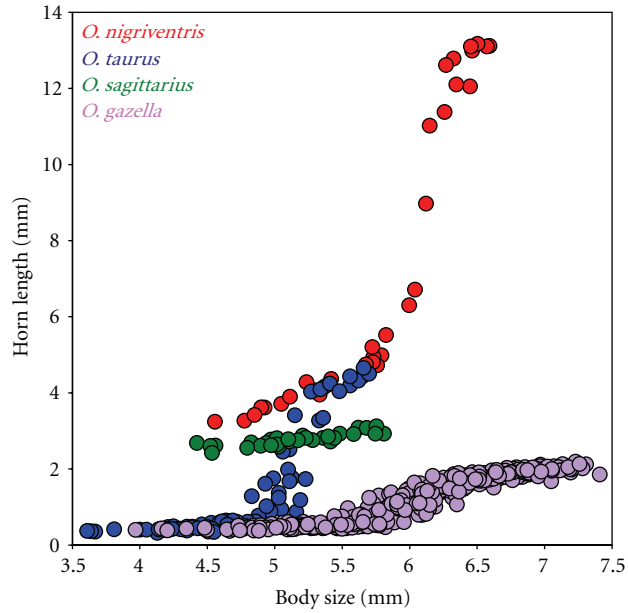

Figure 3: Differences among four *Onthophagus* species in the range of nutrition-mediated plasticity in male horn expression. Shown are the scaling relationships between body size (X-axis) and horn length (Y-axis). Patterns of nutritional plasticity in horn expression range from minimal and linear (*O. sagittarius*) and modestly sigmoidal (*O. gazella*) to strongly sigmoidal with species-specific differences in amplitude (*O. taurus* and *O. nigriventris*).

of a wide range of body sizes, many of which are too small to succeed in aggressive encounters. In many horned beetle species, these conditions have led to the evolution of alternative male phenotypes, with large males relying on the use of horns and aggressive fights to secure mating opportunities, while smaller males rely on nonaggressive sneaking behaviors (discussed in detail below). *Morphologically*, male polyphenism has a range of manifestations.

First, in numerous species horn expression is restricted to, or greatly exaggerated in, large males only, whereas smaller males express greatly reduced or rudimentary horns. On the population level, this results in a bimodal distribution of horn lengths and thus two more or less discrete morphs (Figure 2). Intermediate morphologies do exist, but are rare in most species. As a consequence, populations of conspecific males express a characteristic scaling relationship, or allometry, between body size and horn length (Figure 3). Different species have diversified greatly in the degree of male horn polyphenism and the exact shape of the associated allometry [20], in extreme cases causing alternative conspecific morphs to be classified as different species [21].

Second, smaller males (often referred to as "hornless males" "minor males," or "sneaker males") do not invest in horns and fights as means of securing matings, but instead invest in non-aggressive tactics, including the use of enlarged testes and ejaculate volumes to aid in sperm competition [22]. As with horns, morph-specific differences in testes development differ greatly from one species to the other, but comparative studies have not been able to identify any general relationship between the relative sizes of horns and testes [23, 24].

Third, the facultative enlargement of horns in large males appears to tradeoff with a variety of other structures. The precursors of adult horns develop, just like the precursors of wings, legs, and mouthparts, right before the larval-pupal transition, but *after* all larval feeding has ceased. As such, the development of horns is, like that of all other adult traits, largely enabled by a finite amount of resources accumulated during the larval stage [25]. Structures that develop in the same body location or at the same developmental time may therefore find themselves competing for a limited pool of resources to sustain their growth [26]. When faced with resource allocation tradeoffs, developmental enlargement of one structure may only be possible through the compensatory reduction of another. As such, resource allocation tradeoffs have the potential to not only alter developmental outcomes, but to also bias evolutionary trajectories. In horned beetles, resource allocation tradeoffs have been implicated in antagonistic coevolution of horn length and the relative sizes of eyes, wings [27], and copulatory organs [28], although the exact nature of these tradeoffs remains to be investigated.

4.3. Behavioral Plasticity. Alternative horned and hornless male morphs employ different behavioral repertoires to maximize breeding opportunities [12]. In many species, horned males rely exclusively on fighting behaviors including the use of horns as weapons. Body size is the most important determinant of fight outcome, and among similar-sized males, relative horn length predicts fight outcome in most contests (e.g., [14]). Fights can be long, appear energetically expensive, but are rarely injurious (but see [29]). Horned losers typically withdraw from fights.

Hornless males also engage in prolonged fights when confronted with other hornless males, but quickly withdraw from fights against large, horned conspecifics and switch to a set of non-aggressive sneaking behaviors. For instance, in *Onthophagus taurus*, perhaps the best studied horned beetle species, sneaking behaviors include the use of naturally occurring tunnel interceptions to locate and mate with females without being detected by a guarding male [14]. Small males may also dig their own shallow intercept tunnel to access females underneath guarding males, or wait for females above ground as they emerge periodically to collect dung provisions. Lastly, small males may simply wait next to tunnel entrances for opportunities to temporarily gain access to females while the guarding male is distracted, for instance by fighting off a second intruder. Studies have provided evidence consistent with the hypothesis that hornlessness increases maneuverability inside tunnels, suggesting that the absence of horns may be adaptive in the particular behavioral niche inhabited by small, sneaking males [30].

Male morphs also differ distinctly in nature and extent of paternal investment. Horned males generally assist females in tunneling and brood ball production, whereas small, hornless males invest most to all of their time into tunnel defense and the securing of additional mating opportunities [31].

Lastly, behavioral plasticity is not limited to males but also exists in females. Two contexts are especially relevant. First, females typically reproduce by provisioning food for their offspring in the form of brood balls buried underground. In the process, females of at least some species utilize a wide range of dung types and qualities. For instance, *O. taurus* females routinely utilize horse and cow dung in the field. Both dung types differ substantially in quality, and nearly twice as much cow dung than horse dung is needed to rear an adult of similar body size in the laboratory [32]. Individual mothers respond to this variation in dung quality by roughly doubling brood ball masses when offered cow instead of horse dung. Second, females facultatively switch from brood-provisioning behavior to brood-parasitic behavior and the utilization of brood balls constructed by other females [33]. In most cases, a brood-parasitic female will consume the egg inside and either replace it with one of her own while leaving the remainder of the brood ball intact, or incorporate the brood ball into a new, larger brood ball she is constructing herself. Under benign, *ad lib* laboratory breeding conditions up to 13% of brood balls may be affected by such facultative brood-parasitic behavior. This incidence rate roughly doubles when breeding conditions are made adverse by increasing dung desiccation rates [33].

4.4. Physiological Plasticity. Recent studies have discovered an unexpected amount of plasticity in thermoregulatory properties and preferences among morphs, sexes, and species of horned beetles. Specifically, Shepherd et al. [34] observed that the ability to be active at high temperatures increased substantially with male and female body size in a species with a modest sexual and male dimorphism. This was also observed in a second species except for large males, which express extremely large thoracic horns, yet exhibited the thermoregulatory behavior of small, hornless males and females. Using these and additional observations, Shepherd et al. [34] suggested that horn development and possession adversely affect the thermoregulatory abilities of male beetles, and that the magnitude of this effect depended on the degree of horn exaggeration. Specifically, they proposed that large, heavily horned males lack the thermoregulatory ability of their large female counterparts, possibly due to a tradeoff between horn production and investment into thoracic musculature, which plays an important role in the shedding of excess heat in scarab thermoregulation [35]. If so, large horned males may be forced to be active at lower temperatures to avoid risking overheating. Preliminary biochemical analyses of thorax protein content are at least partly consistent with such a scenario (Snell-Rood, Innes, and Moczek, unpublished).

In summary, developmental plasticity pervades the biology of horned beetles, providing rich opportunities to investigate the epigenetic mechanisms underlying plastic responses alongside the ecological and behavioral contexts within which they function and diversify. One genus in particular, *Onthophagus*, has emerged as an especially accessible study system, in large part due to a growing toolbox of developmental genetic and genomic resources. In the next section, we review what we have learned from the application of these tools in the study of the epigenetic regulation of developmental plasticity in these charismatic organisms.

5. Epigenetic Mechanisms Underlying Developmental Plasticity in *Onthophagus*

5.1. Gene Expression. Microarray applications to *Onthophagus* horned beetle development have been used to quantify and characterize the degree to which the plastic expression of alternative male phenotypes is associated with changes in gene expression [36, 37]. For instance, Snell-Rood et al. [36, 37] used microarrays to examine single-tissue transcriptomes of first-day pupae to contrast male morph-specific gene expression with sex- and tissue-specific gene expression. Several important findings emerged from this work. First, if the same tissue type was examined across alternative morphs (and sexes), transcriptional similarities overall far outweighed differences. Second, for those genes that were significantly differentially expressed across morphs, the frequency and magnitude of differential expression paralleled or exceeded that observed between sexes. In other words, if differential expression is used as a metric of developmental decoupling, the development of alternative morphs appeared just as decoupled as did the development of males and females. Lastly, degree and nature of differential expression varied in interesting ways by tissue type. For instance,

the transcriptomes of developing head horns in *O. taurus* were more similar between hornless males and females than to the corresponding tissue region in presumptive horned males. In other words, the head horn transcriptome of small, hornless males appeared feminized, which may not be surprising as both females and small males inhibit horn expression. In contrast to head horns, thoracic horns are enlarged in all *O. taurus* males compared to females but develop transiently, such that they are only visible in pupae yet become resorbed prior to the pupal-adult molt. Transcriptomes of thoracic horns for both male morphs were more similar to each other compared to that of females, and a similar pattern was observed in developing legs. Lastly, brain gene expression patterns of large horned males were more similar to females than to small hornless males. In other words, opposite to the situation for head horns, brain transcriptomes of *horned* males appeared more feminized. Combined, these data demonstrate that the development of alternative male morphs is associated with an appreciable amount of differential gene expression, the nature and magnitude of which differs significantly by tissue type.

Additional array experiments ([38]; Moczek et al. in preparation) and a growing number of candidate gene studies (e.g., [12, 39–44]) have now begun to investigate the possible functional significance of genes that are expressed in a morph-specific (on/off) or morph-biased (up/down) manner. Several important findings have emerged from these studies. First, the development of horns appears to rely, at least in part, on the function of conserved developmental pathways such as the establishment of proximodistal axis through leg gap genes [39], growth regulation through TGFβ- and insulin-signaling [41, 43, 45], cell-death mediated remodeling during the pupal stage [40], or positioning through Hox- and head gap-genes ([42]; Simonnet and Moczek, unpublished). Second, not all genes expressed during the development of large horns are functionally significant. For instance, the transcription factor *dachshund (dac)* is expressed prominently during the development of both head and thoracic horns, yet RNAi mediated *dac* transcript depletion does not result in any detectable horn phenotypes, despite pronounced phenotypic effects in nonhorn traits [39]. Third, different horn types, whether expressed by different species, sexes, or in different body regions of the same individuals, rely at least partly on different developmental mechanisms and thus may have had different and independent evolutionary histories [46]. Combined, these findings illustrate that the evolution and diversification of horn development have been enabled by the differential recruitment of preexisting developmental mechanisms into new contexts, resulting in a surprising functional diversity within and between species.

5.2. Gene Expression—Future Directions. Except for a few well-studied models such as the honey bee [47] or *Daphnia* water fleas [48], little is known about the overall genome-wide magnitude and nature of conditional gene expression. Similarly, we know little about how conditional gene expression compares to other forms of context-dependent gene expression, such as tissue-, stage-, or sex-specific expression. Such comparative data are critical to evaluate whether (a) differential expression of largely similar or different gene-sets underlie different types of context-dependent changes in gene expression; (b) the extent of pleiotropic constraints that might delineate evolution of context-dependent gene expression; (c) the degree to which environment-specific gene expression may result in relaxed selection and mutation accumulation.

Studies on *Onthophagus* beetles have made a first attempt to address a subset of these questions. As detailed above, preliminary array studies identified that the development of alternative, nutritionally cued male morphs is associated with a considerable amount of morph-biased gene expression, the nature and magnitude of which exceeded that of sex-biased gene expression for some tissue but not others, a level of complexity likely to be overlooked by whole-body array comparisons [36]. Furthermore, genes with morph-biased expression were more evolutionarily divergent than those with morph-shared expression, consistent with predictions from population-genetic models of relaxed selection [36, 49, 50] as well as results from other studies (*Drosophila*: [51]; aphids: [52]; bacteria: [53]).

Additionally, recent work has raised the possibility that conditional gene expression, rather than resulting in relaxed selection, is instead enabled by it. Studies on both Hymenoptera [54] and amphibians [55] show that genes expressed in a morph-biased manner exhibit patterns of sequence evolution consistent with relaxed selection not only in polyphenic taxa, but also related taxa lacking alternative morphs. This suggests that genes exhibiting relaxed selection (for whatever reason) may preferentially be recruited into the expression of alternative phenotypes. If correct this would suggest the possibility for positive feedback, as conditional expression would further relax selection, hence further increasing the probability of recruitment into a plasticity context. Lastly, it is conceivable that the initial relaxation of selection that might enable recruitment of genes for the expression of alternative morphs was facilitated by more subtle forms of plasticity and conditional-gene expression in ancestral, monomorphic taxa, such as season- or sex-biased expression. Ultimately, evaluating the relative significance of the *plasticity-first* versus the *relaxed selection-first* hypotheses (and the potential interplay between them) will require a more thorough sampling of transcriptomes across clades, and most importantly, a more thorough understanding of the developmental functions and fitness consequences of conditional gene expression. Research on *Onthophagus* beetles has the potential to contribute to these efforts through the use of recently developed next-generation transcriptomes and corresponding microarrays [56] as well as studies currently under way to analyze patterns of SNP diversity and sequence evolution within and between species.

5.3. Endocrine Regulation. Endocrine mechanisms play a critical and well-established role in the epigenetic regulation of insect plasticity (reviewed in [57, 58]). Findings supporting a role of endocrine factors in the regulation of polyphenism in *Onthophagus* are derived primarily from hormone manipulation experiments, hormone titer profiling,

and more recently, gene expression and gene function manipulation studies, as summarized below.

Juvenile hormone (JH) is a sequiterpenoid hormone secreted by the insect *corpora allata* that maintains the current developmental stage across molts. Applications of a JH analog, methoprene, during *Onthophagus* development provided some of the first evidence that endocrine factors may regulate the expression of alternative nutritionally cued male morphs. Specifically, applications of JH analogs induced ectopic horn expression in *Onthophagus taurus* larvae fated to develop into small, hornless males [59]. In addition, *O. taurus* populations that have diverged in the body size threshold for horn induction showed corresponding changes in the degree and timing of JH sensitivity [60]. Subsequent work on other species has provided additional evidence that JH applications can alter aspects of horn expression, and do so differently for different species, sexes, and horn types [61].

Ecdysteroids play a critical role in initiating the onset of the molting cycle, and for this class of hormones direct titer measurements do exist for a single *Onthophagus* species, *O. taurus* [59]. Expectedly, ecdysteroid titers were observed to increase in male and female *O. taurus* approaching the larval-pupal molt. However, Emlen and Nijhout [59] also observed a small ecdysteroid peak several days earlier during the feeding phase of the last larval instar. This particular peak in ecdysteroid titers was found in female larvae and male larvae fated to develop into the small, hornless morph, but not in males fated to develop into the large, horned morph. Ecdysteroids have been shown to play a major role in inducing changes in gene expression in developing tissues [62] and Emlen and Nijhout [59] therefore suggested that the low ecdysteroid titers observed in female and small male larvae may facilitate development of a hornless morphology in both groups of individuals via a shared endocrine regulatory process. However, ecdysteroid titers have never been replicated in this or any other *Onthophagus* species, and functional tests using ectopic ecdysteroid applications failed to confirm a function of the early ecdysteroid peak in both females and small males (D.J. Emlen, personal communication).

Most recently, transcriptional profiling combined with candidate gene studies have provided additional, albeit somewhat indirect support for a role of endocrine regulators during horned beetle development. For instance, Kijimoto et al. [40] investigated the dynamics of programmed cell death during horn remodeling using cell death-specific bioassays. Integrating findings from a companion microarray study, the authors also showed that several genes known to be associated with ecdysteroid signaling in *Drosophila* were expressed in a manner consistent with a role of ecdysteroid signaling in the regulation of horn-specific programmed cell death. Similarly, a combination of candidate gene expression data [45] and array-based transcriptional profiling [37, 40] has begun to implicate signaling via insulin-like growth factors in the regulation of male horn polyphenism. A subsequent functional analysis of *FoxO* [43], a key growth inhibitor in the insulin pathway, has now provided the first functional data in support of such a role (and see below).

5.4. Endocrine Regulation—Future Directions. Despite the progress summarized above, our understanding of how endocrine mechanisms influence *Onthophagus* development and behavior lag far behind what is known in other insect model systems, such as photoperiodically cued wing dimorphism in crickets (reviewed in [63–65]) and nutritionally cued caste-development in honey bees (reviewed in [66, 67]). Furthermore, most insights, in particular pertaining to juvenile hormone, have been derived solely from hormone manipulation experiments, whose lack of precision and possible pharmacological side effects limit confidence in the results [63]. While these data are consistent with a functional role of JH in the regulation of developmental plasticity in horned beetles, it is worth noting that direct JH titer profiles have yet to be empirically determined across morphs and sexes for any *Onthophagus* species. Furthermore, direct functional interactions between JH and potential targets relevant for the development of alternative male morphs have yet to demonstrated. Consequently, existing models of JH's role in the development and evolution of horn polyphenism remain largely hypothetical and await critical experimental validation. A recent study by Gotoh et al. [68] is now the first to combine observations of hormone titers with manipulation experiments to demonstrate the role of juvenile hormone in promoting mandible length in a stag beetle, a group of beetles closely related to the Scarabaeidae. These findings motivate complementary studies in horned beetles, which now appear particularly feasible given the recent development of many critical resources.

Research advances in determining gene function and comparative gene expression have raised the possibility that work in the near future will be able to ascertain more clearly the role of hormones in *Onthophagus* ontogeny, characterize the interplay between genetic and endocrine regulators of development, and examine their respective evolution across species that have diverged in nature and magnitude of developmental plasticity. For example, RNA interference protocols now work routinely and reliably in *Onthophagus* beetles and have already permitted comparative gene function analyses of a variety of key developmental regulators [39, 41, 42, 69], including components of endocrine pathways [43], providing numerous avenues for future research. Furthermore, next-generation transcriptomes [56] of at least two species have massively increased access to relevant sequence information, with additional transcriptomes of other *Onthophagus* species forthcoming.

5.5. DNA Methylation. The role of DNA methylation in development and developmental plasticity of *Onthophagus* beetles is still poorly understood, but preliminary evidence suggests that these organisms could be an important system in which to better understand the genetic underpinnings and evolutionary consequences of methylation. First, *O. taurus* has joined the ranks of other emerging insect models, including honeybees, aphids, and parasitic wasps, in containing a complete set of methylation machinery, such as the *de novo* methyltransferase (dnmt3) and the maintenance methyltransferase (dnmt1) [56, 70–72]. Second, a pilot

study now suggests that differential methylation is associated with nutritional environment in at least one species, *O. gazella*, and correlated with performance across nutritional environments [73]. This study used a methylation-specific AFLP analysis to survey methylation patterns in family lines derived from a wild population and reared in two different dung types across successive generations. Two major findings emerged. First, methylation state was most heavily influenced by genotype (family line), then rearing environment (dung type), as well as genotype-by-environment interactions (different lines tended to be methylated at different sites when reared on different dung types). Second, methylation state had a significant effect on performance, measured as body size, but in a surprisingly sex- and environment-specific manner: methylation state affected the performance of males (but not females) on cow dung, with the reversed pattern observed on horse dung. Intriguingly, the family line with the greatest flexibility in methylation across environments also showed the highest consistent performance across those environments. Combined, these data are consistent with the hypothesis that facultative methylation underlies adaptive, plastic responses to variation in nutritional environment.

5.6. DNA Methylation—Future Directions. The patterns, function, and phenotypic consequences of DNA methylation in insects have received increased attention in recent years, in part for two major reasons. First, insects were once thought to be devoid of methyltransferase enzymes as found in mammals due to the lack of such machinery in the model insect *D. melanogaster*. Subsequent studies have shown that DNA methylation is also absent in two other major invertebrate models, the beetle *T. castaneum* and the nematode *C. elegans* [74]. Phylogenetic reconstructions now suggests rather than reflecting ancestral states, all three lineages have lost aspects of DNA methylation independently [75]. This now provides a unique opportunity to determine the relevance of DNA methylation in development and evolution of phenotypic diversity, plasticity, and integration. Second, genomic methylation patterns and their impact upon transcription in insects are very different from patterns in other taxa. In mammals, genomes are heavily methylated, both in intergenic and intragenic regions, and are generally associated with gene silencing (reviewed in [76, 77]). In many invertebrates, however, genomes appear to be mosaically methylated, with methylation occurring disproportionately in intragenic regions of constitutively expressed housekeeping genes (reviewed in [77]). Thus, studies in emerging and nonmodel insects could allow further understanding of the function of DNA methylation in transcriptional and posttranscriptional regulation [78].

In establishing a correlation between methylation patterns, diet, and performance (body size), the study by Snell-Rood et al. [73] summarized above raised the intriguing possibility that methylation patterns influenced by diet could mediate plastic responses during development in *O. gazella*. If correct, the incredible diversity in nutritional responses that exist within and among *Onthophagus* species would provide a remarkable opportunity to explore the evolutionary diversification of methylation-mediated nutritional plasticity. Such studies would be especially powerful if methylation patterns could be linked to gene regions (e.g., through the use of bisulfite sequencing approaches) and replicated separately for different tissue types, such as gut, epithelium, and brain tissue.

5.7. Conditional Crosstalk between Developmental Pathways. The growing number of studies investigating the genetic regulation of horned beetle development has begun to provide the first insights into how different developmental pathways and processes might interact, including facultative interactions depending on nutritional conditions. For instance, Kijimoto et al. [44] investigated the role of *Onthophagus doublesex (dsx)*, a transcription factor known to regulate the sex-specific expression of primary and secondary sexual traits in diverse insects (reviewed in [79]). As in other taxa, *Onthophagus dsx* is alternatively spliced into male- and female-specific isoforms, and consistent with findings from other studies, *male-dsx (mdsx)* and *female-dsx (fdsx)* isoforms promote horn development in male and inhibit it in female *O. taurus*, respectively. Remarkably, *O. taurus mdsx* appears to have evolved the additional function to regulate the development of male horn polyphenism, as evidenced by the following observations. First, *mdsx* is expressed at much higher levels in the head and thoracic horn primordia of large males compared to their legs or abdomen, or when compared to any tissue examined in smaller males. Second, *mdsx*RNA*i* dramatically reduced horn expression in large males only, but left smaller males unaffected. Intriguingly, downregulation of *fdsx* in female *O. taurus* resulted in the nutrition-dependent *induction* of ectopic head horns. Combined, these data suggest that sex- and tissue-specific *dsx* expression and function underlie not only sexual dimorphism, but also male polyphenism in horn expression [44]. The utilization of *dsx* as a regulator of both sexual and male dimorphism may also explain the tight coevolution of both patterns of phenotype expression as reported by earlier phylogenetic studies [20], which found that 19/20 instances of gain or loss in sexual dimorphism were paralleled by a corresponding gain or loss of male dimorphism. Exactly how *dsx* expression and function may be coupled to nutritional input, however, is presently unclear, though several promising candidate mechanisms exist.

One such candidate is signaling via insulin-like peptides, a pathway well-known for its role in coupling nutritional variation to a wide range of developmental responses, including growth [80]. Differential expression of members of the insulin signaling pathway during facultative horn development have been documented by both a candidate gene study on the insulin receptor [45] as well as array-based transcriptional profiling [37, 40]. The latter studies identified a particularly intriguing member of this pathway, the *forkhead box subgroup O* gene, also known as *FoxO*, as being differentially expressed across several tissue types and nutritional responses. *FoxO* is a growth inhibitor which is typically activated during poor nutritional conditions. Array-based expression evaluations suggested that, relative to abdominal

tissue of the same individual, the horn primordia of insipient large males showed much lower *FoxO* expression than the horn primordia of small males, consistent with a role of *FoxO* inhibiting horn growth in small, but not large, males. More detailed qRT-PCR-based expression analyses revealed that contrary to these initial inferences, *FoxO* was not differentially expressed in the horn primordia of large and small male *O. taurus*, but was instead overexpressed in the abdomen of large males, in particular in regions associated with the development of genitalia, including testes. In comparison, the abdomen of small males showed reduced *FoxO* expression. Thus, *FoxO* expression differences in the abdomen of large (high) and small (low) males, rather than expression differences in their horn primordia, accounted for the initial array-based expression data.

Recall that small males, while reducing investment into horns, invest heavily into genital development, in particular testes mass and ejaculate volumes [23, 81, 82]. Low *FoxO* expression in presumptive testes tissue is consistent with a role of *FoxO* in the upregulation of testicular growth in small males relative to more inhibited growth, marked by elevated *FoxO* expression, in large males. Subsequent RNAi-mediated depletion of *FoxO* transcripts resulted in extended development time and larger body size at eclosing, consistent with a general disinhibition of growth. Moreover, *FoxO*-RNA*i* disrupted the proper scaling of male body size with copulatory organ size, further supporting that *FoxO* may regulate morph-specific genitalia development in horned beetles [43]. In particular, small male genitalia lost their body size dependence whereas large male genitalia exhibited reduced development. Lastly, *FoxO*-RNA*i* modestly but significantly increased the length of horns in large males. Since *FoxO* is *not* differentially expressed in different horn primordia, this finding suggests that elevated horn development observed in large RNAi males might be a secondary consequence of *FoxO*-RNAi-mediated reduction in genitalia development in those same males. More generally, these results raise the possibility that *FoxO* regulates relative growth and integration of nutrition-dependent development of body size, horn length, and genitalia size.

5.8. Conditional Crosstalk—Future Directions. How different body parts and tissue types communicate with each other during development, and how their varied scaling relationships are enabled along a continuum of body sizes and in the face of nutritional variation, represent long-standing questions at the interface of developmental and evolutionary biology. Answering these questions is critical to our understanding of the nature of phenotypic integration. Horned beetles are now uniquely positioned as a model taxon in which to identify, on one side, nutrition-responsive developmental pathways and the nature of their interactions with other pathways during development of different body parts and tissues. On the other, the diversity of nutritional responses that exist within and among sexes, populations, and species all provide fantastic substrate for future research efforts into the developmental causes and evolutionary consequences of phenotypic integration.

6. Opportunities and Challenges in *Onthophagus* Epigenetics

6.1. Stepping Back. Adaptive developmental plasticity allows organisms to modulate their phenotype in response to external environmental cues, permitting developing organisms to better cope with variation in resource availability, physical environment, and social contexts [2]. Plasticity has been of interest to biologists for over a century, and the increased accessibility of molecular data and technology is now enabling an exploration of the molecular underpinnings of this developmentally, ecologically, and evolutionarily central phenomenon [83]. Epigenetic processes have emerged as a diverse and important collection of mechanisms that mediate the interaction between environment and the genome at multiple scales, enabling the expression of developmentally plastic phenotypes (reviewed in [83, 84]). Studies of traditional model organisms have provided powerful insights into the nature and consequences of epigenetic mechanisms. For example, through murine models we have learned that endocrine disruptors, such as the pesticide vinclozolin, can impact not only an exposed individual, but can lead to physiological and behavioral changes in unexposed offspring and grand-offspring. Furthermore, gene knockout lines have subsequently allowed researchers to elucidate some of the molecular underpinnings of this particular phenomenon, mainly epimutations in the germline (reviewed in [85, 86]). Although model organisms are clearly useful for investigating mechanisms underlying epigenetic processes, studies in these organisms have limited power to investigate the relative significance of epigenetics in naturally occurring populations. For instance, many laboratory strains of model organisms are highly inbred, and likely fail to capture the richness of genetic and epigenetic variation found in natural populations [87]. Similarly, one reason that many model organisms were initially selected is that they are phenotypically resilient to variation in the environment, making the study of plasticity in these organisms difficult [87]. New models will thus be important in addressing questions regarding the role of various epigenetic processes in regulating developmental plasticity.

Here, diet-induced plasticity stands out as a particularly important and widespread form of plastic development. Variation in diet quality represents a challenge faced by most, if not all, heterotrophic organisms, and numerous diverse developmental strategies have evolved to cope with diet variation. Moreover, understanding how diet and genes interact during development to form adult phenotypes is essential to understanding how experiences in early life can promote trajectories toward disease later on. Here, we contrast these findings to what is known about the epigenetic control of plasticity in other emerging and established insect models, and close by highlighting several research areas in which future research on *Onthophagus* beetles could potentially contribute to the growing knowledge of the role of epigenetics in regulating developmental plasticity in general and diet-induced plasticity in particular.

6.2. The Development and Evolution of Shape. Much variation in organismal shape is the product of evolutionary tinkering in the location, allometry, or function of preexisting structures. Thus, the ultimate factors that promote diversification of shape, as well as the proximate underpinnings that coordinate adaptively proportioned traits, are both of fundamental interest in evolutionary-developmental biology. Adaptive radiations, textbook examples of extensive phenotypic variation stemming from a single ancestral phenotype, have long been used as models to address questions of both ultimate and proximate causes of shape evolution (reviewed in [88]). For instance, the flexible stem hypothesis, pioneered by West-Eberhard [2], suggests that phenotypic diversification observed in adaptive radiations results from selection upon ancestral phenotypes made possible by developmental plasticity. Specifically, ancestral plasticity links the expression of conditional phenotypic variants to particular inducing conditions, thus delineating the nature of phenotypic variation that selection can later act upon in different environments. The flexible stem hypothesis therefore has the potential to explain the common observation of very similar phenotypes arising repeatedly yet independently during adaptive radiations (e.g., [11, 89]). More generally, this hypothesis highlights the potential importance of preexisting plasticity in enabling any kind of evolutionary change, including changes in shape and scaling, by creating the potential for facultatively expressed trait variants to become genetically stabilized and accommodated in descendent generations (see also next section).

Onthophagus beetles provide several interesting opportunities to explore the role of plasticity in the diversification of shape and scaling relationships. For instance, adult thoracic horns emerge during development from pupal precursors that originally carried out a very different function [90]. Ancestrally, pupal thoracic horns were resorbed prior to the adult molt, yet descendent species have evolved various ways of partially or fully retaining thoracic horns into adulthood and shaping them into sex- and species-specific weapons. In a subset of species, degree of resorption itself is nutrition dependent [91]. Furthermore, spontaneous retention of thoracic horns also can be observed on occasion in laboratory colonies of species that normally constitutively resorb horns, possibly in response to stressful environmental conditions [40]. This raises the possibility that the diversification of thoracic horn shape and size may have been made possible by harnessing some of the condition dependency of horn retention that existed in ancestral taxa.

A second example involves the well-defined body size thresholds separating alternative horned and hornless male morphs in many species. The exact location of this threshold has diversified greatly among species (Figure 3) as well as some populations. In *O. taurus*, for instance, exotic populations in the Eastern United States, Eastern Australia, and Western Australia have diverged remarkably from their Mediterranean ancestor since introduction approximately 50 years ago [92]. Some of these divergences are similar in magnitude to those observed between well-established species. Intriguingly, body size thresholds are also subject to seasonal or geographic fluctuations in larval nutrition [60, 93] brought about by changes in dung quality and/or changes in the intensity of competition over breeding resources. Again, this raises the possibility that some of the threshold divergences observed between populations and species may have been facilitated initially by conditional responses to altered growth or social conditions.

6.3. Evolution via Genetic Accommodation. Genetic accommodation posits that environmental conditions interacting with developmental processes generate phenotypic transformations that can subsequently be stabilized genetically through selection operating on genetic variation in a populaztion. Genetic accommodation does not require new mutations to occur, but will take advantage of them alongside standing genetic variation. Evolution of novel traits and norms of reaction by genetic accommodation have been demonstrated repeatedly and convincingly in artificial selection experiments (reviewed in [5]). Similarly, studies on ancestral plasticity and cases of contemporary evolution provide growing evidence consistent with a role of genetic accommodation in diversification of natural populations (e.g., [11, 94]). However, exactly how important environmental induction really is in the origin and diversification of novel phenotypes remains largely to be determined, in particular in natural populations. Similarly, the proximate mechanisms underlying plasticity-mediated diversification are largely unknown.

The preceding section highlighted two examples, the diversification of thoracic horn size and shape and the diversification of size thresholds, where research on horned beetles has the potential to generate valuable case studies on the mechanisms and consequences of genetic accommodation of initially environment-induced phenotypic variation. Many additional opportunities exist. For instance, female *Onthophagus* facultatively engage in intra- and possibly interspecific brood parasitism [33]. Interspecific brood parasitism is the dominant reproductive strategy in other dung beetle genera, raising the possibility that it may have evolved initially as a conditional alternative that became subsequently stabilized in a subset of descendent lineages [13]. Similarly, extent of maternal care (brood ball size and depth of burial) vary greatly among females, in part as a function of female body size and thus the nutritional conditions a mother herself experienced when she was a larva. Importantly, *O. taurus* populations obtained from different latitudes within the Eastern US have diverged significantly in the extent of investment mothers provide, again raising the possibility that some of these divergences were enabled initially by plastic responses to environmental conditions (Snell-Rood and Moczek, unpublished data). As highlighted in the last section, *Onthophagus* beetles also provide great opportunities to begin exploring some of the proximate genetic, developmental, and physiological mechanisms that may facilitate accommodation of conditionally expressed phenotypes.

6.4. The Origin of Novel Traits. How complex novel traits, such as the eye, the firefly lantern, or the turtle shell, originate is among the most fundamental yet unresolved questions in evolutionary biology [46]. Evolution operates within

a framework of descent with modification—anything new and novel must have descended from something old and ancestral. Yet novelties are generally defined as lacking obvious correspondence, or homology, to preexisting traits. How then, do novel traits originate from within the confines of ancestral variation? Studies of epigenetic mechanisms in general, and those focusing on non-model organisms in particular, have likely much to offer to address this question.

Traditional developmental biology and evo-devo are focused on the identification of genes and gene networks that regulate development and developmental outcomes. At times, this view is expanded to make room for environmental influences by viewing gene function as environment dependent, and viewing genotypes as possessing a reaction norm—that is, the range of phenotypes produced across a range of environmental conditions. The study of epigenetics takes a radically broader and far less gene-centric view. Here, phenotypes (from nucleotide sequences to cells, tissues, organisms, and social groups) emerge as the products of developmental processes to which genes contribute important interactants. In this view, genes are critical and genetic changes can make important differences, but they do not make traits or organisms. Instead, those emerge through the actions of development. This more integrative perspective has many important consequences, three of which are especially critical here. First, epigenetic processes facilitate the production of integrated and functional phenotypes through a wide variety of mechanisms operating well above the sequence level [95, 96]. Second, the integration put in place by epigenetic mechanisms allows development—when confronted with environmental perturbations—to give rise to possibly novel but nevertheless integrated, functional, and on occasion adaptive phenotypes. Third, the same integration enabled by epigenetic mechanisms allows random and modest genetic change to give rise to nonrandom, functional phenotypic changes. In short, the integrity and functionality of phenotypes in development and evolution are facilitated through the chaperoning action of epigenetic mechanisms. As such epigenetics likely plays a central role in facilitating innovation and diversification in nature.

Onthophagus beetles have begun to contribute to our understanding of innovation through epigenetic mechanisms through a series of studies focused on the origin and diversification of horns, themselves novel structures lacking any obvious homology to other insect traits (reviewed in [97]). Through a combination of observational, comparative, and manipulation studies it has now become clear that at least some horns originated from pupal-specific structures that originally functioned in completely unrelated contexts (reviewed in [13]). Innovation was enabled initially through the potentially accidental maintenance of normally pupal-specific projections into the adult stage. Similar events can be observed at low frequency in laboratory cultures of species lacking adult horns [40]. Diversification between species, sexes, and morphs was then made possible through the recruitment of preexisting developmental pathways and their targets into a novel context, for instance enabling morph-specific elaboration of horns via preexisting endocrine mechanisms [59, 61, 92, 98] or sex-specific horn expression via sex-specific activation of programmed cell death [40]. Exactly how such recruitment was made possible and by what kind of genetic and environmental variation (and what interactions between them) remain unclear, however, posing some of the many intriguing question for future research in these organisms and the field in general.

7. Conclusions

The study of epigenetic mechanisms in development and evolution promises to fill an otherwise abstract genotype-phenotype map with biological reality. Epigenetic mechanisms feature especially prominently in developmental plasticity and its evolutionary consequences. We hope to have shown in this review that the study of horned beetles provides rich and promising opportunities to investigate the role of epigenetics in the evolution of adaptations, phenotypic diversification, and the origin of novel traits. The remarkable degree of plasticity inherent in the biology of horned beetles, combined with the stunning phenotypic diversity that exists both within and among species, and the growing experimental toolbox available for a subset of these organisms makes horned beetles a promising emerging model system in the study of epigenetic mechanisms, their nature, causes, and consequences.

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Research Article

Ontogenetic Survey of Histone Modifications in an Annelid

Glenys Gibson,[1] Corban Hart,[1] Robyn Pierce,[1] and Vett Lloyd[2]

[1] *Department of Biology, Acadia University, 33 Westwood Avenue, Wolfville, NS, Canada B4P 2R6*
[2] *Department of Biology, Mount Allison University, 63B York Street, Sackville, NB, Canada E4L 1G7*

Correspondence should be addressed to Glenys Gibson, glenys.gibson@acadiau.ca

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Histone modifications are widely recognized for their fundamental importance in regulating gene expression in embryonic development in a wide range of eukaryotes, but they have received relatively little attention in the development of marine invertebrates. We surveyed histone modifications throughout the development of a marine annelid, *Polydora cornuta*, to determine if modifications could be detected immunohistochemically and if there were characteristic changes in modifications throughout ontogeny (surveyed at representative stages from oocyte to adult). We found a common time of onset for three histone modifications in early cleavage (H3K14ac, H3K9me, and H3K4me2), some differences in the distribution of modifications among germ layers, differences in epifluorescence intensity in specific cell lineages suggesting that hyperacetylation (H3K14ac) and hypermethylation (H3K9me) occur during differentiation, and an overall decrease in the distribution of modifications from larvae to adults. Although preliminary, these results suggest that histone modifications are involved in activating early development and differentiation in a marine invertebrate.

1. Introduction

One of the central questions in biology is how differences in gene expression during development lead to the generation of form. Epigenetic mechanisms such as histone modifications activate or silence gene expression and thereby provide rapid, reversible mechanisms that regulate gene expression in embryonic development. The importance of histone modifications in development has been extensively studied in model systems. As this approach is gradually extended to nonmodel species, histone modifications are being discovered as mechanisms that are highly conserved in a wide variety of eukaryotes and critically important in regulating fundamental developmental processes, including meiosis [1], cell differentiation [2], organ development in plants [3], sexual and asexual reproduction in fungi [4], genomic imprinting in plants and insects [5], and X-inactivation in mammals [6].

Despite the clearly established importance of histone modifications in the development of many eukaryotes, they have received almost no attention in the development of benthic marine invertebrates. Benthic marine invertebrates represent an exciting group for epigenetic research as they not only are morphologically diverse as adults, but their larvae are morphologically and behaviorally distinct from adults and form the basis for an impressive diversity of life-history patterns. Our objectives are to determine if histone modifications can be detected in a marine worm using immunohistochemistry, if modifications differ among differentiating tissues, and if changes in modifications correlate with ontogenetic transitions. We chose the worm *Polydora cornuta* Bosc, 1802 (Annelida, Spionidae) for this study. *P. cornuta* is a small, opportunistic detritivore that is common in intertidal mudflats and has a wide distribution in temperate and subtropical coastal areas [7, 8]. Fertilization in *P. cornuta* is internal, and females deposit zygotes in a string of egg capsules that they brood in their mud tubes. Larval development for this species has been described by several authors and is strongly influenced by the presence of nurse eggs in the egg capsules [8–12]. Some broods contain only a few nurse eggs and young hatch as small, swimming larvae that feed on phytoplankton (a trophic mode termed planktotrophy). In other broods, most eggs are nondeveloping nurse eggs which provide extraembryonic nutrition for encapsulated larvae (termed adelphophagy), and as a result, young hatch as large, advanced larvae which

settle soon after hatching. Although two developmental morphs are observed for *P. cornuta*, the present study focuses on epigenetic similarities between morphs. Our goal is to establish a foundational understanding of changes in the epigenome throughout development, as the first step in a larger project that investigates the potential for histone modifications to influence plasticity in larval development in this species.

We surveyed histone modifications throughout ontogeny using immunohistochemistry. The survey included oocytes, embryos, early larvae, and adults. This allowed us to correlate histone modifications with specific developmental events (e.g., completion of meiosis, tissue formation) and life history stages (i.e., embryos, larvae, and adults). We focused on histone modifications as histones are among the most highly conserved proteins in eukaryotes [13]. Their modifications are equally conserved and are an important aspect of epigenetic gene regulation in many different organisms [5, 14–17]. We used antibodies for core histones as well as for four commonly studied histone modifications including antihistone H3 acetyl Lys14 (referred to in this paper as H3K14ac), antihistone H3 dimethyl Lys4 (or H3K4me2), antihistone H3 monomethyl Lys9 (or H3K9me), and antihistone H4 dimethyl Lys20 (or H4K20me2). Generally, H3K14ac is associated with transcription, as acetylation loosens the nucleosomes and allows transcription factors to bind to promoter regions; H3K9me and H3K4me2 are associated with both transcription and gene silencing; H4K20me2 is associated with gene silencing [2, 18–20]. Because we do not know the transcriptional outcome of a change in histone modifications in *Polydora cornuta*, and also because of the overall complexity of the epigenome, we follow the advice of Turner [18, 21] and interpret an ontogenetic change in histone modifications as a change within the histone code, rather than a specific indicator of gene expression. We show that histone modifications were detected throughout ontogeny in *Polydora cornuta*. Similarities in the distribution of three histone modifications suggest that certain phases of development (i.e., early cleavage and possibly metamorphosis) represent transition points during which widespread changes in histone modifications occur.

2. Materials and Methods

2.1. Collection and Culture. Adult *Polydora cornuta* were collected from intertidal mudflats at West Marsh, Halifax Co., Nova Scotia (N44.6456, W-63.3744) in early summer (May to July) of 2010 and 2011. Adults were cultured in 250 mL Pyrex crystallizing dishes which contained enough sand to cover the bottom. Each dish contained approximately 10–16 worms, including some males to ensure sperm availability. Cultures were immersed in seawater at approximately 14-15°C, provided with continuous aeration, and maintained on a 15 : 9 LD photoperiod.

After spawning, broods were removed from the females' tubes and cultured. As *P. cornuta* is poecilogonous [11], broods were identified under a compound microscope by determining the trophic morph of young (planktotrophy or adelphophagy) and counting the number of nurse eggs per egg capsule. We use the term *P-brood* to refer to broods in which there are few or no nurse eggs (<5% of the total number of eggs per brood), most eggs develop (approximately 80 embryos/capsule) and young hatch as small (3 to 5 segments), planktotrophic larvae. The term *A-brood* is used for broods in which most eggs (>90%) are nurse eggs, few young develop (approximately 5/capsule), and most young are adelphophagic while in the egg capsule (data from MacKay and Gibson) [11]. Individual egg capsules were placed in 3.5 mL Falcon well plates containing filtered seawater with antibiotics (1000 mL seawater : 1 mL penicillin-streptomycin; Sigma P4333). Well plates and culture water were changed daily until broods reached desired ontogenetic stages. Stages examined were oocyte, cleavage (2- to 32-cell stages), blastula, gastrula, trochophore, metatrochophore, early larva (3-4 chaetigers), and for adelphophagic morphs only, advanced larvae (5–12 chaetigers).

Forty-eight A-broods and sixteen P-broods were examined. Unequal sample sizes reflect the fact that P-broods were relatively uncommon in the West Marsh population. Approximately eight egg capsules were fixed per brood per ontogenetic stage. We processed two to three egg capsules per assay and examined all embryos per capsule for consistency in epifluorescence (i.e., presence and relative intensity of epifluorescence in specific cells or tissues). A complete examination was done for young from both A- and P-broods at all ontogenetic stages, and observations of histone modifications common to both morphs are presented.

2.2. Fixation and Immunohistochemistry. Embryos and larvae of both morphs were fixed and labeled with commercially available primary antibodies for histones and histone modifications. Egg capsules were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 45 minutes on ice, rinsed in PBS, dehydrated to absolute methanol, and stored at −20°C for up to two months.

Immunohistochemistry was performed using a procedure modified from Sagawa et al. [22] and kindly provided by Shiga. Specimens were rehydrated to phosphate buffered saline containing 1% (v/v) Tween 20 (PT), and under a dissecting microscope, a small hole was then torn in each egg capsule to allow further reagents to enter. Specimens were blocked in PT containing 2% (w/v) bovine serum albumin (2% BSA/PT) for 2 hours at 4°C and then incubated in one of the following primary antibodies (1/500) in 2% BSA/PT overnight at 4°C. Five primary antibodies were used: antihistone, histone 1 and core histones (monoclonal mouse, Millipore MAB052), antihistone H3 acetyl Lys14 (monoclonal rabbit, Abcam ab52946), antihistone H3 dimethyl Lys4 (polyclonal rabbit, Abcam ab32356), antihistone H3 monomethyl Lys9 (polyclonal rabbit, Abcam ab9045), and antihistone H4 dimethyl Lys20 (polyclonal rabbit, Abcam ab9052-25). Histone proteins and modifications are highly conserved [13], and the primary antibodies used here have also been used to detect the same histone modifications in a wide variety of eukaryotes, ranging from yeast to plants

[23–25]. Specimens were then washed ten times over 1 hour with PT. Secondary antibodies were FITC-goat anti-rabbit IgG (1/50, Invitrogen 65-6111) for specimens incubated with H3K14ac, H3K4me2, H3K9me, and H4K20me2; TRITC-goat anti-mouse IgG (1/50; Invitrogen T-2762) for specimens incubated with antihistone. All specimens were also colabeled with DAPI (1/500; Sigma 32670) in 2% BSA/PT for 2 hours at 4°C. Specimens were washed 12 times over 1 hour with PT and mounted onto glass slides using Vectashield (Vector Laboratories H-1000). Coverslips were sealed with nail polish.

Gravid females were examined in whole mounts of individual segments ($n = 8$ females) or in paraffin section ($n = 5$). Whole-mounted segments were processed as for embryos. For paraffin sectioning, females were placed in filtered seawater for 24 h to allow the gut to void of sand and then fixed and dehydrated as described for egg capsules. After females were embedded in paraffin, they were sectioned at 10 μm and 5-6 sections from each female placed on poly-L-lysine coated slides. Sections were deparaffinized in xylene, rehydrated to PT, and processed as described for egg capsules. Sections were ringed with a liquid blocker pen to avoid loss of reagents, and the immunohistochemistry protocol was done in a humid, sealed chamber.

Negative controls were processed using the typical protocol but with the primary antibody replaced with 2% BSA/PT during the first incubation, followed by labeling as usual with FITC- or TRITC-conjugated secondaries during the second incubation. Epifluorescence was not detected in the negative controls.

Samples were examined using a Zeiss Axioplan II compound fluorescence microscope and micrographs taken using an SPOT-2 camera (Diagnostic Instruments, Inc.). Micrographs were adjusted for size and contrast using Corel PhotoPaint 11.0.

3. Results and Discussion

3.1. Detection of Histones (Antihistone 1 and Core Histones). Epifluorescence of TRITC-conjugated antihistone indicated that histones were present in the nuclei of all cells throughout development (Table 1). TRITC epifluorescence colocalized with that of DAPI, providing evidence that the TRITC signal was restricted to nuclear chromatin (Figures 1(a) and 1(b)). TRITC-conjugated antihistone was detected in all cells of embryos and larvae throughout development and is shown here for a gastrula from a P-brood (Figure 1(c)). These results demonstrate that this antibody appears to recognize and bind to worm antigens, and also that we can detect histones in all blastomeres, even at early stages when the blastomeres are very yolky.

3.2. Antihistone H3 Acetyl Lys14. Acetylation of H3K14 was not detected in oocytes located within the coelom of gravid females although it was detected in a few of the follicular cells associated with the oocytes (Figures 2(a) and 2(b)). The earliest embryo that was surveyed for H3K14ac was at a two-cell cleavage stage. H3K14ac was not detected in blastomeres although it was evident in the polar bodies (Figures (2(c)–2(e); Table 1). Four-cell embryos were similar: H3K14ac was not detected in blastomeres, but it was detected in polar bodies (not shown). H3K14ac was first detected in blastomeres in embryos that were entering the eight-cell stage. In these embryos, H3K14ac was present in all blastomeres except the large D macromere (Figures 2(f)–2(h), shown in an embryo in which the D blastomere is undergoing mitosis; H3K14ac was detected in mitotically active cells in other embryos, described below). Acetylation of H3K14 was also absent in the D blastomere of 12- to 16-cell embryos (not shown). By late cleavage (roughly 32 cells), H3K14ac appeared to be present in most, if not all, blastomeres although epifluorescence in the inner, yolky macromeres was sometimes difficult to observe.

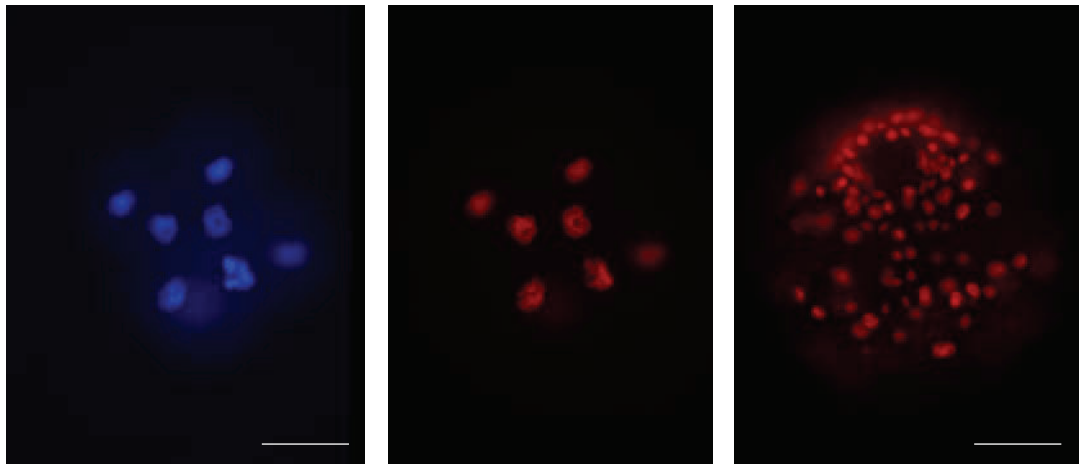
In gastrulae, H3K14ac was detected as weak epifluorescence throughout the epidermis and as strong epifluorescence in a few cells associated with the mouth (shown below). In trochophores and early larvae, bright epifluorescence was detected in the mouth and also on the ventral surface and pygidium (Figures 3(a)–3(c), shown for a three-chaetiger adelphophagic larva). As larvae developed, this pattern of H3K14ac was retained: weak epifluorescence was detected throughout the epidermis (Figures 3(d)–3(f), dorsal view shown in a four-chaetiger adelphophagic larvae), and strong epifluorescence was present in a few cell lineages, specifically the mouth, ventral cells, and pygidium. Bright epifluorescence was also observed in cells during mitosis, indicating that H3K14ac persists or is restored through karyokinesis (Figures 3(g) and 3(h)). Older larvae had a similar distribution of H3K14ac-positive cells (not shown). In contrast, gravid females had detectable levels of H3K14ac in relatively few cells, including scattered cells of the epidermis, nephridia, and chaetal sacs (Figures 3(i) and 3(j)). Importantly, the differential brightness observed in specific cell lineages was consistent among young from the same egg capsules and across multiple (in some cases, up to four) broods per ontogenetic stage.

Histone acetylation is generally associated with transcription and in eukaryotes is common in undifferentiated cells, while differentiated cells often contain hypoacetylated chromatin [26]. Our observations fit with that general pattern as H3K14ac was acquired in most blastomeres (i.e., all except the D macromere) in early development (around the 8-cell stage), rapidly growing larvae had detectable levels of H3K14ac throughout the epidermis, and the relative number of H3K14ac-positive cells decreased in adults. One result that differed from that reported in model systems (specifically, mammals and *Drosophila*) is the lack of detectable H3K14ac in oocytes and early embryos (2- to 4-cell stages). Histone acetylation is important in oogenesis in mammals [1] and while lack of H3K14ac is reported in mammalian zygotes, H3K14ac is often restored in cleavage [27]. H3K14ac is also important in meiosis in oocytes of *Drosophila* and has been shown to vary at different points in the meiotic cycle [28]. Our detection of H3K14ac in polar bodies is consistent with Endo et al. (2005) who detected a strong signal of histone acetylation in polar bodies in mammals [1]. In *P. cornuta*, it appears that H3K14ac is important in at least some aspects

Table 1: Summary of histones and epigenetic modifications during development of *Polydora cornuta*. The modifications listed were common to both morphs (i.e., were observed in both adelphophagic and planktotrophic young). + = modification present; − = modification absent; blank = no sample. h = head, m = mouth, p = pygidium, vc = ventral cells.

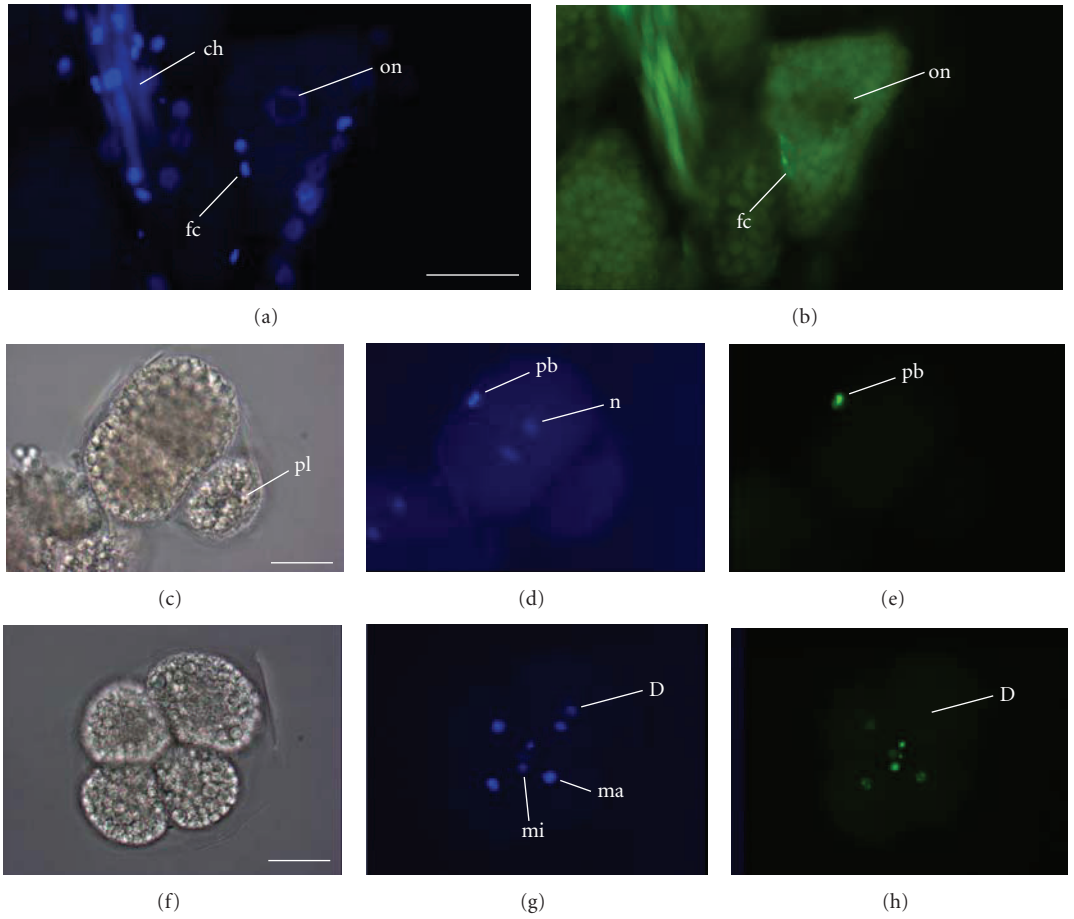
Modification	Developmental stage										
	Oocyte	2–4 cells	8 cells	32 cells	Blastula	Gastrula	Trochophore	Metatrochophore	3 chaetigers	5–12 chaetigers	Adult
Histones	+	+	+	+	+	+	+	+	+	+	+
H3K14ac	−	−	+ (not D)	+	+	+* (m)	+* (m, vc, p)	+* (m, vc, p)	+* (m, vc, p)	+* (m, vc, p)	Some tissues
H3K9me	−	−	+	+	+* (h)	+* (h)	+	+	+	+	Some tissues
H3K4me2	−		+	+	+	+	+	+	+	+	Some tissues
H4K20me2							−		−		−

* interpreted as hyperacetylation or hypermethylation in indicated cell lineages (see text).



(a) (b) (c)

Figure 1: Distribution of core histones in embryos of *Polydora cornuta*. (a, b) Companion micrographs of an eight-cell embryo from an A-brood, showing the distribution of DNA (a, DAPI) and histones (b, TRITC-conjugated anti-histone). (c) Gastrula from a P-brood labeled with TRITC-conjugated antihistone. Scale bars = 50 μm.



(a) (b)

(c) (d) (e)

(f) (g) (h)

Figure 2: H3K14 acetylation in early development of *Polydora cornuta*. (a, b) Companion micrographs of an oocyte inside the coelom of a female in paraffin section showing nuclear DNA (a, DAPI) and nuclei that are acetylated at H3K14 (b, FITC-conjugated anti-H3K14ac). The remaining images are bright field (left) and companion images showing DNA (DAPI, middle) and nuclei with H3K14ac (FITC-conjugated anti-H3K14ac, right). (c–e) Two-cell stage with polar bodies and a polar lobe (P-brood). (f–h) Eight-cell embryo, shown from the animal pole (P-brood). ch: chaetae, D: D macromere, fc: follicular cell, ma: macromere, mi: micromere, n: nucleus, on: oocyte nucleus, pb: polar body, and pl: polar lobe. Scale bars = 50 μm.

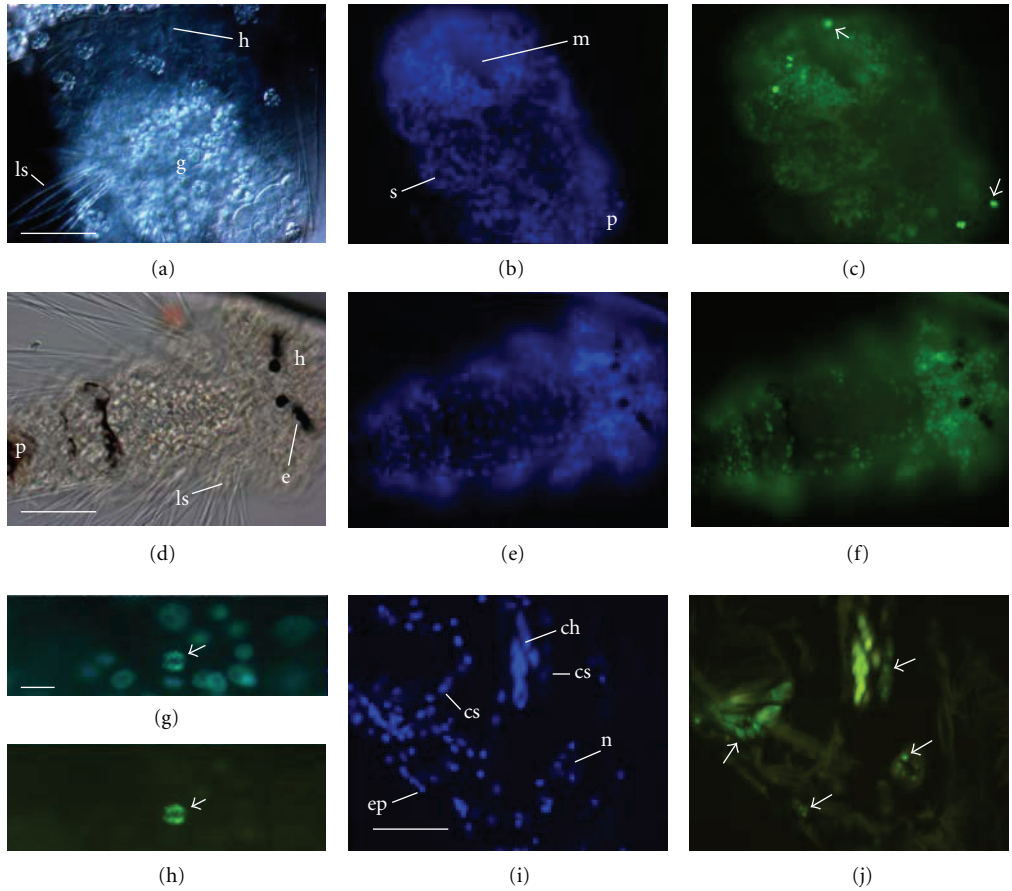


Figure 3: H3K14 acetylation in larvae and adults of *Polydora cornuta*. (a–c) Companion micrographs of the ventral surface of a three-chaetiger larva (A-brood) in bright field (a), showing nuclear DNA (b, DAPI), and showing nuclei that are acetylated at H3K14 (c, FITC-conjugated anti-H3K14ac). Note the strong epifluorescence that is typical of cells of the mouth and pygidium (small arrows in c). (d–f) Dorsal view of a four-chaetiger larva (A-brood) in bright field (d), with DAPI (e) and with FITC-conjugated anti-H3K14ac (f). (g, h) Images of an ectodermal cell from a gastrula (P-brood) that is undergoing mitosis, double labeled with DAPI (g) and FITC-conjugated anti-H3K14ac (h). (i, j) Paraffin section through the body wall of a female that is double labeled with DAPI (i) and FITC-conjugated anti-H3K14ac (j). cs: chaetal sac, ch: chaetae, e: eye, ep: epidermis, g: gut, h: head, ls: larval spines, m: mouth, n: nephridium, s: segment, and p: pygidium. Arrows indicate the presence of FITC-conjugated H3K14ac in the indicated cells. Scale bars = 10 μm (g, h) or 50 μm (a–f, i, j).

of meiosis, as it was detected in polar bodies and suggests a potential pathway by which polar bodies may be determined.

The onset of acetylation of H3K14 occurred in early embryos (8-cell stage), when it was detected in all blastomeres except for the D macromere; this pattern was retained at least through the sixteen-cell stage but was difficult to follow in later development (from 32 cells on) given the techniques used here. In polychaetes, the D macromere gives rise to most of the segmented tissue including ectoderm and mesodermal derivatives [29]. Lack or delayed onset of acetylation of H3K14 in the D blastomere suggests a delay in transcription of some genes within this lineage, but this remains to be confirmed.

3.3. Antihistone H3 Monomethyl Lys9. H3K9me was not detected in oocytes located within the coelom of gravid females (Figures 4(a) and 4(b)). The earliest stage of development in which H3K9me was detected was the eight-cell stage where it was detected in all blastomeres (Figures 4(c)–4(e); note that the distribution of H3K9me is shown for planktotrophic embryos and larvae throughout this section; Table 1). In blastulae, H3K9me was detected as weak epifluorescence in all blastomeres but gave a characteristically bright signal in a ring of cells around the presumptive head (Figures 4(f)–4(h)), a distribution pattern that was retained in gastrulae. We interpreted this bright signal as due to increased histone modification (i.e., hypermethylation) rather than altered nuclear structure (i.e., micromeres giving brighter signal in their nuclei simply because they were small and concentrated). Our interpretation is founded on the argument that other micromeres (e.g., the two in the centre of the blastula in Figures 4(g) and 4(h)) also had small and concentrated nuclei, but did not possess the same bright signal as those forming the ring around the presumptive head.

In trochophores and metatrochophores, moderate levels of H3K9me were detected throughout the ectoderm with bright epifluorescence in the head and laterally in the region

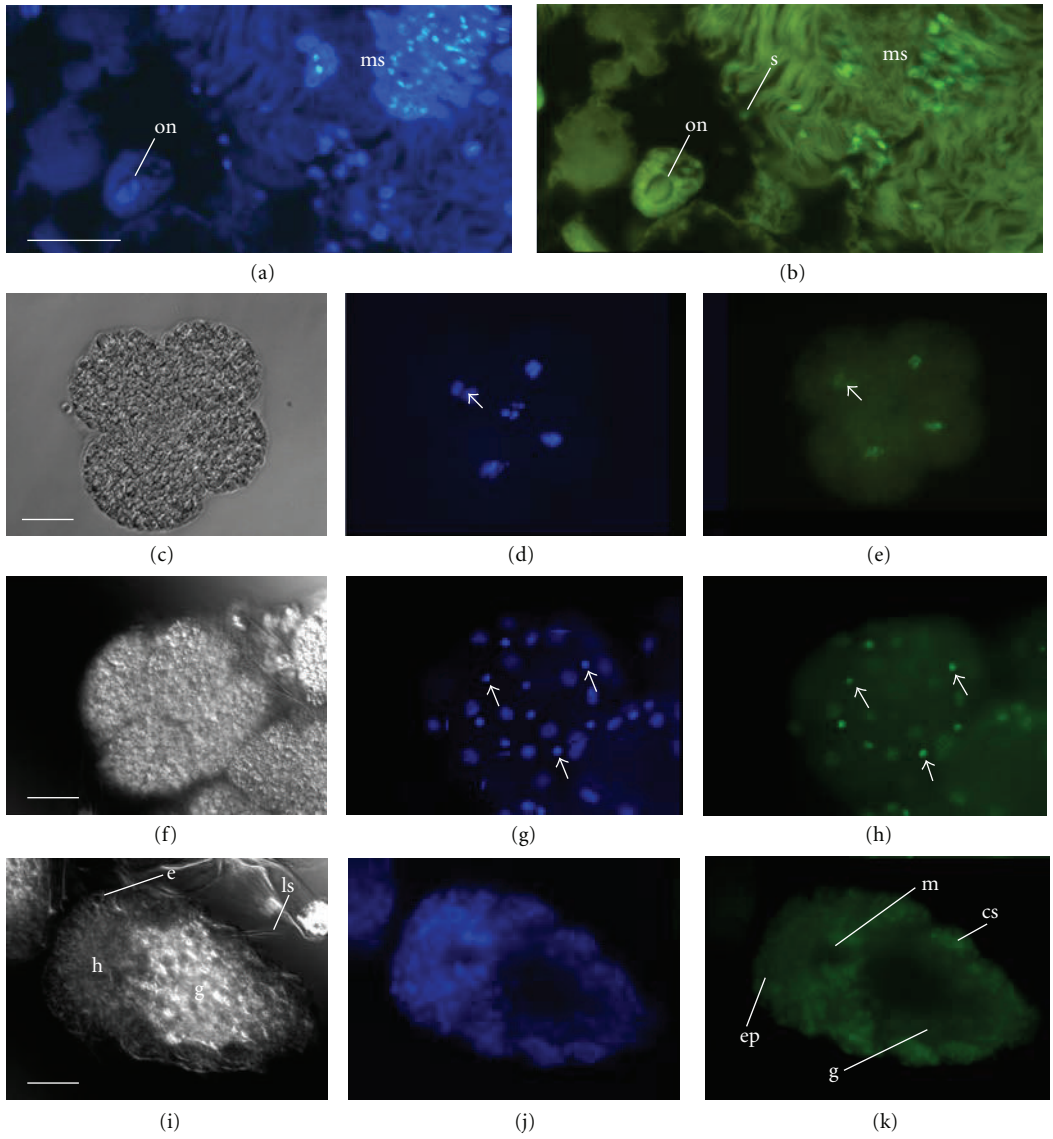


Figure 4: H3K9 monomethylation in *Polydora cornuta*. (a, b) Companion micrographs of an immature oocyte inside the coelom of a female in paraffin section showing nuclear DNA (a, DAPI) and nuclei that are monomethylated at H3K9 (b, FITC-conjugated anti-H3K9me). The remaining images are bright field (left) and companion images showing DNA (DAPI, middle) and nuclei with H3K9me (FITC-conjugated anti-H3K9me, right) for embryos and larvae from planktotrophic broods. (c–e) Eight-cell embryo shown from the animal pole. The small arrow indicates the presence of H3K9me in a dividing blastomere. The micromeres are out of the plane of focus and are difficult to see in (e). (f–h) Blastula, shown from the animal pole. The small arrows indicate the ring of hypermethylated micromeres surrounding the presumptive head. (i–k) Ventral view of a three-chaetiger larva. H3K9me is visible throughout the epidermis, chaetal sacs, and gut. cs: chaetal sac, e: eye, ep: epidermis, g: gut, h: head, ls: larval spines, m: mouth, ms: muscle, on: oocyte nucleus, and s: septa. Scale bars = 50 μm.

of the presumptive chaetal sacs. Epifluorescence was also detected in the mesoderm and endoderm (demonstrated below). Early larvae had moderate FITC signal throughout the epidermis, chaetal sacs and mesoderm, and weak epifluorescence in the gut (shown in a three-chaetiger larva; Figures 4(i)–4(k)), a pattern of distribution that was retained at least to the six-chaetiger stage. In gravid females, H3K9me was detected in ectodermal and mesodermal derivatives including many epidermal cells, nephridia, muscle, and a few cells of the chaetal sacs (shown for muscle and septa; Figures 4(a) and 4(b)).

These observations suggest that the distribution of H3K9me is in many ways similar to H3K14ac; H3K9me was not detected in oocytes, it was first detected in early cleavage, and it was widely distributed in cells of embryos and larvae and varied in intensity among cell lineages (e.g., in the blastula stage), and H3K9me-positive cells decreased in distribution in adults. Here, we interpret the presence

or relative intensity of H3K9me as indicating a change in histone modifications (onset, loss, or hypermethylation) rather than specifically indicating gene activation or repression, as H3K9me has been associated with both [19, 25, 30, 31]. Differences among cell lineages in epifluorescence intensity were consistent from blastulae to larvae, with low levels of epifluorescence throughout the ectoderm and bright signal in some nuclei of the head. This suggests that hypermethylation occurs in these cells and may affect differential levels of gene expression as differentiation occurs. Adults showed a decrease in methylation of H3K9; in larvae, H3K9me was broadly found in most, if not all, cells of the epidermis, mesoderm, and, gut but in adults, H3K9me was restricted to relatively few cells of the epidermis and mesodermal derivatives. This suggests that a transition in histone modifications occurs between larvae and adults that involves a shift from detectable levels of H3K9me in most cells to methylation in few cell lineages only and is consistent with loss of methylation of H3K9 during differentiation that has been observed elsewhere [19].

3.4. Antihistone H3 Dimethyl Lys4. H3K4me2 was not detected in oocytes located within the coelom of gravid females (Figures 5(a) and 5(b)). H3K4me2 was detected in both micromeres and macromeres in early cleavage although epifluorescence was difficult to detect in the macromeres because of the large amount of yolk in these cells (six- to eight-cell embryos; Figures 5(c)–5(e); Table 1). In blastulae and gastrulae, H3K4me2 was present in most, if not all, blastomeres. At the trochophore stage, H3K4me2 was detected throughout the ectoderm and also in some of the deeper cells of the underlying mesoderm (demonstrated below). The distribution of H3K4me2 was similar in early larvae (i.e., three to four chaetigers in length) and was generally detected throughout the epidermis and underlying muscle and was also detected as weak epifluorescence in the developing gut (Figures 5(f) and 5(g)). Only adelphophagic larvae were observed at later ontogenetic stages. In later larval development (i.e., five chaetigers) and at hatching (roughly twelve chaetigers), adelphophagic larvae still had strong FITC signal associated with the epidermis and in the underlying muscle (not shown). In gravid females, H3K4me2 was detected throughout the epidermis, septa, and muscle but not the gut (shown for septa; Figure 5(a) and 5(b)).

These observations suggest that H3K4me2 was similar in distribution to H3K14ac and H3K9me: H3K4me2 was not detected in oocytes, had an onset in early cleavage, was broadly distributed in embryos and larvae, and was detected in adults in specific tissues only. The major difference between H3K4me2 and the modifications described above was the lack of hypermethylation in specific cell lineages. These observations suggest that widespread changes in H3K4me2 occur at roughly the eight-cell stage (i.e., onset) and possibly also with metamorphosis (i.e., change in tissue-specific expression). As with H3K9me, the specific functional implications of H3K4me2 are not yet known for *P. cornuta*, as dimethylation of H3K4 is associated with varying transcriptional activity depending on interactions with other histone modifications as differentiation occurs [20, 32].

3.5. Antihistone H4 Dimethyl Lys20. We attempted to detect H4K20me2 in female tissue (specifically the body wall and palps), in trochophores, and in three-chaetiger larvae. H4K20me2 was not detected at any of these ontogenetic stages, and therefore, our search for it was discontinued.

3.6. Changes in Histone Modifications throughout Development. In many metazoans, histone modifications are reprogrammed during meiosis and embryos gradually acquire modifications during differentiation [33]. Our results suggest that this general pattern also occurs in polychaetes. H3K14ac, H3K9me, and H3K4me2 were not detected in oocytes, but all three were detected in early cleavage embryos (at roughly the eight-cell stage in the planktotrophic morph), had a widespread distribution in larvae within the derivatives of certain germ layers, and were detected in adults but in specific tissues only.

Collectively, these observations suggest that global changes in gene expression occur at about the eight-cell stage with the onset of three modifications that affect gene transcription (i.e., H3K14ac, H3K9me, and H3K4me2). The onset of histone modifications was consistent but not uniform among blastomeres; for example, the onset of H3K14ac was delayed in the D blastomere relative to other cells of the same embryo. While the importance of histone modifications in the early development of marine invertebrates has not received much attention, the importance of histone variants has been demonstrated by Arenas-Mena and colleagues for the polychaete *Hydroides elegans* and the sea urchin *Strongylocentrotus purpuratus* [34]. Both species express the histone variant H2A.Z in early cleavage where it is specifically associated with undifferentiated cells, and as cellular differentiation occurs in larvae, the expression of H2A.Z declines [34]. Our observations suggest that histone modifications (in addition to histone variants) may be associated with determination of cell fate in polychaetes, given the common onset of three modifications in early cleavage in *P. cornuta*. Additionally, histone modifications are associated with differentiation as they were also detected in specific larval and adult tissues.

The potential for histone modifications to be associated with tissue differentiation is supported by the presence of hyperacetylation of H3K14 in cells of the mouth and pygidium of larvae, of hypermethylation of H3K9 in cells of the presumptive head of embryos (blastulae and gastrulae), and of the restriction of some modifications to specific organs (e.g., H3K9me and H3K4me2 were detected in the larval gut, but H3K14ac was not). Thus, lineage-specific modifications also occur and suggest that histone modifications may influence not only early specification of cell fate but also cell differentiation as tissues (such as the gut) specialize and become functional in larvae.

All three histone modifications had patterns of distribution that differed between larvae and adults. In larvae, H3K14ac was broadly distributed throughout the epidermis,

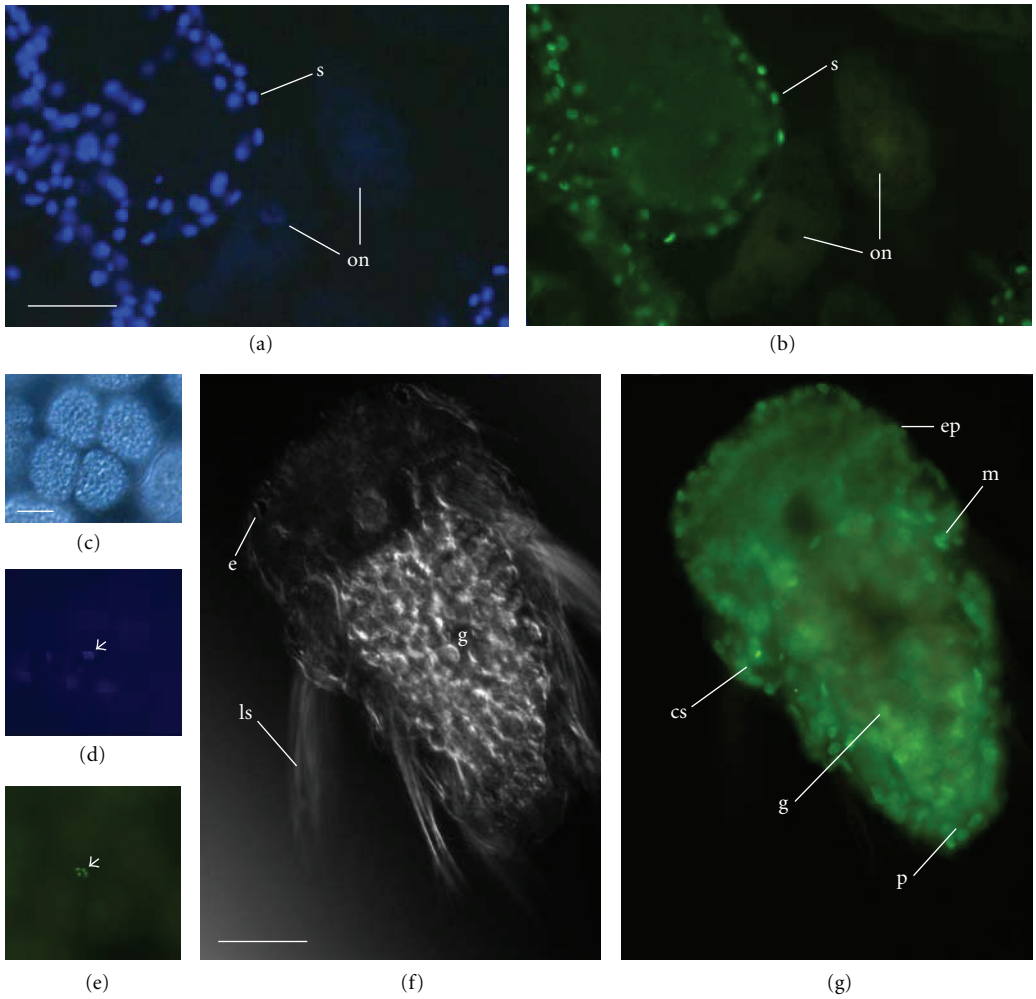


Figure 5: H3K4 dimethylation in *Polydora cornuta*. (a, b) Companion micrographs of an oocyte inside the coelom of a female in paraffin section showing nuclear DNA (a, DAPI) and nuclei that are dimethylated at H3K4 (b, FITC-conjugated anti-H3K4me2). Note that H3K4me2 was not detected in the oocyte nucleus. (c–e) Early cleavage stages of a planktotrophic embryo in bright field (c) and with epifluorescence for DAPI (d) and FITC-conjugated H3K4me2 (e). The small arrows indicate the presence of H3K4me2 in the micromeres. (f, g) Three-chaetiger larva from a P-brood in bright field (f) and with epifluorescence for FITC-conjugated H3K4me2 (g). e: eye, ep: epidermis, g: gut, ls: larval spines, m: mesoderm, on: oocyte nucleus, s: septa, and p: pygidium. Scale bars = 50 μm.

and H3K9me and H3K4me2 were detected throughout derivatives of ectoderm, mesoderm, and endoderm. In contrast, the distribution of all three modifications was restricted in adults, in terms of being detected in relatively few cells within a tissue (e.g., H3K14ac in the adult epidermis) or no longer being present at detectable levels (e.g., H3K4me2 in the adult gut). This suggests that a transition in the histone code may occur as larvae undergo metamorphosis. Metamorphosis was not a focus of this study, but this general pattern suggests two hypotheses. One is that histone modifications affect a change in gene expression that correlates with changes in growth from rapidly growing larvae to more slowly growing adults. The other hypothesis is that changes in histone modifications correlate with a developmental reprogramming at metamorphosis. Both hypotheses have merit. Most larval tissues contribute directly to adult tissues in spionid polychaetes, suggesting that the first hypothesis may be more valid, but settlement involves widespread behavioural and morphological changes; thus, the potential for a global reprogramming of gene expression at metamorphosis is also to be considered.

4. Conclusions

We surveyed histone modifications in the development of a polychaete, *Polydora cornuta*, using immunohistochemistry. We found that three of the four tested primary antibodies for histone modifications appeared to recognize and bind to antigens of this species. H3K14ac, H3K9me, and H3K4me2 colocalized with DAPI and were consistently detected throughout development. The fourth primary antibody, H4K20me2, did not react with the tissue. The three detected modifications collectively suggest that these histone modifications are first present in early cleavage,

are widely distributed throughout larval development, and also are found in some adult tissues but with a more restricted distribution. The observed common onset in histone modifications suggests that a global change or activation of gene expression occurs in early embryos. Two modifications showed a generally low level of epifluorescence in most cells but a very strong signal in a few cell lineages, indicating a role in tissue differentiation. Finally, differences in the distribution of three modifications between larvae and adults suggest a second transition in histone modifications may occur at metamorphosis. Although preliminary, this research indicates that histone modifications are present in a marine invertebrate and show characteristic changes with tissue differentiation and also with specific life stages.

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Review Article

Epigenetic Mechanisms of Genomic Imprinting: Common Themes in the Regulation of Imprinted Regions in Mammals, Plants, and Insects

William A. MacDonald[1, 2]

[1] *Department of Biology, Dalhousie University, Halifax, NS, Canada B3H 4R2*
[2] *Departments of Biochemistry and Obstetrics & Gynecology, University of Western Ontario's Schulich School of Medicine and Dentistry, Children's Health Research Institute, London, ON, Canada N6C 2V5*

Correspondence should be addressed to William A. MacDonald, wmacdon7@uwo.ca

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Genomic imprinting is a form of epigenetic inheritance whereby the regulation of a gene or chromosomal region is dependent on the sex of the transmitting parent. During gametogenesis, imprinted regions of DNA are differentially marked in accordance to the sex of the parent, resulting in parent-specific expression. While mice are the primary research model used to study genomic imprinting, imprinted regions have been described in a broad variety of organisms, including other mammals, plants, and insects. Each of these organisms employs multiple, interrelated, epigenetic mechanisms to maintain parent-specific expression. While imprinted genes and imprint control regions are often species and locus-specific, the same suites of epigenetic mechanisms are often used to achieve imprinted expression. This review examines some examples of the epigenetic mechanisms responsible for genomic imprinting in mammals, plants, and insects.

1. Introduction

Epigenetic regulation of the genome is a critical facet of development. Epigenetic control of gene expression allows heritable changes in gene expression without the need for alterations in DNA sequence. This is achieved through the recruitment of molecular processes that assist transcription, block transcription, or degrade existing transcripts. Genomic imprinting is an epigenetic process that marks DNA in a sex-dependent manner, resulting in the differential expression of a gene depending on its parent of origin. Achieving an imprint requires establishing meiotically stable male and female imprints during gametogenesis and maintaining the imprinted state through DNA replication in the somatic cells of the embryo. Erasure of the preceding generation's imprint occurs in the germ line, followed by imprint reestablishment, in accordance with the sex of the organism. Each step in this imprinting process requires epigenetic marks to be interpreted by the genome and acted upon accordingly to result in parent-specific gene expression.

Genomic imprinting has been widely reported in eutherian mammals and marsupials [1–3]. Mice comprise the primary research model organism for the study of genomic imprinting. Approximately one hundred imprinted genes have been identified in mice with many more predicted to be present [2, 4]. This review considers imprinting to include chromosomal domains that direct imprinted epigenetic regulation, even if endogenous transcriptional units have yet to be identified as imprinting targets. Many imprinted genes in mice are developmentally important, linked to the formation of the placenta, or involved in brain function [2, 5, 6]. Noncoding transcriptional units, such as noncoding RNA, can also be imprinted and often form imprinted domains with developmentally important imprinted genes [7]. Imprinted genes found in mice are often used as candidates for investigating imprinted genes in other mammals. While some imprinted genes are conserved in mammals, many imprinted genes do not retain their imprinted status, even across eutherian mammals [1, 2]. For example, only a portion of the imprinted genes identified

in mice are also known to be imprinted in humans [2], with placental-specific imprinted genes standing out in this discordance [8]. This demonstrates that imprinting cannot be predicted in nonmodel species simply by monitoring homologous genes. Additionally, this does not preclude the presence of imprinted genes or imprinted chromosomal regions being present in species outside of the existing documented examples. Determining how imprinting is lost in orthologous genes and what epigenetic changes are found within these regions can lead to a better understanding of how imprinted domains might be regulated.

In addition to mammals and marsupials, imprinted genes have also been identified in flowering plants [9, 10]. Imprinted chromosomes and chromosomal regions have been reported in insects [11], while transgenes have identified imprinted chromosomal regions in fish [12] and nematodes [13, 14]. Imprinted domains in chromosomal regions with unidentified target genes are seemingly dissociated from significantly influencing the development of these organisms, however, they are still subject to parent-specific epigenetic modifications and provide insight into the overall organization and mechanisms of genomic imprinting. While the function and characteristics of imprinted loci vary, both between and within organisms, there are some common themes of genomic imprinting. Many imprinted regions are either arranged in restrictive chromosomal areas or regulated as multigene clusters, indicating imprinted regions are contained as distinct structural domains. This organization may be related to the close association of imprinted domains to regions of the chromosome containing tandem repeats or transposable elements [9, 11, 15, 16]. It has further been suggested that these distinct imprinted domains could have a broader function to maintain genome integrity and assist in chromosome pairing, possibly contributing to the presence of such domains in diverse organisms [17].

In this review, the epigenetic mechanisms involved in the regulation of imprinted domains in mammals, *Arabidopsis,* and *Drosophila* are explored. Mice represent the archetypal model for genomic imprinting and will be used to illustrate the differing roles of epigenetic mechanisms involved in regulating distinct imprinted domains. *Arabidopsis* is an emerging model organism for the study of genomic imprinting, where imprinting is pronounced in the endosperm but not the embryo proper. *Drosophila* are a model organism with a rich history in epigenetic research that have been utilized for transgenic imprinting element experiments while also having characterized imprinted chromosomal regions, despite not having any identified endogenously imprinted genes. Much remains to be understood about epigenetic regulation of genomic imprints. As epigenetic research expands to diverse model and nonmodel organisms, comparisons can be made between the structure and mechanisms of imprinted domains.

2. Common Epigenetic Mechanisms

The imprinted domains of mammals, plants, and insects represent distinct imprint events that do not share conserved sequence origins. While there are no universal templates that can be applied adequately to explain the regulation of all imprinted domains, either within or between organisms, there are common themes in the epigenetic mechanisms utilized and the multiple levels of regulation required to execute this parent-dependent mode of inheritance. As an epigenetic process, genomic imprinting alters gene expression without altering DNA sequence. However, DNA sequences are important in demarcating an imprinted domain. Imprinting control regions (ICRs) are often composed of repetitive DNA sequences found flanking, or internal to, imprinted genes, and in most cases, removal of an ICR will result in a loss of imprinting. Epigenetic modifiers of gene expression such as DNA methylation, histone modification, non-RNA, and higher-order chromatin formation act within ICRs to establish and maintain the imprinted state. ICRs act as nucleation sites for gene silencing or activation and are able to regulate expression of a single gene or an entire gene cluster. Enhancers and boundary elements are often associated with ICRs to restrict imprinted regulation to specific domains.

3. DNA Methylation

DNA methylation, the first epigenetic mechanism to be associated with imprinting, is an epigenetic modification that is applied directly to a strand of DNA [18, 19]. DNA methyltransferases (Dnmt) are highly conserved classes of enzymes that transfer methyl groups onto cytosine-C5 and are essential for both mammal and plant genome stability [20, 21], while being dispensable for the viability of *Drosophila*, which have low levels of genomic DNA methylation [22]. In plants and mammals, many ICRs contain differentially methylated regions (DMRs) that direct the epigenetic regulation of imprinted domains. Methylation within DMRs is often applied during gametogenesis and subsequently maintained throughout development, demonstrating the importance of DNA methylation for both the establishment and maintenance of many imprinted domains.

4. Histone Modification

Histone proteins and the modifications applied to them are highly conserved and comprise the most pervasive elements of imprinting across all taxa. Nuclear DNA is wrapped around nucleosomes, histone octamers composed of histones H2A, H2B, H3, and, H4, to form the basic repeating unit of chromatin. Various epigenetic modifications can be applied to the histones that affect chromatin conformation. Histone acetylation generally creates an accessible chromatin conformation while histone deacetylation, often coupled to histone methylation, initiates a compressed chromatin conformation that promotes silencing and the formation of heterochromatin [23]. Histone methylation can confer both an active or repressed transcriptional state depending upon which lysine is methylated. Histone 3 lysine 9 (H3K9), histone 4 lysine 20 (H4K20), and histone 3 lysine 27 (H3K27) are silencing modifications, while histone 3 lysine 4 (H3K4)

methylation produces active chromatin [24]. Histone modifications and DNA methylation are often intertwined, each epigenetic mark can influence the other's recruitment to reinforce differential epigenetic states [25, 26]. Histone modifications at imprinted regions can also facilitate the formation of higher-order chromatin structures.

5. Higher-Order Chromatin Structures

Maintaining transcriptional inactivation of an imprinted allele often involves the formation of heterochromatin, a compacted chromatin structure that can spread in *cis* and generally impose transcriptional silencing. Heterochromatic regions remain stable throughout development and are propagated through cell division by late replication in S phase of the cell cycle [27]. Heterochromatic protein 1 (HP1) is a highly conserved nonhistone chromatin protein that is able to recruit other heterochromatic proteins and accessory factors, such as histone methyltransferases, to reinforce the structure of heterochromatin and initiate spreading in *cis* [28–30]. Polycomb group proteins form a silencing pathway largely parallel to heterochromatic silencing that targets homeotic genes [31]. Polycomb group silencing also involves histone deacetylases and histone methyltransferases, however, there is only modest overlap between Polycomb group and heterochromatic silencing.

6. Noncoding RNA, Antisense RNA, and RNA Interference

RNA interference (RNAi) is a highly conserved posttranscriptional silencing mechanism in which double-stranded RNA (dsRNA) are processed to form guides for the degredation of complementary RNA transcripts through an RNA silencing complex (RISC) [32, 33]. The production of noncoding RNA has been described at multiple imprinted regions in both mammals and plants [7, 34]. In many organisms, components of the RNAi silencing pathway are found to be involved in the recruitment DNA methyltransferases and other factors that facilitate higher-order chromatin structure [35]. As more imprinted domains in diverse organisms become characterized, noncoding RNA and RNAi may be found to have a significant role in the regulation of genomic imprinting.

7. Imprinting in Mammals

In mammals, most known imprinted genes are organized into clusters that share common ICRs to direct the parent-specific regulation of multiple genes within the cluster. Many mammalian ICRs contain differentially methylated regions (DMRs) that gain parent-specific DNA methylation marks either in the germline for imprint establishment, or in somatic cells for imprint maintenance. A survey of both human and mouse genomes found more tandem repeats in methylated regions of imprinted genes than methylated regions of nonimprinted genes [36]. The presence of these repeats may represent additional structural elements in imprinted regions that could direct chromatin alterations or recruit additional epigenetic mechanisms. The presence of noncoding RNA is another common feature of mammalian imprinting. In mice, extensive transcription of noncoding RNA has been reported at multiple imprinted loci, with many of these transcripts extending beyond the previously established boundaries of imprinted regions [37].

8. DNA Methylation and *Igf2-H19* Imprinting in Mammals

The mouse *insulin-like growth factor 2* (*Igf2*) and *H19* genes were among the first imprinted genes to be characterized in detail [38, 39]. Subsequently, the same imprinting pattern was found for the human *Igf2* and *H19* genes [40, 41], leading to the imprinted status of *Igf2* becoming a standard assay for determining the presence of genomic imprinting in other vertebrates such as fish, birds, marsupials, sheep, and cattle [42–46]. The reciprocal imprinting of the *Igf2* and *H19* genes is mechanistically coupled. *H19* is maternally expressed and *Igf2* paternally expressed (Figure 1(a)). Two ICRs exist for *Igf2* and both are paternally methylated. DMR1, which is upstream of *Igf2* promoter 1, is a silencer that is inactivated by methylation [47]. DMR2 is located in exon 6 of *Igf2* and is an enhancer activated by methylation [48]. *H19* has one ICR which is located upstream of the *H19* gene and is also paternally methylated [49]. Regulation of the *Igf2* and *H19* imprinted domains is dependent on paternal-specific DNA methylation within the DMRs to maintain monoallelic expression; deletions of the *H19* DMR and *Igf2* DMR1 or alterations to Dnmts result in biallelic expression of both *H19* and *Igf2* [50]. Passage through the germline is required to establish *Igf2*/*H19* DMR methylation [51], which is carried out by the Dnmt3a methyltransferase assisted by the Dnmt cofactor, Dnmt3L [52, 53]. Once established, paternal-specific methylation is then identified and maintained in somatic cells by Dnmt1 [54]. Dnmt1 cannot reestablish parent-specific DNA methylation patterns if prior methylation marks are lost [51].

During mouse preimplantation development, both paternal and maternal genomes undergo extensive demethylation a few hours after fertilization. The paternal genome is demethylated rapidly by active demethylation while the maternal genome passively looses DNA methylation during each cell cycle [55, 56]. Imprinted DMRs must escape demethylation during preimplantation development to preserve any methylation marks established in the germline and this is achieved through the recruitment of maintenance methyltransferases to retain their methylated status [57]. In comparison to mice, sheep embryos have lower levels of genome reprogramming through preimplantation DNA demethylation [58], and only limited levels of active paternal genome demethylation [59]. An investigation into the epigenetic regulation of imprinted genes in sheep has found that parent-specific gene expression is not initiated until after the blastocyst stage, suggesting a later embryonic onset of parent-specific DNA methylation patterns [46]. Furthermore, *Igf2* and *H19* remain the only imprinted genes

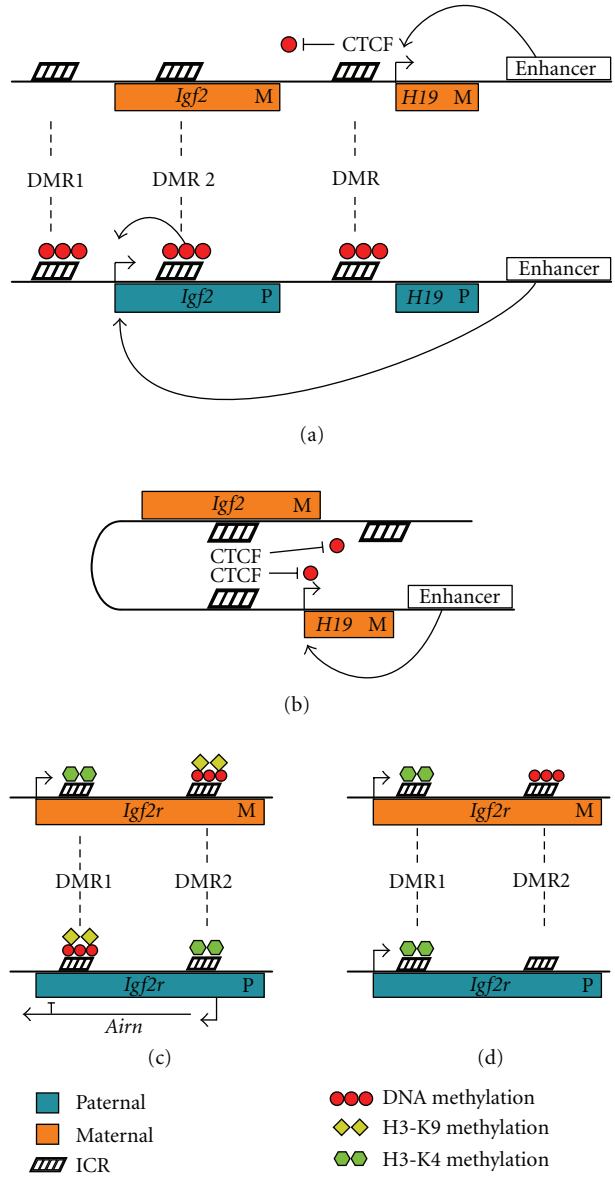

Figure 1: Imprinted regulation of *Igf2/H19* and *Igf2r/Airn* in mice and humans. (a) The *Igf2* and *H19* genes are reciprocally imprinted, with *H19* and *Igf2* being expressed maternally and paternally, respectively. CTCF binds the maternal *H19* ICR and acts as an insulator sequestering enhancers to initiate maternal *H19* transcription while also protecting the *H19* ICR from methylation. Methylation on the paternal *H19* ICR prevents CTCF binding and silences paternal transcription. *Igf2* is only expressed paternally as a lack of CTCF binding in the paternal *H19* ICR allows enhancers to activate the *Igf2* promoter. DMR1 is a silencer that is inactivated by methylation while DMR2 is an enhancer that is activated by methylation. DMR1 and DMR2 are both methylated on the paternal allele, facilitating paternal *Igf2* transcription and blocking maternal transcription. (b) CTCF mediates an intrachromosomal loop, which prevents DNA methylation of the *H19* DMR and *Igf2* DMRs, while facilitating *H19* expression. (c) In mice, *Igf2r* is maternally expressed while the overlapping *Airn* antisense transcript is paternally expressed. Histone H3K4 methylation in the maternal *Igf2r* promoter (DMR1) initiates transcription, while DNA methylation and histone H3K9 methylation in the downstream *Airn* promoter region (DMR2) silences maternal *Airn* transcription. Activating H3K4 methylation at the paternal *Airn* promoter region initiates paternal transcription of the *Airn* transcript. The *Airn* transcript overlaps the *Igf2r* promoter and contributes to the silencing of the paternal *Igf2r* allele along with DNA methylation and histone H3K9 methylation. (d) In humans, *Ifg2r* is biallelically expressed. Activating H3K4 methylation is found in both the maternal and paternal promoter regions of *Igf2r*. While maternal-specific DNA methylation of DMR2 is maintained, there is no H3K4 methylation of paternal DMR2, preventing the transcription of the *Airn* transcript.

in sheep that have identifiable germline DMR methylation, the DMRs of other investigated imprinted genes only acquire parent-specific methylation marks later in embryonic development [46, 60]. Together, these results demonstrate that DNA methylation can be recruited to maintain silencing at imprinted regions that lack germline parent-specific DMRs, and that species-specific differences in genome regulation are reflected in the differential timing and recruitment of epigenetic mechanisms to maintain imprinted domains.

The *Igf2* and *H19* imprinted domains remain one of the most studied examples of imprinting but much remains to be elucidated about the involvement DNA methylation at this imprinted domain. Ectopic localization of the *H19* DMR to a nonimprinted domain still results in paternal-specific DNA methylation of the DMR after fertilization despite the lack of germline establishment DNA methylation during spermatogenesis [61]. In order to achieve germline methylation of the ectopic *H19* DMR, additional DNA elements downstream of the endogenous *H19* DMR need to be included with the ectopic element [62]. These results suggest that more than DNA methylation alone is required to establish imprinting of this domain. Furthermore, in rare cases following DNA methylation disruption, a reversal of parent-specific imprinting patterns has been observed, including the H19 DMR gaining maternal DNA methylation and the paternal allele remaining unmethylated [63, 64]. These rare events may be due to the disruption of intra-chromosomal connections or nuclear localization of the parental alleles. The DMRs of *Igf2* and *H19* can physically interact, potentially initiating parent-specific chromosome loops separating the two domains into active or repressed nuclear compartments [65]. Such separation of maternal and paternal alleles into different nuclear compartments may provide additional reinforcement for the maintenance of parent-specific expression [66, 67].

9. Chromatin Domains and the CTCF Insulator

The evolutionarily conserved CCCTC-binding factor (CTCF) is also involved in *Igf2* and *H19* imprinting. Within the *H19* ICR, there is a CCCTC binding site that is only functional on the maternal, unmethylated, allele. When CTCF binds the maternally unmethylated *H19* ICR, it acts as an insulator, blocking access of the *Igf2* promoter to enhancers [68]. Paternal methylation of the *H19* ICR inhibits CTCF binding, allowing enhancers access to the *Igf2* promoter on the paternal chromosome [69, 70]. Silencing of the *Igf2* maternal allele is also facilitated by CTCF, which insulates maternal DMR1 and DMR2 from methylation when bound to the maternal *H19* ICR [71]. A loss of CTCF function results in *de novo* methylation of the maternal *H19* ICR, which effectively erases imprinted expression of *H19* and *Igf2* [72]. Recent phylogenetic and mutational analysis has shown that the CTCF binding sites, and not DNA methylation of ICRs, are the more reliable predictor of the imprinted expression of *Igf2*. CTCF binding sites are conserved in humans, mice, and marsupials, which all have imprinted *Igf2* and *H19*, while they are lacking in monotremes that do not imprint *Igf2* or *H19* [73]. Furthermore, *Igf2* DMR2 is biallelically methylated in both marsupials and monotremes, even though it is only biallelically expressed in monotremes, showing that methylation alone does not cause imprinted expression [73].

CTCF binds numerous sites within mammalian genomes, where it is identified both as a transcriptional regulator and a chromatin insulator able to block the spread of heterochromatin and mediated long-range chromosomal interactions [74]. CTCF-directed intrachromosomal loops are thought to contribute to parent-specific expression of *Igf2* and *H19* (Figure 1(b)). Self-association between CTCF proteins bound to ICRs can initiate a chromosomal loop that isolates *H19* to maintain maternal expression, while reinforcing *Igf2* silencing through the creation of a repressive domain [75]. Disruption of CTCF binding to the maternal *H19* ICR results in *de novo* DNA methylation of maternal *Igf2* DMR1 and DMR2, suggesting that intrachromosomal looping mediates regulation of the entire maternal *Igf2*/*H19* imprinted region [76]. Isolation of imprinted alleles by CTCF has been reported at various other mammalian imprinted domains, where parent-specific binding of CTCF is critical for maintaining active expression from an imprinted allele [77]. However, it remains to be determined if the initiation of higher-order chromatin structures via CTCF-mediated intrachromosomal looping is a common feature of other imprinted domains.

10. Histone Modification and Mammalian Imprinting

Although DNA methylation has been the focus of the majority of studies on genomic imprinting in mammals, it is becoming clear that histone modification and RNA-based processes also play a critical role. The receptor of Igf2, *Igf2r*, is another well-characterized imprinted gene [78]. Rodents and marsupials imprint their *Igf2r* gene, while monotremes, birds, and primates (including humans) do not, and thus they have biallelic *Igf2r* expression [79]. In mice, *Igf2r* is maternally expressed, displaying a reciprocal pattern of imprinting to that of *Igf2* (Figure 1(c)). Two ICRs are present in *Igf2r*; the first, DMR1, is located in the *Igf2r* promoter region and is paternally methylated, and the second, DMR2, lies within the second intron of *Igf2r* and is maternally methylated. DMR2 corresponds to the promoter of an antisense RNA transcript *Airn* (formally *Air*), a large transcript that overlaps the promoter region of *Igf2r* [80]. The *Airn* transcript is exclusively paternally expressed and not only contributes to the silencing of paternal *Igf2r*, but also to the silencing of the genes which are in the same region as *Igf2r* yet do not overlap the *Airn* transcript [80].

Histone methylation patterns are critical components of the parent-specific expression of *Igf2r* and *Airn* genes. In mice, the expressed maternal *Igf2r* allele and paternal *Airn* allele are both marked by H3K4 di- and trimethylation marks, while the repressed paternal *Igf2r* allele and maternal *Airn* allele are both marked by H3K9 trimethylation within the promoter region [81]. Indeed, histone methylation marks

are more reflective of the imprinted state of *Igf2r* than the presence of *Airn* transcripts or DNA methylation patterns. In the mouse brain, *Igf2r* is biallelically expressed. This correlates with the presence of activating H3K4 methylation in both the paternal and maternal *Igf2r* DMR1 promoter region, despite the retention of paternal *Airn* transcription [81]. In humans, activating H3K4 methylation is present within both the maternal and paternal *Igf2r* promoter regions (Figure 1(d)) yet is absent from the *Airn* promoter region, eliminating *Airn* expression while facilitating biallelic *Igf2r* expression [81]. Recently, H3K4 demethylation is shown as a requirement for establishing imprinted silencing at some maternally repressed genes in mice, where the disruption of H3K4 demethylation prevented *de novo* DNA methylation of DMRs [82]. H3K4 demethylation appeared critical for imprinted genes that undergo *de novo* DNA methylation at later stages in embryonic development, suggesting the interaction between histone modifications and DNA methylation may be dependent on the developmental timing of epigenetic regulatory activity.

A comprehensive survey of the histone modification present at imprinted regions compared to nonimprinted regions in mice determined three modifications closely associate with imprinted genes; repressed alleles contained H3K9 trimethylation and H4K20 trimethylation, while active alleles contained H3K4 trimethylation [83]. The chromatin state of imprinted regions was found to closely resemble heterochromatin and may be distinct from the general developmental silencing of genes, as H3K27 trimethylation was not present at all imprinted genes. The enrichment of H3K4, H3K9, and H4K20 trimethylation was present in imprinted genes regardless of whether the gene contained a DMR within its IRC, demonstrating both the importance and consistency of histone modification at imprinted domains. Broad enrichment of H3K27 trimethylation has been reported across some imprinted gene clusters [84]. This enrichment is occasionally biallelic and can be associated with both imprinted and nonimprinted genes alike within the same cluster [84]. Additionally, H3K27 trimethylation can also be disassociated from DNA methylation, or even antagonistic to DNA methylation within imprinted DMRs [85]. The complex association of H3K27 trimethylation with specific imprinted domains may be due to the secondary recruitment of H3K27 during development and tissue differentiation.

11. Antisense Transcripts and Mammalian Imprinting

The presence of noncoding RNA transcripts, such as the *H19* and *Airn* RNAs, is associated with imprinted regions in mammals. Deletion of the DMR2 *Airn* promoter [86], or the truncation of the *Airn* transcript [80], results in paternal activation and biallelic expression of *Igf2r* and the neighboring gene clusters. Additionally, the *Airn* transcript is capable of maintaining paternal silencing in this gene cluster even if the paternal *Igf2r* promoter is experimentally activated [87] or if DNA methylation of DMR2 is lost [78]. Part of the silencing function of *Airn* may be the ability to recruit additional silencing complexes to the imprinted region. In the mouse placenta *Airn* can recruit the histone H3K9 methyltransferase G9a, which contributes to the imprinted silencing of the gene *Slc22a3* within the *Igf2r* imprinted cluster [88]. Another important aspect of regulation by noncoding RNAs is the act of transcription itself and the interference such transcription can cause. It has been proposed that transcription of *Airn* through neighboring genes in *cis* contributes to their silencing [89]. Furthermore, the *Airn* transcript overlaps its own promoter and active transcription of *Airn* is required to prevent *de novo* methylation of this promoter on the paternal allele [90]. Recently, the transcriptional importance of noncoding RNAs been shown for the *Kcnq1* imprinted domain. In stem cells, targeted depletion of the *Kcnq1ot1* noncoding RNA did not relieve silencing of the paternally silenced genes, suggesting transcription through these genes during the production of *Kcnq1ot1* contributes to their silencing more so than the presence of the *Kcnq1ot1* transcript [91].

MicroRNAs (miRNAs) are endogenous 21–25 nt RNA transcripts that target complementary sequences for silencing [92]. Two miRNA genes, *miR-127* and *miR-136*, have been shown to be part of an imprinted domain responsible for the imprinted expression of the retrotransposon-like gene *Rtl1* in mice and the orthologous *PEG11* gene in sheep and humans [93, 94]. Imprinted expression is associated with an unmethylated maternal ICR, leading to the miRNA genes only being maternally expressed which drives maternal-specific silencing of *Rtl1* [95]. In sheep, *PEG11* produces a functional protein as well as an antisense *PEG11* transcript [96]. Imprinted silencing is directed by maternally produced antisense miRNA acting as guides for RISC-mediated destruction of maternal *PEG11* transcript [97]. However, complex modulations of maternal miRNA generation suggest that maternal gene expression levels are balanced for dosage and not completely silenced [96, 97]. It is unclear if RNAi processing of *PEG11* transcripts by RNAi machinery recruits additional chromatic remodelers to regulate expression from the maternal allele.

Genomic imprinting has been linked to dosage compensation in some mammals, where the silencing is directed towards the paternal X chromosome [98]. In female mice, the paternal X chromosome is selectively silenced in extraembryonic tissues, in part by the production of the noncoding RNA *Xist*. Transcription of *Xist* spreads from an initial transcription site to cover most of the paternal X chromosome, leading to the recruitment of additional epigenetic silencing factors, such as histone methyltransferases and heterochromatic proteins [99]. Preferential silencing of the paternal X chromosome still occurs if *Xist* noncoding RNA is lost, however, silencing is destabilized [100]. This may be related to the finding that the RNAi component Dicer is required for the spread of *Xist* and recruitment of the H3K27 trimethylation silencing in somatic cell X inactivation [101]. It is possible that imprinted silencing of the paternal X chromosome in extraembryonic mouse tissues originates from the imprinted silencing of specific target genes or regions, which then act as nucleation sites for RNAi-directed spreading of silencing across the whole chromosome.

12. Imprinting in Plants

Imprinting in plants was first documented in 1970, when it was found that a gene in maize produced fully colored kernels when maternally inherited and variegated kernels when paternally inherited [102]. In more recent years, genomic imprinting in angiosperms has been investigated extensively in *Arabidopsis*. Angiosperms experience double fertilization, with one sperm fusing the egg cell to produce the embryo proper, and the other fusing with the central cell to produce endosperm. The endosperm acts largely as support structure of the developing embryo and is terminally differentiated.

13. DNA Methylation in *Arabidopsis* FWA and FIS2 Imprinting

The *Arabidopsis* gene FWA encodes a homeodomain-containing transcription factor involved in the regulation of flowering and is a well-characterized imprinted gene expressed solely from the maternal allele [103]. FWA imprinting involves DEMETER (DME), a DNA glycosylase able to excise modified nucleotide bases and the MET1 methyltransferase (Figure 2(a)). MET1 methylates tandem repeats in the FWA promoter and DME acts to remove methylated cytosines from the maternal FWA allele, leaving only the paternal FWA allele methylated [103, 104]. If DME demethylation is lost, the imprint is also lost, as both maternal and paternal FWA alleles remain methylated by MET1 [103, 104]. This scenario implies methylation is the default state and active demethylation is required to imprint an allele. DME is primarily expressed in the female central cell before fertilization and is not expressed until long after fertilization or in the male sporophyte [105]. This disparity in DME expression provides a window during which the imprint can be established on the maternal FWA allele prior to fertilization but requires additional mechanisms to maintain expression after fertilization. FWA, FERTILIZATION INDEPENDENT SEED 2 (FIS2) is also maternally expressed and is regulated through the antagonistic action of DME and MET1 (Figure 2(b)). A distinct 200 bp region upstream from FIS2 acts as the nucleation center for FIS2 paternal methylation but, unlike the MET1 methylation site in the FWA gene, there are no tandem repeats in this region [106]. For both FWA and FIS2, active MET1 methylation is required during male gametogenesis to produce paternal-specific silencing [106].

14. RNAi and Heterochromatin Formation in *Arabidopsis* FWA Imprinting

RNA-directed DNA methylation (RdDM) is a process that produces locus-specific heterochromatin formation in angiosperms and is attributed to the need to silence transposons. Initially, dsRNA is processed by RNAi machinery into small interfering RNAs (siRNA). These siRNA then guide site-specific DNA methylation and heterochromatinization [107]. Methylation produced by RdDM does not spread significantly in *cis* so silencing is precisely targeted to the region producing the dsRNA [108]. Heterochromatin formation arising from the RdDM pathway involves the ATPase chromatin-remodeling factor DECREASE IN DNA METHYLATION1 (DDM1), an SWI/SNF homologue involved with the maintenance of H3K9 histone methylation and DNA methylation [107].

The FWA promoter contains tandem repeats that produce dsRNA from the paternal FWA allele, which guides DDM1 methylation and heterochromatin formation [107]. The function of DDM1 is exclusively in the maintenance of silencing as FWA methylation cannot be reestablished by DDM1 after siRNA or DNA methylation is lost [109]. Mutations in genes involved in the RNAi pathway of *Arabidopsis*, including *dicer-like3* and *argonaute4*, result in a loss of paternal FWA methylation It has been proposed that the siRNA generated from the FWA promoter tandem repeats also guides DOMAINS REARRANGED METHYLTRANSFERASE (DRM), a Dmnt3 homologue, to perform *de novo* methylation [110]. This shows that the RNAi pathway in *Arabidopsis* can initiate silencing of targeted imprinted domains.

15. Histone and Polycomb Group Proteins in *Arabidopsis* Imprinting

The *Arabidopsis* Polycomb group protein MEDEA (MEA) gene is imprinted, resulting in expression exclusively from the maternal allele in the endosperm (Figure 2(c)). Similar to FWA and FIS2 imprinting, MEA regulation also involves DME activation and MET1 DNA methylation [111]. However, while DNA methylation is found in the promoter region of the paternal MEA allele, it likely does not play a large role in the initial regulation of the imprint [112]. Transcriptional activation of maternal MEA is maintained in the female central cell by DME [105], while the paternal MEA allele is silenced by H3K27 histone methylation [106]. Paternal MEA silencing is maintained by a Polycomb group complex, which includes FERTILIZATION INDEPENDENT ENDOSPERM (FIE), FIS2 and the maternally produced MEA [106, 113]. This Polycomb group complex is able to initiate a self-reinforcing loop of silencing, maintaining H3K27 methylation and recruiting additional Polycomb complexes.

MEA not only assists in regulating its own imprinted expression but also causes a cascade of imprinted expression in the genes that it regulates. The gene PHERES1 (PHE1) is regulated by the imprinted MEA protein and, as a consequence, is also imprinted [114]. PHE1 encodes a type I MADS-box protein, a protein family typically involved in DNA binding, and leads to uncontrolled endosperm proliferation when overexpressed. MEA, acting as part of a multiprotein complex with other Polycomb group proteins, forms condensed chromatin structures at its binding site within the PHE1 promoter which silences the PHE1 gene (Figure 2(d)) [115]. As only the maternal MEA allele is active prior to fertilization in the endosperm, PHE1 Polycomb silencing is also limited to the maternal allele [114]. The imprinting of both MEA and PHE1 demonstrates that the

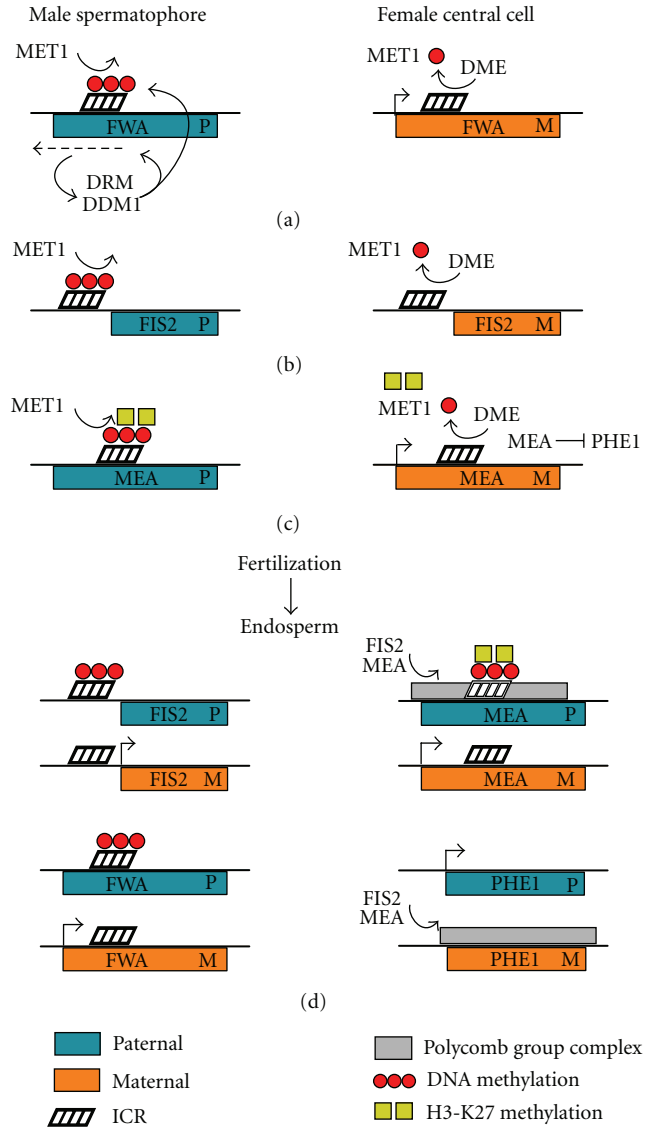

Figure 2: Imprinted regulation of the *Arabidopsis* genes FWA, FIS2, MEA, and PHE1. (a) Imprinted FWA is only expressed from the maternal allele. Prior to fertilization, MET1 methylates the paternal FWA promoter. In the male spermatophores, tandem repeats in the promoter produce siRNA (represented by the dashed arrow), which recruit DRM and DDM1 to the promoter region to maintain the methylated state. In the female central cell, DME demethylates the maternal FWA promoter maintaining maternal expression. (b) The antagonistic relationship between MET1 and DME is also involved in the imprinting of FIS2. MET1 methylates a region upstream of the paternal FIS2 allele that initiates silencing while DME demethylates the maternal allele. (c) The imprinted regulation of MEA also involves MET1 and DME; however, histone modification plays a key role in initiating parent-specific expression. Histone H3K27 methylation is present in the promoter region of the paternal MEA allele in addition to DNA methylation. DME protects the maternal promoter from both DNA and histone methylation. Transcribed maternal MEA, which encodes a member of the Polycomb group silencing complex, initiates the parent-specific silencing of maternal PHE1. (d) In the endosperm, the Polycomb group gene MEA contributes to its own imprinted expression in the endosperm, with maternally produced MEA involved in the silencing of the paternal MEA allele. FIS2, which is also part of a Polycomb silencing complex, contributes to silencing the paternal MEA allele. PHE1, which is regulated by Polycomb group silencing, is only expressed from the paternal allele. Maternally produced FIS2 and MEA combine to maintain the silencing of the maternal PHE1 allele.

imprinting of a regulatory gene can produce a cascade of parent-specific gene expression. Recently, the gene *Phf17* (*Jade1*), which encodes for a component of the HBO1 histone 4 acetylation complex, has been found to be imprinted in the mouse placenta [116]. This finding is interesting as it suggests the possibility of similar downstream imprinting events in the mouse placenta as those found in *Arabidopsis* endosperm.

16. The *mee1* Gene Is Imprinted in the Maize Embryo

While all imprinted genes in *Arabidopsis* have so far been found to be monoallelically expressed only in the endosperm, a gene in maize, *maternally expressed in embryo 1* (*mee1*), is reported to have parent-specific expression in both the

endosperm and embryo [117]. Maternal-specific expression of *mee1* in the endosperm is regulated in a manner similar to that described for *Arabidopsis,* with maternal-specific active DNA demethylation and protection from DNA methyltransferases. The paternal *mee1* allele is methylated in gametes and remains methylated at all stages of development, preventing paternal transcription. The maternal allele is also methylated DNA in gametes; however, active demethylation of a DMR located near the transcriptional start site of *mee1* occurs after fertilization, suggesting that the initial parent-specific demarcation of the alleles is independent of DNA methylation. During gamete production, the maternal allele regains DNA methylation within the DMR. It remains to be determined which epigenetic mark establishes the maternal imprint but, it appears as though the *mee1* DMR is in fact a differentially demethylated region, which may be a reflection of species-specific epigenetic reprogramming dynamics. Regardless, this finding illustrates the ability of the maize genome to maintain parent-specific demarcation of genes in the developing embryo, and predicts the identification of further genes with imprinted embryonic expression in plants.

17. Imprinting in Insects

The investigation of imprinting in insects has progressed quietly since early studies in *Sciara* and *Coccids* revealed that gene silencing induced by whole chromosome heterochromatinization was dependent on the parental origin of the chromosome [118, 119]. It was the study of chromosome elimination in the fungus gnat, *Sciara,* which leads to the use of the descriptive term "imprint" [120]. Crouse reported that X chromosomes acquire an "imprint" which directs paternally derived X chromosomes to be eliminated from somatic cells and ensures that only the female X chromosomes remain in the gametes [120]. This work provided explicit evidence of parent-specific silencing. Whole chromosome imprinted regulation such as this is not uncommon in insects [121]; however, parent-specific transcriptional silencing of smaller chromosome regions, similar to that found in mammals and plants, has also been described in *Drosophila*.

18. Genomic Imprinting in *Drosophila*

Thus far, all imprinted domains in *Drosophila melanogaster* have been found only in chromosome regions that are heterochromatic [11]. In *Drosophila*, most heterochromatin is compartmentalized into large blocks such as those flanking the centromeres, the entire Y chromosome, and in a few discrete regions that are developmentally controlled. The relegation of imprinted domains to gene poor chromosomal regions is advantageous as it limits parent-specific silencing to relatively few genes [122]. This property also has made identifying endogenous imprinted genes in *Drosophila* difficult as these regions are mostly uncharacterized. Most known imprinted domains in *Drosophila* have been detected through position-effect variegation (PEV), which causes variegated transcriptional silencing of gene clusters placed adjacent to heterochromatic regions. Using transgenes or reporter genes placed into heterochromatic regions, imprinted domains have been identified by the display of parent-specific PEV silencing of the marker gene. The majority of the *Drosophila* Y chromosome is imprinted, as inserted transgenes are silenced in a parent-specific manner [123, 124], while distinct imprinted domains have been reported in heterochromatic regions of the X chromosome and the autosomes [11, 125, 126].

19. Imprinting of the *Drosophila Dp(1;f)LJ9* Mini-X Chromosome

The *Drosophila Dp(1:f)LJ9* mini-X chromosome is the result of an X chromosome inversion and deletion which juxtaposes euchromatic genes to a heterochromatic *Drosophila* imprinting center [126, 127]. One of the euchromatic genes that falls under control from the imprinting center is the eye color gene *garnet.* This gene is uniformly expressed when maternally inherited and exhibits variegated silencing when paternally inherited, and so acts as a reporter for the imprint. Mutations which alter PEV by either enhanced silencing (*E(var)*) or suppressed silencing (*Su(var)*) do so by affecting proteins and accessory factors involved in heterochromatin formation. An extensive screen of the effects of *Su(var)* mutations on imprinted *garnet* expression revealed that both HP1 (*Su(var)2–5*) and the H3K9 histone methyltransferase (*Su(var)3–9*) were required for the maintenance of the paternal imprint (Figure 3(a)) [128]. Additionally, a mutation of *Su(var)3-3*, responsible for H3K4 demethylation [129], also disrupted the silencing of the paternally inherited *Dp(1:f)LJ9* [128]. This suggests active removal of the activating H3K4 methylation mark is required before H3K9 methylation can direct HP1 recruitment and the formation of heterochromatin. While Polycomb group proteins have been implicated in the regulation of both mammalian and plant imprinting [6, 130], they do not appear to have any role in epigenetic regulation from the *Dp(1:f)LJ9* imprinting center. Mutations in Polycomb group genes, including *Enhancer of zeste E(z)* which initiates H3K27 methylation, have no effect on paternal-specific silencing [128].

None of the *Su(var)* mutations tested on *Dp(1:f)LJ9* had any effect on the stability of the maternal imprint, demonstrating that maternal inheritance of *Dp(1:f)LJ9* allows a stable boundary to form between the marker gene and the ICR to counteract heterochromatinization. The compact *Drosophila* genome utilizes many insulator proteins to create regulatory domains, but only the CTCF insulator protein is highly conserved [131, 132]. Similar to the role of CTCF in maintaining mammalian imprinted domains, CTCF also acts to protect maternally inherited *Dp(1:f)LJ9* by acting as a boundary element against the spread of heterochromatin (Figure 3(b)) [133]. Other insulator proteins remain to be fully tested for their involvement in the *Dp(1:f)LJ9* maternal-specific boundary, however, Suppressor of Hairy-wing (Su(Hw)) and the *Drosophila*-specific Boundary Element-associated Factor (BEAF-32) do not appear to be necessary

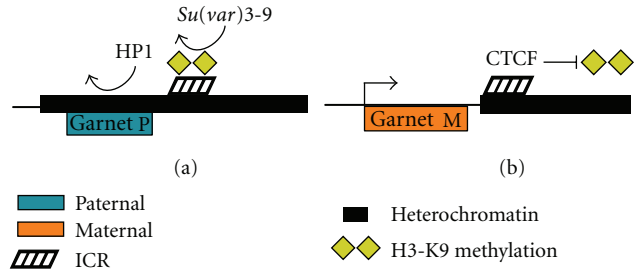

Figure 3: Creation of the *Drosophila* mini-X chromosome and the resulting imprinted expression of the garnet marker gene. The *Dp(1:f)LJ9* mini-X chromosome was generated through an inversion followed by a large deletion by X-ray irradiation. In the resulting mini-X chromosome, *garnet* is placed next to a region centric of heterochromatin containing an imprinting center. (a) Paternal transmission of the mini-X chromosome results in silencing of *garnet*, as a result of H3K9 methylation and heterochromatin formation. (b) Maternal transmission of the mini-X chromosome results in active transcription of the *garnet* gene, maintained by CTCF counteracting heterochromatin formation.

[134]. In *Drosophila,* many non-CTCF insulator proteins depend on PcG and Trx group proteins for proper function [135]. The failure of PcG and Trx group mutations to modify maternal *Dp(1:f)LJ9 garnet* expression [128] suggests non-CTCF insulators are not likely to be recruited to the maternal boundary. The specific involvement of CTCF with the *Dp(1:f)LJ9* imprint is intriguing as it raises the possibility that the imprint was acquired prior to *Drosophila* speciation or that the factors contributing imprint maintenance are more likely to involve conserved epigenetic mechanisms.

The role of heterochromatin at the *Dp(1:f)LJ9* imprint center is limited to imprint maintenance; no *Su(var)* mutations, Polycomb group protein mutations, or chemical heterochromatin modifiers impacted either the maternal or paternal establishment of the imprint [126, 128]. Similarly, CTCF is not involved in establishment of the maternal imprint [133], mirroring of its role in mammalian imprinting where it is also dispensable for imprint establishment [68, 136]. These findings illustrate the fact that distinct epigenetic mechanisms are used for the establishment and maintenance of parent-specific expression from the *Dp(1:f)LJ9* ICR. Establishment of the imprint requires correct passage through the germline, as evidenced by the loss of the *Dp(1;f)LJ9* paternal imprint in cloned *Drosophila* [137].

Regulation of the *Dp(1;f)LJ9* imprinting center demonstrates features of both discrete mammalian ICRs and whole chromosome imprinting characteristics found in other insects. Paternal inheritance of the disrupted imprinting region results in the spreading of heterochromatic silencing to proximal areas; a similar spreading of silencing from an imprinted region has also been described in mammals [138]. However, a secondary effect of the exposed paternal *Dp(1;f)LJ9* ICR is a chromosome-wide decrease in transcription, similar to the imprinted silencing of whole chromosomes in *Coccids* [122]. The stable maternal boundary generated from the *Dp(1;f)LJ9* ICR prevents both the local spreading of heterochromatin and the chromosome-wide reduction of transcription [122]. This finding suggests that silencing initiated from a heterochromatic imprinted domain is able to impose long-range *cis* alterations in regulation when not properly insulated within a heterochromatic region.

20. Noncoding RNA and Imprinting in *Drosophila*

Drosophila dosage compensation involves an increase in male X chromosome expression instead of the silencing of one female X chromosome, as occurs in mammals [139]. Increased transcription of the male X chromosome coincides with the binding of the male-specific lethal (MSL) complex, which is recruited to specific chromosome sites by the noncoding RNAs *roX1* and *roX2* [139]. Deletion of both *roX* genes eliminates compensated expression from genes on the X chromosome, resulting in male lethality [140]. Similar to the stabilization role of *Xist* in spreading of X chromosome silencing in mice, the MSL complex is still able to colocalize to specific X chromosome sites and direct limited activation in the absence of roX [139]. The spreading of MSL transcriptional activation, however, is dependent on *roX* RNA transcription [141]. Recently, it has been reported that experimental manipulation causing maternal inheritance of the Y chromosome significantly relieves male lethality caused by *roX* mutations, suggesting imprinted regions on the Y chromosome augment *roX* expression [142]. This suggests that correct passage of the Y chromosome through the male germline results in the establishment of epigenetic marks that influence dosage compensation in *Drosophila*. It has been proposed that the imprinted regions of the Y chromosome may contribute to hybrid incompatibility between *Drosophila* species [142], a phenomenon previously associated with imprinted genes in both mammals and plants [143, 144].

21. DNA Methylation and Imprinting in Insects

There is a precedent for the involvement of DNA methylation in insect imprinting in the mealybug *Planococcus citri*. Complete silencing of paternally inherited chromosomes in males is associated with DNA hypomethylation [145]. In this case, hypomethylated chromosomes, which have been inherited paternally, become silenced in males, while chromosomes inherited maternally remain hypermethylated and active. The epigenetic imprint marking paternal chromosomes for silencing appears to be H3K9 di- and trimethylation,

which is established during gametogenesis, while the lack of H3K9 di- and trimethylation on the maternal chromosomes may simply reflect a default imprinted state [146]. Heterochromatic spreading reinforces the silent state of paternal chromosomes, as HP1-like and HP2-like complexes are recruited to chromosomes with H3K9 di- and trimethylated histones [147]. It is proposed that silencing of entire paternal chromosomes is nucleated from discrete ICRs marked by H3K9 di- and trimethylation, which escape early embryonic activation signals, and propagates chromosomal silencing [146]. Such spreading of silencing, originating from discrete ICRs to cover the entire chromosome, corresponds to the mechanisms guiding parent-specific chromosomal regulation described in *Drosophila* and mouse extraembryonic tissues.

Drosophila possess a single DNA methyltransferases, Dnmt2, and only have low genome-wide levels of DNA methylation that peak early in embryogenesis and decline towards adulthood [22, 148]. The presence of DNA methylation in the developing embryo is defined developmentally, as nuclear concentrations peak in the early embryo then begin to decline as development progresses [22, 149]. *Drosophila* with *Dnmt2* mutations remain fertile and viable with no observable phenotype [22], however, overall lifespan is diminished [150]. Recently, Dnmt2 has been implicated in the genomic regulation of retrotransposons, suppressing retrotransposon transcription in somatic cells of the early embryo [151]. Loss of Dnmt2 resulted in the mislocalization of the H4K20 methyltransferase, resulting in the elimination of H4K20 trimethylation and reduced retrotransposon repression. Dnmt2 was also shown to be associated with heterochromatin formation at repeat transgene arrays, illustrating the potential for DNA methylation to assist in the recruitment and stabilization of heterochromatic factors in *Drosophila* [151].

The role of Dnmt2 in retrotransposon repression does not extend to the germline [151]. This finding is supported by research involving transgenic *Drosophila* with mammalian Dnmts; flies overexpressing mammalian Dnmts are not viable [152], however, germline-specific expression of mammalian Dnmts does not effect fertility or the viability of progeny [153]. Together, these findings suggest that genomic regulation by DNA methylation in *Drosophila* is restricted to somatic cells, and unlike mammals and plants, does not have an essential role in in the germline. While the role of DNA methylation in *Drosophila* development is still an area of great debate [154, 155], current research would suggest that DNA methylation is not a candidate for a germline establishment epigenetic mark in *Drosophila* imprinting.

22. Recognition of Mammalian Imprinting Elements in Transgenic *Drosophila*

Various transgenic *Drosophila* lines have been produced that contain either mouse or human ICRs [156–158]. These ICRs function as silencers in *Drosophila* but do not confer parent-specific silencing. Similar experiments involving human ICRs introduced into transgenic mice also resulted in a loss of parent-specific regulation [159, 160]. Transgenic studies involving the mouse *H19* ICR exemplify remarkable conservation of epigenetic function between the mouse and *Drosophila* genomes. A specific region of the upstream *H19* ICR was identified as a silencing element in mice by first being identified as a required sequence for silencing in transgenic *Drosophila* [161]. Furthermore, the production of noncoding RNA transcripts from the upstream *H19* ICR was also first discovered in transgenic *Drosophila*, where noncoding RNA production from the transgenic insert was associated with reporter gene silencing [162]. The upstream *H19* ICR is necessary for proper repression of paternal *H19* expression in mice [163], where the noncoding transcripts are thought to be involved in the recruitment of other silencing mechanisms [162]. Both of these studies involving the transgenic mouse *H19* ICR identified endogenous silencing mechanisms using a transgenic system, demonstrating the potential for epigenetic regulatory fidelity between two distinct organisms.

The *Drosophila* insulator Su(Hw) and Polycomb group proteins, Enhancer of zeste (E(z)) and Posterior sex combs (Psc), were found to regulate the transgenic *Igf2/H19* ICR construct [164]. These results show that imprinted transgenes are able to recruit histone modifiers and chromatin remodelers to direct silencing of a chromosomal domain. The binding of Su(Hw) to the transgenic *Igf2/H19* ICR construct is reminiscent of CTCF binding to the endogenous *H19* ICR in mice [68]. In mice, CTCF protects *H19* from methylation and silencing, whereas in *Drosophila* Su(Hw), binding to the *H19* ICR initiates downstream silencing, possibly by the recruitment of heterochromatic factors. The involvement of Su(Hw) with silencing from the *H19* ICR is specific to this imprinted element. Typically, Su(Hw) protects transgenes from silencing in *Drosophila* [165] and other ICRs are not dependent on Su(Hw) for silencing in transgenic *Drosophila* [164]. This unexpected involvement of Su(Hw) with the *H19* ICR suggests that elements within the ICR are eliciting a genomic response from *Drosophila* that are beyond that of a nondescript repetitive element.

An intriguing finding from the mammal-*Drosophila* transgenic imprinting experiments is that silencing activity is often maintained, but the insulator/boundary activity necessary for maintaining gene expression is lost. Expression from an imprinted domain requires the parent-specific recruitment of both silencing and activating chromatin remodelers, which includes insulators. Binding of *Su(Hw)* to the transgenic *H19* ICR did not produce the same insulator properties as endogenous CTCF binding provides, but, rather, acted as a silencer [164]. Furthermore, multiple transgenic constructs, produced from sections of both human and mouse *H19* ICRs, all acted as silencing elements in *Drosophila* but did not retain any of their insulator functions [166]. These findings could suggest that the maintenance of the active component of imprinted regions might be equally as complex as the silenced component and may require species-specific recognition of epigenetic marks. The complexity of imprinted large domains and their association with repressed repetitive elements could favor robust regulatory mechanisms to ensure the maintenance of active imprinted alleles, as exemplified by the

complex intrachromosomal folding associated with maternal activation of *H19* (Figure 1(b)). Together, these transgenic experiments show that while many epigenetic mechanisms utilized for silencing genes are highly conserved, the elements that superimpose the parental specificity of silencing are more specialized and tailored to the regulatory needs of each species.

23. Common Epigenetic Mechanisms Regulate Diverse Imprinted Domains

Producing parent-specific expression requires independent regulation of the maternal and paternal alleles. Histone modification and DNA methylation, leading to heterochromatin formation, are common regulators of imprinted silencing. Noncoding RNA and RNAi are emerging as critical components for the early recruitment of silencing mechanisms to ICRs. Boundary elements have also been shown to be necessary to maintain discrete regulatory domains by protecting active alleles, in a parent-specific manner, from silencing by blocking either the recruitment or spreading of silencing mechanisms. In all cases, genomic imprinting relies on multiple epigenetic mechanisms acting in concert to maintain and reinforce silencing.

The recent identification of H3K4, H3K9, and H4K20 trimethylation as an epigenetic marks common to imprinted genes in mice is a significant step in understanding the epigenetic code that constitutes the demarcation of a genomic imprint [83]. As high-throughput screening of genome-wide epigenetic modifications is explored in more organisms, it will be interesting to see if a similar, concise pattern of epigenetic modifications emerges. In *Drosophila*, both H3K9 and H3K4 methylation are associated with the *Dp(1;f)LJ9* imprinted domain, while H3K27 methylation is not [128]. The finding that H3K27 trimethylation was found at some, but not all, imprinted genes in mice [83], yet is the primary histone modification associated with imprinting in *Arabidopsis*, may reflect the role of H3K27 trimethylation as a ubiquitous epigenetic modification in *Arabidopsis* [167]. This highlights that species-specific variations in the use of epigenetic regulators such as DNA methylation or RNAi will be reflected in how an imprinted region is regulated. Variation in the structure of an imprinted domain, and the organism in which it found, will result in differential reliance on specific epigenetic mechanisms and, possibly, the order in which they are recruited. Evolutionary pressures and the species-specific arrangement of chromosomes also factor into the construction of large imprinted domains or novel genes acquiring imprinted regulation. Nevertheless, in all species examined here, common suites of epigenetic processes appear to be employed to regulate genomic imprinting.

The study of genomic imprinting has progressed for the better part of a century but it is still very much in its infancy. Complex regulatory patterns continue to be revealed within known imprinted regions and new imprinted genes continue to be discovered. Assessing imprinting in diverse model and nonmodel organisms can broaden the understanding of what epigenetic processes are necessary to achieve an imprint. Despite the fact that specific imprinted genes are not often conserved between diverse species, the epigenetic mechanisms and gross structural features of imprinted regions are often similar. Recognizing the common processes of genomic imprinting will aid our understanding of the epigenetic mechanisms required to distinguish maternal and paternal genomes during development in both model and nonmodel organisms.

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Research Article

Epigenetic Variation May Compensate for Decreased Genetic Variation with Introductions: A Case Study Using House Sparrows (*Passer domesticus*) on Two Continents

Aaron W. Schrey,[1] Courtney A. C. Coon,[1] Michael T. Grispo,[1] Mohammed Awad,[1] Titus Imboma,[2] Earl D. McCoy,[1] Henry R. Mushinsky,[1] Christina L. Richards,[1] and Lynn B. Martin[1]

[1] *Department of Integrative Biology, University of South Florida, SCA 110, 4202 East Fowler Avenue, Tampa, FL 33620, USA*
[2] *Ornithology Section, Department of Zoology, National Museum of Kenya, Museum Hill Centre, Museum Hill Road, Westlands, Nairobi, Kenya*

Correspondence should be addressed to Aaron W. Schrey, aschrey@usf.edu

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Epigenetic mechanisms impact several phenotypic traits and may be important for ecology and evolution. The introduced house sparrow (*Passer domesticus*) exhibits extensive phenotypic variation among and within populations. We screened methylation in populations from Kenya and Florida to determine if methylation varied among populations, varied with introduction history (Kenyan invasion <50 years old, Florida invasion ~150 years old), and could potentially compensate for decrease genetic variation with introductions. While recent literature has speculated on the importance of epigenetic effects for biological invasions, this is the first such study among wild vertebrates. Methylation was more frequent in Nairobi, and outlier loci suggest that populations may be differentiated. Methylation diversity was similar between populations, in spite of known lower genetic diversity in Nairobi, which suggests that epigenetic variation may compensate for decreased genetic diversity as a source of phenotypic variation during introduction. Our results suggest that methylation differences may be common among house sparrows, but research is needed to discern whether methylation impacts phenotypic variation.

1. Introduction

Epigenetic variation may be important to ecology [1–4], and understanding its mechanistic basis will provide insights into population processes at both ecological and evolutionary time scales [5]. Epigenetics is the study of stably heritable phenotypes that occur without alterations in the DNA sequence [6]. Molecular epigenetic modifications, such as methylation, histone deacetylation, and small interfering RNAs, regulate gene expression and are a significant contributor to phenotypic variation in diverse taxa [7]. Epigenetic modifications may vary between genome regions, over time, and in response to environmental stressors [1, 8, 9] and even among individuals and populations [10–15]. Epigenetic modification of gene expression may enable organisms to adjust their phenotypes to match novel environments or provide them the ability to quickly respond to a changing environment [16].

Epigenetic variation potentially has an ecologically relevant role in the adaptation of introduced or invasive species to novel environments. Typically, introduced or invasive species will not be adapted to their new environments and will be hampered by reduced genetic variation associated with bottlenecks or founder effects, which creates a genetic paradox [17]. Over the ecological time scales of invasions, mutation and recombination would rarely provide sufficient sources of variation for the often extensive phenotypic differentiation that is observed among populations [4]. Epigenetic variation may be one mechanism that compensates for the lack of genetic variation in the successful introduced species,

allowing the short-term adaptation to the new environment and allowing the new environment to influence genome function in the introduced species [17]. Also, if some species (or populations) are better able to regulate expression of genes via epigenetic mechanisms, which then affects the expression of ecologically important phenotypes, their ability to colonize new areas or expand their native ranges may be enhanced [3, 18–20].

The most studied molecular epigenetic mechanism is DNA methylation [21], usually of 5′methylcytosine where cytosine is followed by guanine (CpG sequences). These sequences are particularly common in gene regulatory sequences [4]. DNA methylation can cause phenotypic variation in flower shape and fruit pigmentation [22, 23], mouse tail shape, adult body size, and coat color [24, 25], and in numerous traits differentiating queen and worker honeybees [26]. DNA methylation is also known to be important in imprinting (differential gene expression depending on the parent of origin) [27], X-inactivation [28], silencing transposable elements [27], and response to environmental stressors [1, 8]. Importantly too, traits modified by DNA methylation have been stably inherited for at least eight generations [29].

DNA methylation is also a potential source of interindividual phenotypic variation [4] because of its propensity to alter gene expression contingent on environmental change [1], which could generate phenotypic variation even in cases of reduced genetic variation. There are several studies of ecological epigenetics using DNA methylation in plants. Different amounts of methylation were observed between an elite rice hybrid and its parentals [30], in phenotypically variable strains of *Brassica oleracea* [31], and among *Arabidopsis thaliana* accessions [10, 12]. The formation of the hybrid species *Spartina anglica* involved a large number of methylation changes, which as the authors noted, could play a role in the increased ecological breadth and morphological plasticity displayed by this species compared to the parental species [32]. Recently, a high level of inter-individual DNA methylation variation was detected in the violet (*Viola cazorlensis*), and variation among individuals was related to the amount of damage caused by herbivory [15]. Also, genetically identical dandelion (*Taraxacum officinale*) plants developed variation in DNA methylation in response to stressors (i.e., chemical induction of pathogen and herbivory responses), and many of these changes were stably inherited in the next generation [8]. These findings suggest that DNA methylation may provide an ecologically important source of phenotypic variation among individuals.

The house sparrow (*Passer domesticus*) is a promising organism in which to study the ecological importance of DNA methylation in wild vertebrates in response to introductions into new locations. The house sparrow has been successfully introduced throughout the world [33]. Phenotypic differentiation is extensive with populations exhibiting latitudinal and altitudinal clines in morphology, physiology, behavior, and life history characteristics in the native and introduced ranges [33–35]. Such extensive phenotypic diversification is surprising given that the short periods of time populations have had to adapt to new environments and the founder effects and/or bottlenecks that likely occurred with introductions (typically <150 years) [36].

Our study is the first yet for a wild bird species and the first to empirically investigate the role of epigenetic variation in introduced species. Relatively little information is available concerning DNA methylation in birds. However, several DNA methyltransferase enzymes and several putative DNA methyltransferase enzymes are present in the chicken genome [37], which suggests that DNA methylation is an active mechanism in birds. Our objective was to determine whether DNA methylation is variable in the house sparrow and if this variation could compensate for decreased genetic variation associated with introductions. This research is part of an ongoing effort to understand the causes of phenotypic variation among native and introduced populations [36, 38, 39]. We screened genomic CpG methylation using methylation-sensitive AFLP (MS-AFLP) among multiple individuals from Nairobi and Tampa. The MS-AFLP technique detects the methylation state of a particular recognition sequence. Thus, we were able (i) to establish if variation in DNA methylation occurs in a wild avian species, (ii) to characterize the variation that occurred among individuals and between populations, and (iii) to determine whether DNA methylation patterns differ between populations. We also could determine if DNA methylation in a wild vertebrate is similar to that observed among plants.

We screened individuals from Nairobi and Tampa because the two populations differed in time since introduction [33, 40, 41]: one introduced less than 50 years ago (Nairobi, Kenya) and one resident for about 150 years (Tampa, Florida, USA). House sparrows from these populations have different levels of genetic diversity at multiple microsatellite loci [36]. A sample from Nairobi had less genetic diversity than samples from the native European and introduced North American ranges [36], while the introduced North American populations screened, including Tampa, had similar genetic diversity as native populations. All populations screened were genetically differentiated, and the Nairobi sample was more strongly differentiated from the remaining sites, potentially because of a recent founder effect reducing genetic diversity in this area. Thus, the Nairobi sample has the genetic characteristics of a recent founder effect or bottleneck, likely associated with introduction, while the Tampa sample now has similar genetic diversity as native populations. We compared the relative amounts of epigenetic variation to genetic variation between Nairobi and Tampa. If Nairobi and Tampa have similar amounts of variation in DNA methylation, given that Nairobi has less genetic variation, it is possible that this epigenetic mechanism compensates for the decrease in genetic diversity associated with introductions as a source of phenotypic variation.

2. Materials and Methods

We collected house sparrow adults in Tampa, Florida, USA ($n = 16$) in spring 2008 and in Nairobi, Kenya ($n = 14$) in summer 2008. We bled individuals at capture, preserved the collected blood in a saline solution, and kept them at room temperature until DNA extraction with the DNeasy Kit

(Qiagen, Valencia CA). Our objective was to determine how DNA methylation varied among individuals. DNA methylation could differ among tissues, so we used the same type of sample, blood, extracted with similar methods, for all individuals. We selected blood to match ongoing research in the Martin Lab [33–35] focused on the role of the immune and stress response in house sparrow population expansion. We performed methylation-sensitive-amplified fragment length polymorphism (MS-AFLP) following a previously described protocol [42]. For MS-AFLP, we modified an AFLP protocol [43] by substituting methylation sensitive isoschizomeric enzymes MspI and HpaII for MseI. The enzymes MspI and HpaII have different sensitivities to cytosine methylation. Thus, if the AFLP protocol is performed independently for each enzyme for each individual, the resulting banding pattern indicates the methylation state of a particular restriction site. Both enzymes cut at a CCGG restriction sequences, but *Msp*I does not cut when the inner C is methylated, while *Hpa*II does not cut when the outer or both cytosines are methylated. Together, four different types of variation can be scored [31]; Type I both enzymes cut indicating that the restriction site is not methylated, Type II *MspI* does not cut, and *HpaII* does cut indicating that the restriction site has a methylated internal C, Type III *MspI* does cut and *HpaII* does not cut indicating that the restriction site has a methylated outer C, and Type IV neither enzyme cuts indicating that either both Cs are methylated or the restriction site has mutated.

We digested DNA extracts with both EcoRI/MspI and EcoRI/HpaII enzyme combinations independently by combining approximately 200 ng DNA with 10 U of both EcoRI and MspI, and 10 U of both EcoRI and HpaII independently, in a 20 μL reaction and incubated at 37°C for 6 hours (all restriction enzymes were from New England Biolabs Ipswich, MA, USA). We then ligated double-stranded EcoRI and Msp/HpaII adaptors to the digested fragments with T4 DNA ligase (New England Biolabs Ipswich, MA, USA). We conducted preselective PCR with primers designed for the EcoRI and MspI/HpaII adaptors (EcoRI 5′GACTGCGTACCAATTC; MspI/HpaII: 5′ATCATGAGTCCTGCTCGG) at a final volume of 25 μL. Preselective PCR products were diluted to 100 μg/μL. We used one primer combination for selective PCR (MspI/HpaII: 6-FAM-CATGAGTCCTGCTCGGTCCA, EcoRI: GACTGCGTACCAATTCCGCTG). We conducted selective PCR at a final volume of 10 μL; the thermal cycle was 95°C 2 m, 95°C 30 s, 53°C 30 s, 72°C 30 s, 70°C 5 m, repeated 40 times. We labeled the MspI/HpaII-selective primer with 6-FAM for visualization. We diluted PCR products 1 : 1 with loading buffer (deionized formamide, blue dextran EDTA, and MRK 500, The Gel Company San Francisco, CA, USA) and electrophoresed them on an ABI 377 (Applied Biosystems Foster City, CA, USA). We used GENESCAN 3.2.1 and GENOTYPER v 2.5 (Applied Biosystems Foster City, CA, USA) to analyze gel images and define band sizes.

We scored individuals at each enzyme combination and identified the type of epigenetic variation for each individual at each identified restriction site. We iterated the entire protocol twice for at least two individuals from each population in order to determine which restriction sites were reliably detectable. We adopted a conservative approach to scoring the gel images as AFLP-type reactions can generate variable banding among and within individuals. For a scored position to be considered reliable, the bands had to be identical and clearly distinguishable in each replicate of a given sample. Also, if subsequent reactions on additional samples generated inconsistent or unclear bands, or bands occurred at highly variable intensities at a site, that site was dropped from the analysis. We pooled data into two categories [31]: methylated (Type II and Type III) or not methylated (Type I, Type IV) restriction sites and constructed epi-haplotypes to characterize the state of DNA methylation at each site for each individual. Type IV variation at MS-AFLP could be generated either by epigenetic modification or a change in DNA sequence at the restriction site or by the gain/loss of an adjacent restriction site. Because it is not possible to accurately diagnose the underlying change, we did not include this state as methylated in our analysis.

We performed an AMOVA using GENALEX-6 [44] to calculate Φ_{ST} to characterize the amount of epi-haplotypic differentiation between Tampa and Nairobi. We conducted AMOVA over all restriction sites and independently for each restriction site. We also used BAYESCAN [45] to identify outlier loci as those potentially under selection. BAYESCAN compares a model with selection to one without selection for each restriction site. Bayes factors are calculated for each restriction site, and sites with positive Bayes factors are potentially under selection [45].

3. Results

Variation in DNA methylation was present among individual house sparrows (Table 1). Every individual had a unique epigenotype when all scored restriction sites were considered. We could confidently score 23 variable restriction sites from the 50 banding positions between 70 and 250 base pairs in length for both restriction enzymes. There were differences in DNA methylation among individuals at each of the 23 restriction sites. Type I (no methylation) and Type II variation occurred in certain individuals at all of the 23 restriction sites in both Nairobi and Tampa. Type III variation only occurred in certain individuals at 11 restriction sites, while Type IV variation occurred in certain individuals at 19 restrictions sites.

When the type of variation in DNA methylation was considered between locations, each type occurred in differing proportions (Table 1). Type I variation was more frequent at 14 restriction sites in Nairobi and 9 in Tampa. Type II variation was more frequent at 12 restriction sites in Nairobi and 11 in Tampa. Type III variation was in higher frequency at 5 restriction sites in Nairobi and 6 in Tampa. One restriction site had Type III variation only in Nairobi and 6 restriction sites only in Tampa. Type IV variation was more frequent at 5 restriction sites in Nairobi and at 12 sites in Tampa. One restriction site had Type IV variation only in Nairobi, and five restriction sites had Type IV variation only in Tampa.

Table 1: Frequency of epigenetic variation detected by MS-AFLP at the restriction site CCGG. The type of epigenetic variation is presented following Salmon et al. [31]; Type I = restriction site no methylation, Type II = methylation of internal C, Type III = methylation of external C, and Type IV = hypermethylation or mutation in restriction site.

	Restriction site																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Nairobi																							
Type I	0.79	1	0	0.64	0.21	0.79	0.93	0.29	0.64	0.71	0.86	0.29	0.21	0.43	0.86	0.5	0.5	0.07	0.86	0.71	0.64	0.79	0.71
Type II	0.21	0	0.21	0.14	0.5	0.21	0.07	0.5	0.21	0.14	0.14	0.64	0.14	0.29	0.14	0.14	0.36	0.21	0.07	0.21	0.36	0.21	0.14
Type III	0	0	0.14	0.07	0	0	0	0	0	0.07	0	0	0	0	0	0	0	0.07	0	0	0	0	0.14
Type IV	0	0	0.64	0.14	0.29	0	0	0.21	0.14	0.07	0	0.07	0.64	0.29	0	0.36	0.14	0.64	0.07	0.07	0	0	0
Tampa																							
Type I	0.88	0.94	0.38	0.31	0.5	0.88	0.75	0.25	0.75	0.44	0.88	0.5	0.19	0.13	0.63	0.69	0.63	0	0.81	0.63	0.31	0.69	0.44
Type II	0.13	0.06	0.19	0.38	0.38	0.06	0.19	0.63	0	0.38	0.06	0.25	0.19	0.19	0.25	0.06	0.25	0.06	0.13	0.13	0.63	0.31	0.38
Type III	0	0	0.13	0	0.06	0.06	0	0	0	0.06	0	0	0.06	0	0	0.06	0	0	0.06	0.06	0	0	0.06
Type IV	0	0	0.31	0.31	0.06	0	0.06	0.13	0.25	0.13	0.06	0.25	0.56	0.69	0.13	0.19	0.13	0.94	0	0.19	0.06	0	0.13

All restriction sites had different proportions of methylation among individuals between Nairobi and Tampa (Figure 1). Twelve restriction sites ($n = 12$) had a higher frequency of methylation in Nairobi, while 11 had a higher frequency of methylation frequency in Tampa. Restriction site 2 was only methylated in Tampa, and restriction site 9 was only methylated in Nairobi.

The AMOVA performed over all restriction sites did not detect significant differentiation between Nairobi and Tampa ($\Phi_{ST} = 0.001$, $P = 0.420$; Table 2). When AMOVA was calculated restriction site-by-restriction site however, two restriction sites had stronger Φ_{ST} estimates (site 9 $\Phi_{ST} = 0.17$, $P = 0.09$; site 12 $\Phi_{ST} = 0.22$, $P = 0.06$). The two restriction sites had higher proportions of methylation in Nairobi (Figure 1, sites 9 and 12). BAYESCAN also identified these two loci as the strongest outliers (Site 9 Bayes Factor = 0.04; Site 12 Bayes Factor = 0.03); however, the Bayes Factors were relatively weak.

Table 2: Summary AMOVA table for the comparison among all sites between house sparrows from Florida and Kenya (d.f.: degrees of freedom).

Source	d.f.	Sum of squares	Mean square	Estimated variance
Among populations	1	4.107	4.107	0.004
Within populations	28	113.393	4.050	4.050
Total	29	117.500		4.054
Φ_{ST}	0.001			
P	0.420			

4. Discussion

Epigenetic variation, in the form of DNA methylation at CpG sites, occurred in wild house sparrows. This variation could be screened with a simple and reliable MS-AFLP technique. A great deal of variation occurred among individuals, and all screened individuals had unique epigenotypes. All types of methylation were present in both populations, indicating that both epigenetic and genetic variation (indicated by Type IV variation) exists within and between Nairobi and Tampa populations. We observed more methylation overall in the Nairobi population; however, some restriction sites were more methylated in Tampa, and most restriction sites had different frequencies of methylation between Nairobi and Tampa. Nairobi and Tampa had more similar levels of variation in DNA methylation than at microsatellite loci, where Nairobi had fewer alleles, lower heterozygosity, and more private alleles than Tampa [36]. Thus, it is possible that epigenetic variation may provide a source for the phenotypic diversity found in the more recently introduced populations and compensate for the decreased genetic variation.

When the frequency of methylation was compared between populations at all restriction sites, no significant difference was detected, and the amount of within-population variation was much greater than the between-population variation. Given the amount of variation detected and that the state of DNA methylation could change in opposing ways at each site (i.e., from methylated to nonmethylated at one site, yet from unmethylated to methylated at another), our results have suggested it would take a great deal of statistical power to detect differences among all restriction sties. However, we identified two restriction sites with a stronger signal differentiating Nairobi and Tampa, and these sites approached statistical significance, indicating that screening additional MS-AFLP selective primer combination could identify sufficient restriction sites to discriminate locations. These findings suggest that there may be a complicated relationship among variable restriction sites and that only a few of the variable sites may be ecologically important (sensu [14]). Thus, the amount of within-population variation observed across presumably mostly neutral loci was so great that we were not able to detect an overall signal of differentiation between populations. As in the recent study of *V. cazorlensis* populations [14, 15], our results suggest that MS-AFLP data may require attention to detect atypical outlier loci, which are important for a particular trait, yet are only a subset among several variable restriction sites. Our analysis suggests

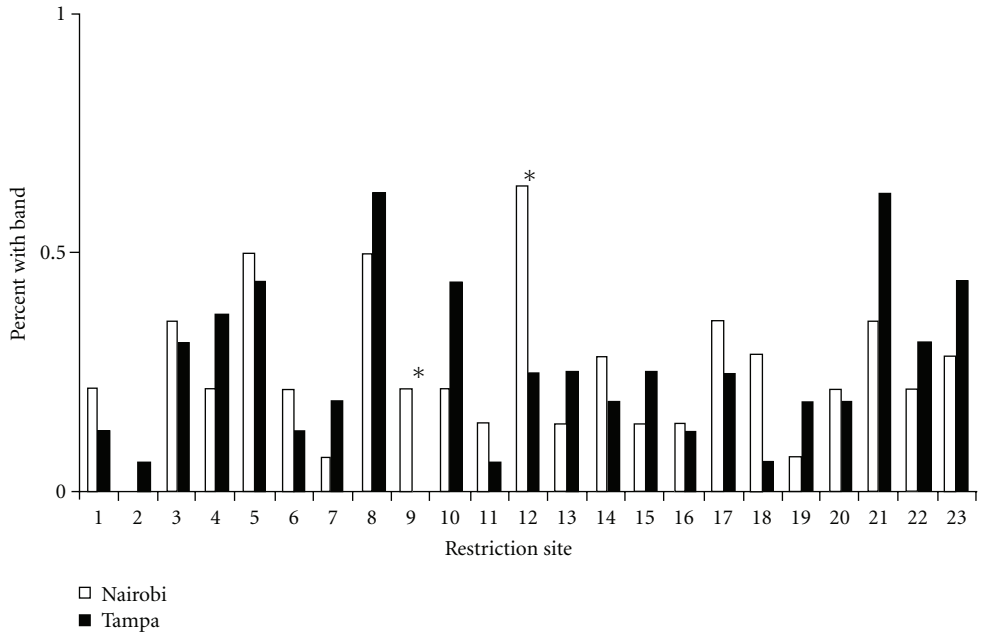

Figure 1: Comparison of percentage of house sparrow individuals with methylated CpG sites (Type II and Type III combined) at 23 restriction sites between Tampa ($n = 16$, black bars) and Nairobi ($n = 14$, white bars). An asterisk indicates the two outlier loci, restriction sites 9 and 12.

that it would be very difficult to detect differences between populations without scanning for outliers.

It is possible that in some cases the differences between MspI/EcoRI and HpaII/EcoRI reactions could have been generated by inconsistent restriction digests rather than differential methylation. However, inconstant digestion could generate false methylated or false unmethylated results, and we performed a long restriction digestion (6 h) and used a conservative scoring approach to minimize potential errors. Thus, given the high level of variation detected, the main conclusions of this study would not be affected greatly by a modest error rate in restriction digests.

Substantial phenotypic divergence has occurred among introduced house sparrows within 150 years [33–35]. Epigenetic variation is a potential mediator of rapid evolution of introduced species to new environments [17] and has been linked to phenotypic variation [4, 46]. Together, the presence of MS-AFLP variation in house sparrows and the persistence of latitudinal patterns of phenotypic variation among introduced populations predominantly coming from western European sources [35] support the possible role of epigenetic variation as a mediator of phenotypic diversity in introduced populations.

5. Conclusions

Given the amount of variation observed, it is plausible that epigenetic variation may compensate for decreased genetic variation as a source of phenotypic variation within introductions. It is also plausible that epigenetic variation may be responsible for some of the phenotypic differentiation among individuals. However, the MS-AFLP technique alone does not allow for the identification of the specific underlying genetic elements that are methylated, nor does it identify the effects (i.e., silencing or enhancing) on gene expression. Also, DNA methylation likely has multiple functions in addition to the possible role in phenotypic differentiation (e.g., silencing transposable elements). Thus, it may be that only a small subset of fragments screened with the MS-AFLP technique may regulate expression of genes determining phenotypic traits. Also, we screened DNA methylation in blood samples, and additional variation in DNA methylation may occur in other tissues, and the variation in other tissues could occur in different patterns. However, the level of variation detected in blood suggests that DNA methylation would be variable in other tissues. Additional research will be critical to characterize epigenetic variation at restriction sites that are functionally related to phenotypic differences, but presently only a few examples exist in which gene methylation has demonstrable phenotypic effects in animals [47]. Our labs are presently investigating the effects of methylation of the glucocorticoid receptor promoter on brain and behavior (sensu [48]), and the present study demonstrates that such an effort could be fruitful in house sparrows.

Also, as this study is a two-population comparison, it is premature to conclude that differential methylation is pervasive among sparrow populations. However, the extensive phenotypic variation despite moderate genetic differentiation that exists among populations of introduced house sparrows [36] indicates that epigenetic modification could be important. Simple next steps to assess the relevance of methylation would entail comparisons (i) among species with different levels of introduction success, (ii) populations at the edges and centers of ranges, or (iii) populations that differ

in time since introduction/colonization. It may also be informative to characterize the methylation present in different tissues at different times after a stimulus (i.e., a stressor or immune challenge), as this approach could implicate the restriction sites and hence the genes that contribute directly to phenotypic variability.

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Review Article

Daphnia as an Emerging Epigenetic Model Organism

Kami D. M. Harris, Nicholas J. Bartlett, and Vett K. Lloyd

Department of Biology, Mount Allison University, 63B York Street, Sackville, NB, Canada E4L 1G7

Correspondence should be addressed to Vett K. Lloyd, vlloyd@mta.ca

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Daphnia offer a variety of benefits for the study of epigenetics. *Daphnia's* parthenogenetic life cycle allows the study of epigenetic effects in the absence of confounding genetic differences. Sex determination and sexual reproduction are epigenetically determined as are several other well-studied alternate phenotypes that arise in response to environmental stressors. Additionally, there is a large body of ecological literature available, recently complemented by the genome sequence of one species and transgenic technology. DNA methylation has been shown to be altered in response to toxicants and heavy metals, although investigation of other epigenetic mechanisms is only beginning. More thorough studies on DNA methylation as well as investigation of histone modifications and RNAi in sex determination and predator-induced defenses using this ecologically and evolutionarily important organism will contribute to our understanding of epigenetics.

1. Introduction

The unusual life cycle of the freshwater microcrustacean, *Daphnia*, has been studied for more than 150 years [1]. Most species are cyclic parthenogens able to produce two types of eggs, diploid parthenogenetic eggs or haploid sexual eggs, in response to environmental cues [2, 3]. Sex determination is likewise environmentally controlled; males are produced in response to suitable environmental cues [3]. Additionally, *Daphnia* exhibit a range of spectacular polyphenisms, phenotypic alternations including helmet and neckteeth formation, in response to predators [4, 5]. This makes *Daphnia* an excellent candidate for studying environmental influences on epigenetic developmental programs. Most importantly in the context of epigenetics, clonal lines are genetically identical yet consist of phenotypically divergent individuals. This offers a unique opportunity to separate genetic and epigenetic influences on the phenotype, an invaluable asset when studying epigenetics. The attractiveness of *Daphnia* as a potential epigenetic model organism is further enhanced by the fact that they are easy and inexpensive to maintain and have a rapid life cycle. As a primary consumer and a food source for invertebrates and fish [6], there is an extensive body of literature on their ecological role, development, and the evolution of parthenogenesis. Thus, *Daphnia* is an ecologically important organism well-studied in the context of evolution, ecology, ecotoxicology, predator-induced polyphenisms, and genomics [7, 8] and offers unparalleled opportunities to study epigenetics in these biologically important processes.

Epigenetics is the study of mitotically or meiotically heritable changes in phenotypes that occur without changes in the DNA sequence [9]. Altered gene expression can be caused by DNA methylation, histone modifications, and RNA interference as well as other, less well-studied, epigenetic mechanisms such as variant histones, nucleosome phasing, higher-order chromatin structures, and nuclear localization [4, 9].

DNA methylation, performed by either *de novo* or maintenance DNA methyltransferases, has been associated with transcriptional regulation, chromosome inactivation, and transposable element regulation, among other functions [10]. Although DNA methylation is found in a wide variety of eukaryotes, the amount of methylation and its organization within the genome differ dramatically between species and developmental stages [4]. DNA methylation interacts with other epigenetic processes [11]. Modifications to the amino- or carboxyl-terminal histone tails affect the interactions of histones with DNA, other histones, and other

chromatin-associated proteins [12]. These modifications are performed by specialized enzymes and include acetylation, ubiquitination, sumoylation, phosphorylation, and methylation, all of which can alter gene expression [12]. DNA methyltransferases and histone modifying enzymes can recruit each other by way of a mutual attraction to the modifications imposed by the other [11]. DNA methylation and histone modifications also interact with the RNA silencing system [13]. The RNA silencing system operates through the production of small noncoding RNA molecules (ncRNA) and is referred to as RNA interference (RNAi). Small RNAs, microRNA (miRNA) and short interfering RNA (siRNA) are excised from larger double-stranded molecules can form RNA-induced silencing complexes (RISC) that target complementary nucleic acid sequences and recruit or activate DNA methyltransferases and histone modifying enzymes [14].

Epigenetic marks are modified by external environmental factors such as nutrition and exposure to chemicals, as well as developmental cues [15]; these epigenetic alterations can enhance the cell and organism's ability to respond to its environment and thrive [16]. DNA methylation, histone modifications, and RNAi are all mitotically transmissible. Additionally, as epigenetic changes can be adaptive, selection for meiotic transmission might be expected to allow epigenetic information to be passed between generations [4]. Such transgenerational inheritance has been documented in *Arabidopsis* [17], mice [18], *Drosophila* [19], and humans [20, 21] and is postulated in *Daphnia* [16]. However, identification of transgenerational effects can be problematic when the embryo undergoes development in the mother's body, as is the case in *Daphnia*. In such situations, maternal exposure to environmental factors could affect the offspring either by retention of maternal epigenetic states in the germ line cells that give rise to the embryo, a true transgenerational effect, or more simply by exposure of the somatic cells of the embryo while it is in the mother. To resolve this ambiguity, the persistence of the trait needs to be monitored in the F3 and subsequent generations, those which were not exposed as either the embryos that produce the F1 or the embryonic germ line that produce the F2.

Spurred by the use of *Daphnia* as a subject of ecological and developmental research, numerous techniques have been developed that can equally enhance its use in epigenetic studies. Conventional cytological methods have been employed [22] and more recently these have been extended to include fluorescence *in situ* hybridization (FISH) [23]. This could allow examination of higher-order chromatin structures that have been associated with the epigenetic status of genome regions in other animals [24]. Recently *Daphnia pulex* was the first crustacean to have its genome sequenced, revealing the largest number of genes yet found in a single organism, yet present in a remarkably compact genome [25]. The large number of genes is due to a very high rate of tandem gene duplication events, and approximately 30% of the genes are unique to *Daphnia* [25]. The availability of the genome sequence allows for the development of microarrays for genome-wide transcriptional studies [26]. *Daphnia* embryos are transparent and can develop independently of the mother, and as a result embryogenesis of *Daphnia* has been well documented [2, 27, 28]. With the genomic sequence available, conventional embryology can be extended to look at specific gene products. Methods for *in situ* immunohybridization and immunohistology have been developed so the tissue- and developmental-specific localization of RNAs and proteins can be examined [29]. In the context of epigenetics, this approach could be used to detect developmental and tissue-specific histone modifications. While there are no immortalized cell lines currently available for *Daphnia*, methods for primary culture have been developed [30]. These cells are viable for at least one week and can be transformed to study the role of overexpression of endogenous or foreign genes [30]. More recently, Kato et al. [31, 32] showed that it is possible to insert double-stranded RNA to reduce the expression of genes by RNAi-based gene knockdowns. The same technique can be used to overexpress selected genes [33]. Knockdown of specific genes encoding for DNA methyltransferases, histone modifying enzymes, and their interacting proteins would allow for an assessment of the role of DNA methylation, histone modification, and related epigenetic processes correlated with the well-defined phenotypes that arise from epigenetic alterations.

2. The *Daphnia* Life Cycle and Epigenetic Phenotypic Variation

2.1. The Daphnia Life Cycle. Most *Daphnia* can reproduce either asexually or sexually, depending on environmental cues. In both cases, eggs are produced by stem cells in the ovary [2]. In sexual eggs, meiosis is conventional and the haploid oocytes are fertilized. Parthenogenetic oocytes undergo only the equational meiotic division and so remain diploid and embryogenesis occurs without fertilization. Early embryogenesis commences as the egg matures on route to the brood pouch. Sexually produced eggs are typically produced in pairs, arrest in the blastula stage in the brood pouch, and the carapace overlying the brood pouch is modified into a tough, desiccation-resistant structure called the ephippium, which allows the eggs to survive harsh environmental conditions [2]. Parthenogenetic eggs complete embryogenesis in the brood pouch and are released as miniature versions of the adult [2]. Once hatched, the neonates typically undergo four to six larval instars, depending on species, before reaching reproductive maturity (Figure 1) [7, 38].

2.2. Epigenetic Regulation of the Life Cycle. Epigenetic changes in gene expression can modify an organism's phenotype and these changes are particularly obvious when there are no genetic differences between individuals of any one strain. Sensitivity of the epigenome to environmental cues occurs at different stages of the *Daphnia* life cycle. In general, the embryonic stages appear important for establishing the epigenetic states of genes involved in phenotypic variation, whereas exposure to environmental cues in the postembryonic larval stages is important for maintaining the epigenetic state (Figure 1).

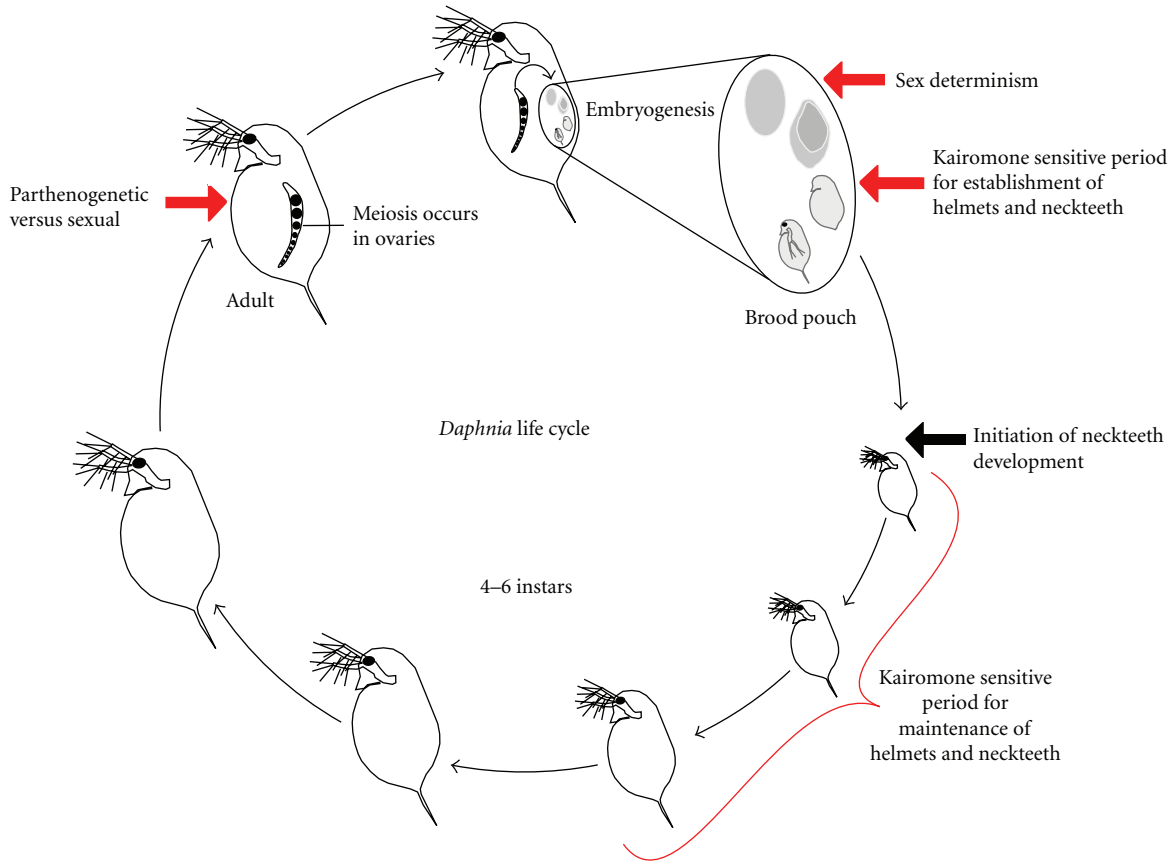

Figure 1: The *Daphnia* life cycle. The life cycle is shown with the stages at which the epigenome is sensitive to the environmental inputs that regulates sexual reproduction, sex determination, helmets, and neckteeth (indicated in red).

The production of sexual versus asexual eggs is environmentally cued by environmental factors such as photoperiod, temperature, food abundance, and crowding [3]. In sexual eggs meiosis is conventional whereas asexual parthenogenetic eggs arise from an abortive first meiotic division, resulting in diploid eggs able to initiate development in the absence of fertilization [2]. In parthenogenetic eggs the first division is abortive; however, many of the same meiotic genes are expressed in parthenogenetic as in sexual reproduction [39] and bivalents are produced [40]. Nevertheless, genes suppressing recombination are overrepresented in the *Daphnia* genome relative to those promoting recombination [39], chiasmata are not observed [40] and genetic evidence of recombination has not been observed [41]. Thus, barring rare conversion, mitotic recombination or mutation events [42] parthenogenetic progeny are genetically identical. Since the ovary can simultaneously contain parthenogenetic and sexual eggs [2], the cues must act during the first meiotic division, as the oocytes form (Figure 1). How these environmental signals are interpreted and the molecular mechanism by which meiosis is regulated, remains unknown. The production of males is triggered by similar environmental cues as sexual egg production [3]; however, the control of male sex determination is independent of the regulation of female meiosis [2, 43]. Males are produced in either mixed or, more typically, all-male broods [3, 44, 45] and at least in some species can emerge from fertilized sexual ephippial eggs [46]. Despite obvious morphological differences—males being smaller, having testes, modified appendages, and carapace—all parthenogenetic offspring, male or female, and their mothers, are genetically identical. The mechanism of sex determination is thus clearly environmental and epigenetic. As juvenile hormone analogs induce males even in the absence of environmental cues, this suggests environmental cues are transduced by the endocrine system [33, 45]. Based on the production of intersexes in *D. magna* and *D. longispina*, induced by altered temperature or intermediate hormone concentrations, respectively, the determinative events in sex determination have been shown to act in oocyte maturation before the first embryonic division [44, 45]. Interestingly, Sanford [44] shows that intersex progeny are produced in broods long after the mother has been moved from the inducing conditions. This underscores the epigenetic nature of sex determination and might represent an example of transgenerational inheritance, but could equally reflect the early developmental action of the sex determination process. Many genes show differential expression between males and females [47], including the core sex-determination gene, *doublesex,* that is expressed at higher levels during embryogenesis in males than in females [33]. This suggests that, in *Daphnia,* environmental sex determination arose by imposing environmentally mediated

regulation on the conserved *doublesex* genetic sex determination pathway. Identification of differences in the epigenetic status of the *doublesex* gene in males and females would further our understanding of environmental sex determination and the role of epigenetics in such a key aspect of the life cycle.

3. Epigenetic Regulation of Helmet Formation

Predators are an important aspect of an organism's environment, and various predator-induced defenses, such as helmets, have been well documented in *Daphnia* [16]. Helmets are cranial extensions of the exoskeleton that have been shown to decrease the daphnids' chances of predation [48]. Helmet growth is induced by kairomones, which are aquatic chemicals released by predators [48]. Circulating kairomones can double the relative helmet size in some daphnids [49].

Agrawal et al. [16] have shown that kairomones induce helmet growth in *Daphnia cucullata* both in the generation exposed to the kairomones and in their nonexposed progeny (Table 1). Newly hatched animals were exposed to kairomone-containing water, or control non-kairomone water and the size of helmets were monitored. Additionally, females were exposed and successive broods of their progeny were monitored for helmet production to detect transgenerational effects.

Exposure of neonates to kairomones induced helmet formation and removal of kairomones reduced helmet size [16]. This shows that kairomones act directly during early larval stages to promote helmet growth. Interestingly, when mothers were exposed, helmets were present in their neonate progeny, even if the progeny were not exposed [16]. Helmet formation in the neonates following only maternal exposure, could arise either from a transgenerational effect, transmission of the altered maternal epigenome to the F1 progeny via the oocyte, or, as the embryos are brooded in the mother, sensitivity of the embryonic somatic cells to kairomones. The latter is suggested by the fact that final helmet size is diminished in successive broods, which would have been younger, with fewer somatic cells, at the time of exposure, and that the F2 was not strongly influenced by grandmaternal exposure [16]. This finding also implies that late embryonic stages are more sensitive than earlier ones.

The effect of kairomone exposure on helmet size was cumulative; the largest helmets were obtained when both the mother and the neonates were exposed [16]. This additive effect indicates that both stages are sensitive. The possibility of different epigenetic events contributing to cuticular growth during embryonic and larval stages is suggested by similar studies on neckteeth formation (see below) [48]. Growth of the helmet is accomplished by mitotic division of diploid epidermal cells, thought to be triggered by signals from adjacent polyploid epidermal cells [50]. It is possible that kairomone exposure during late embryonic stages induces cell fate changes producing more polyploid cells whereas kairomone exposure during the larval stages increases the mitogenic activity of these polyploid cells.

4. Epigenetic Regulation of Neckteeth Formation

Another common predator-induced defense is exhibited by several species, including *Daphnia pulex*. In the presence of kairomones produced by *Chaoborus* (phantom midge) larvae, *Daphnia pulex* produces structures known as neckteeth, small protrusions on the neck region accompanied by a strengthened carapace [48, 51]. Daphnids that have these outgrowths have a higher predator escape rate, presumably due to the thickened exoskeleton that makes handling and consumption more difficult [48]. Development of the neckteeth begins in the first larval instar and growth continues until the third instar [52]. Withdrawal of the predatory cue at the first, second, or third instar reduces the number of neckteeth at successive instars [52]. Thus, the maintenance of epigenetic marks on the genes controlling the growth of neckteeth requires kairomone exposure in the larval stages [52]. However, Miyakawa et al. [51] were able to show that production of neckteeth involves at least two additional critical stages in late embryonic development. Few or no neckteeth form when kairomones are absent during embryogenesis, even if kairomones are present during the postembryonic larval stages [50]. Thus, as for helmet formation, embryonic exposure appears to be required to establish cell fates, while larval exposure is required to maintain and express the phenotype. *Differential Display 1* (*DD1*) is a gene identified as having altered expression in the embryonic stage in kairomone-exposed daphnids [51]. It is proposed that *DD1* plays a role in kairomone reception and/or cell fate determination that establishes the epigenetic state of target genes leading to the formation of neckteeth [51]. Multiple endocrine and morphogenetic genes, such as *Hox3, exd, JHAMT, Met, InR, IRS-1, DD1, DD2,* and *DD3,* were shown to be upregulated in the exposed postembryonic larvae [51]. The *Hox* gene upregulated in kairomone-exposed daphnids encodes a transcription factor associated with chromatin [53]. The *exd* and *met* gene products can similarly act as transcription factors and potentially alter the epigenetic status of downstream genes [54, 55]. Thus, the upregulation of these genes supports the conclusion that the maintenance and growth of neckteeth production is a result of epigenetic changes.

5. Epigenetic Regulation of Growth

In much the same way that external environmental cues such as kairomones can affect the development of helmets and neckteeth, environmental toxicants can affect the body length and growth in *Daphnia magna* [34]. Again, as the animals are all genetically identical, differences between exposed and nonexposed animals must be epigenetic. Among many others, 5-azacytidine, genistein, biochanin A, vinclozolin, and zinc, all of which can alter DNA methylation, were shown to have an effect on body length (Table 1) [34, 56]. This growth effect, however, was transient as it was only seen in 7-day-old animals but not adults [34]. Additionally, zinc exposure significantly reduced body length of 6-day-old animals in the untreated F1 generation [56]. This finding

Table 1: Epigenetic assay systems.

Assay system	Species	F0 treatment	F0 effects	F1	F2	Reference
Helmets	*D. cucullata*	Kairomones	n.d.	Increase	Increase	[16]
	D. cucullata	Kairomones	Increase	n.d.	n.d.	[29]
	D. lumholtzi	Kairomones	Increase	n.d.	n.d.	[29]
	D. ambigua	Kairomones	Increase	n.d.	n.d.	[29]
Neckteeth	*D. pulex*	Kairomones	Increase	n.d.	n.d.	[29]
Growth	*D. magna*	5-azacytidine	Decrease (day 7 only)	Decrease	Decrease (day 7 only)	[34]
		Genistein	Decrease	n.s.	n.s.	[34]
		Vinclozolin	Decrease	n.s.	n.s.	[34]
		Zinc	Decrease (day 6 only)	Decrease (day 6 only)	n.s.	[35]
Reproduction	*D. magna*	5-azacytidine	Decrease	Decrease	n.s.	[34]
		Genistein	Decrease	n.s.	n.s.	[34]
		Vinclozolin	n.s.	n.s.	n.s.	[34]
		Zinc	Decrease	n.s.	n.s.	[36]
Global DNA methylation	*D. magna*	Zinc	n.s.	Decrease	Increase	[37]
		5-azacytidine	Decrease	Decrease	Decrease	[34]
		Genistein	n.s.	n.s.	n.s.	[34]
		Vinclozolin	Decrease	n.s.	Decrease	[34]

Data summarized here is for a treated F0 generation with subsequent generations untreated. n.s. denotes nonsignificant results. n.d. denotes that those trials were not done.

might be an indication of a transgenerationally heritable effect but as it did not persist to the F2 generation, it is more likely the result of embryonic exposure (Table 1).

6. Epigenetic Regulation of Fertility

Fertility was also shown to be affected by chemical treatment. While vinclozolin exposure had no significant effect, 5-azacytidine, 5-aza-2′-deoxycytidine, genistein, biochanin A, and cadmium all reduced reproduction in surviving females, in comparison to nonexposed females (Table 1) [34, 57]. Zinc exposure was found to have complex effects; exposure decreased reproductive success in the F0, but not in the subsequent F1 and F2 generations when these were raised in control medium (Table 1) [36]. When animals were continuously exposed to zinc, reproduction was reduced in the F0 and F1 but not the F2 [36]. These results were interpreted as an acclimation effect [36], which would be interesting; however, this conclusion would be strengthened by results from a larger number of reproducing females and corroborating molecular data.

The effects of chemical exposure occurred in genetically identical individuals and in some cases were heritable between generations, suggesting that the phenotypic variability is epigenetic. This possibility is reinforced by the fact that some of these chemicals have been shown to alter DNA methylation [34].

7. Epigenetic Mechanisms—DNA Methylation

The role of epigenetic mechanisms such as DNA methylation, histone modification, and RNAi in normal *Daphnia* development and the epigenetic adaptations described above is still in its infancy. Vandegehuchte et al. [57] were the first to determine that *D. magna* is capable of methylating DNA. They found genes homologous to the three main human DNA methyltransferases and confirmed that DNA methylation occurred. Through the use of ultraperformance liquid chromatography (UPLC) and microarrays, Vandegehuchte et al. [34] examined the DNA methylation and transcription levels, respectively, in *D. magna* exposed to various chemicals. They measured direct effects on methylation in the exposed generation as well as the effects in subsequent generations (Table 1). Global or localized DNA methylation levels were found to be affected by 5-azacytidine, vinclozolin, genistein, and zinc but were not affected by 5-aza-2′-deoxycytidine, biochanin A, and cadmium [34, 57].

5-azacytidine is known to hinder DNA methylation in humans by inhibiting DNA methyltransferases and, consistent with this, *D. magna* treated with 5-azacytidine showed a decrease in global DNA methylation [34, 58]. Interestingly, the untreated offspring of 5-azacytidine exposed daphnid mothers also showed decreased methylation when compared to nonexposed daphnids of the same generation (Table 1). Vandegehuchte et al. [34] interpreted this as a transgenerational effect; however, the F1 were exposed to the toxicant as embryos, a time shown to be sensitive to epigenetic perturbations in many animals [20, 59, 60] including *Daphnia* [51] so these results are more likely due to exposure of the F1 as embryos rather than a true transgenerational effect. Conclusive evidence for a transgenerational effect would be the persistence of the effect into nonexposed generations beyond the F2, a result not observed in this series of experiments. The sensitivity of this experiment and confirmation

of any transgenerational effects would be enhanced by examination of gene-specific epigenetic alterations as opposed to global DNA methylation levels, and monitoring changes persisting to the F3 and subsequent generations.

In comparison to nonexposed daphnids, when the F0 was exposed to zinc, there was decreased methylation of the F0 and F1 generations followed by a significant increase in the F2 generation (Table 1) [37]. Vandegehuchte et al. [37] attributed the increase in the third generation to acclimation. While possible, this explanation cannot be confirmed until the study is repeated with a larger sample size. Additionally, as age affects DNA methylation levels in *Daphnia* [37] the age of the daphnids would have to be tightly controlled. Treatment with vinclozolin showed a significant decrease in DNA methylation in *D. magna* in the F0 and F1 exposed generations; however, the F2 showed a nonsignificant increase in overall methylation levels [34]. This implies that while the fungicide vinclozolin does alter DNA methylation, evidence for a transgenerational effect is still lacking. Unusual results were seen with genistein treatment. In mammals, genistein causes global DNA hypomethylation [61] but in *D. magna* it yielded hypermethylated DNA [34]. This confounding result could be attributed to differences in genomic organization between mammals and daphnids, the possibility exists that the sequences that are hypermethylated in the much larger daphnid genome do not exist in humans.

The microarray platform used for these studies was originally designed for investigation of developmentally regulated genes and allowed monitoring of only a subset of those genes, so it was not ideal for global transcription assessment [36]. Until the *D. magna* genome is fully sequenced and a more complete microarray can be employed, it would be preferable to monitor specific genes or to use a species with a fully sequenced genome, such as *D. pulex*. Additionally, bisulfite sequencing, methylated DNA immunoprecipitation (meDip), or DNA methylation sensitive restriction enzyme digests, which allow monitoring of the methylation status of individual genes would be more biologically informative. Candidate genes include those that are involved in reproduction and growth since brood size and body length is affected by toxicant exposure in *D. magna* [34, 36, 56, 57], sex determination [47], as well as those involved in helmet and neckteeth formation [16, 51].

8. Conclusion

Daphnia have the potential to be invaluable animals for epigenetic study. They are already well-studied in the context of their important ecological and evolutionary roles, as well as being readily available and inexpensive to maintain. The ability of *Daphnia* to produce clones parthenogenetically allows for the elimination of genetic variability, a valuable resource in the study of epigenetics. Obvious phenotypic assay systems such as sexual reproduction, helmets, neckteeth, growth, and fertility allow correlations to be made between such phenotypic responses and the epigenetic changes that accompany them (Table 1). Further, potential transgenerational effects in the production of polyphenisms, intersex individuals, and other epigenetically determined states remain to be explored [44, 45]. There are also many classical and molecular tools available for use in studying epigenetics in *Daphnia*.

The next steps in establishing *Daphnia* as an epigenetic model organism will be to determine the genetic and epigenetic mechanisms responsible for the establishment and maintenance of phenotypic responses to the environment such as sexual reproduction, helmets, and neckteeth. It is also essential to extend the research on epigenetic mechanisms to include histone modifications, RNAi, and further define the baseline levels and changes in DNA methylation in response to environmental stimuli throughout development. Documenting the epigenetic differences between sexual and asexual *Daphnia* and stressed and unstressed individuals would prove a fruitful area of research with important implications for evolutionary and developmental biology.

Authors' Contribution

All authors contributed to the writing of this review. Kami D. M. Harris and Nicholas J. Bartlett contributed equally.

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Research Article

General-Purpose Genotype or How Epigenetics Extend the Flexibility of a Genotype

Rachel Massicotte[1] and Bernard Angers[1, 2]

[1] *Département de Sciences Biologiques, Université de Montréal, C.P. 6128, succursale Centre-ville, Montréal, QC, Canada H3C 3J7*
[2] *Group for Interuniversity Research in Limnology and Aquatic Environment (GRIL), Trois-Rivières, Qc, Canada G9A 5H7*

Correspondence should be addressed to Rachel Massicotte, rachel.massicotte@umontreal.ca

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This project aims at investigating the link between individual epigenetic variability (not related to genetic variability) and the variation of natural environmental conditions. We studied DNA methylation polymorphisms of individuals belonging to a single genetic lineage of the clonal diploid fish *Chrosomus eos-neogaeus* sampled in seven geographically distant lakes. In spite of a low number of informative fragments obtained from an MSAP analysis, individuals of a given lake are epigenetically similar, and methylation profiles allow the clustering of individuals in two distinct groups of populations among lakes. More importantly, we observed a significant pH variation that is consistent with the two epigenetic groups. It thus seems that the genotype studied has the potential to respond differentially via epigenetic modifications under variable environmental conditions, making epigenetic processes a relevant molecular mechanism contributing to phenotypic plasticity over variable environments in accordance with the GPG model.

1. Introduction

Over the years, the debate about the evolutionary advantage of sexual over asexual reproduction has focused in part on the higher adaptive potential of populations with standing genetic variation [1] (and references therein). Each generation, the reproduction of amphimictic organisms results in genetic mixing, thus creating a multitude of new genotypes (and potentially novel phenotypes) in natural populations. While in sexually reproducing organisms each individual possesses a different genotype, asexually reproducing individuals from the same clonal lineage are presumed to be genetically identical.

On the other hand, asexuality has some advantages of its own; there is no need to produce males, and asexual populations can double their size each generation [2]. This twofold advantage of asexual reproduction is thought to be constrained by their limitation in colonizing new environments and/or when living in temporally unstable or heterogeneous environments. In such conditions, the survival, flexibility, and adaptive potential of asexual lineages are aspects that are not well understood. The general-purpose genotype (GPG) model [3] (Figure 1(a)) proposed that evolutionary success of asexual organisms could be possible via generalist lineages selected for their flexible phenotypes utilizing wide ecological niches. Such phenotypic flexibility enables a given genotype to be successful in many different and variable environments [4, 5]. Other models, such as the frozen niche variation (FNV) model [6], rely on the existence of genetic diversity among multiple highly specialized clonal lineages within a population each having respective narrow ecological subniches to explain the maintenance of asexual lineages. Each specialist lineage persists through time by partitioning of available ecological space so as to avoid clonal competition. However, microniche models do not provide explanations for how single clonal lineages can be successful across different and temporally variable environmental conditions.

One of the process underlying the GPG model is the concept of phenotypic plasticity, an environmentally induced phenotypic difference that occurs within an organism's lifetime in the absence of genetic variation [7] (but see [8]). Epigenetic variation potentially represents a molecular mechanism that can generate phenotypic plasticity under natural environmental conditions [9]. The modification of

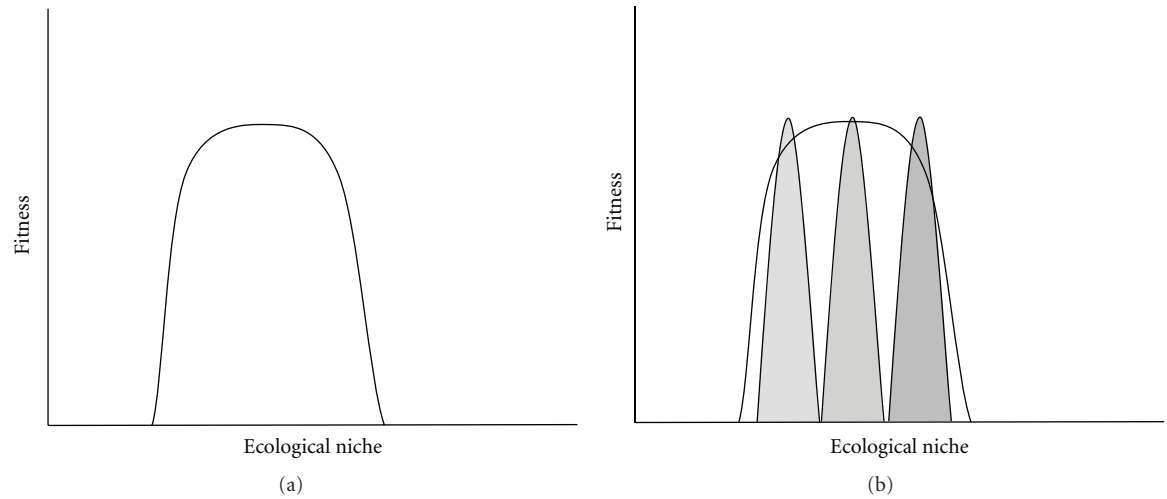

Figure 1: Graphic representation of the general-purpose genotype (GPG) model and the flexibility hypothesis. (a) GPG model, a flexible genetic lineage (unfilled distribution) with a wide ecological niche and a high fitness under variable environmental conditions. (b) Epigenetic as a mechanism extending the flexibility of a genome, environmentally induced epigenotypes (grey distributions) from a single genetic lineage (unfilled distribution from (a)).

the epigenome of an organism by variable methylation of DNA sequences has been shown to play a role in the regulation of some genes expression [10]. There are now numerous examples of epigenetically driven phenotypic variations that are not related to DNA sequence encoded genetic polymorphisms [11–14]. Such phenotypic variation can also be caused by an inability to maintain the original epigenetic state during embryogenesis [15]. Environmental cues (extrinsic signal) such as the diet [11, 16], temperature [17], maternal behaviour [14] and chemicals exposure [18], have been shown to influence the epigenetic profile of individuals.

The fact that the genome is able to integrate extrinsic signals from the environment to vary gene expression is a potentially important mechanism for producing phenotypic plasticity. This stands in sharp contrast with better understood mechanisms which are based on sequence encoded genetic variation. More importantly, some epigenetic variation has been shown not to be related to genetic polymorphism in natural populations [19]. While the genome provides the material to work upon, it is the epigenetic regulation that in part enables genomic flexibility. Finally, recent studies have argued that some naturally occurring epimutations can be adaptive [11, 20].

This project aims at investigating the link between individual epigenetic variability (not related to genetic variability) and the variation of natural environmental conditions. In accordance with the general-purpose genotype (GPG) model, a flexible genotype under different environmental conditions would exhibit distinct methylation patterns due to alternate gene expression profiles necessary to produce flexible phenotypes (Figure 1(b)). As a result, DNA methylation would represent a molecular mechanism extending the plasticity and flexibility of phenotypes produced by a given genotype. As a model, we used the clonal fish hybrid *Chrosomus eos-neogaeus* (Cyprinidea and Pisces). We chose this system because a given clonal lineage of *C. eos-neogaeus* can be present over a large geographic distribution [21], is found in many different types of habitats [22], is thought to be generalist [23, 24], and, more importantly, has been shown to be epigenetically variable [19].

2. Materials and Methods

2.1. Biological Model and Sampling. The all-female *C. eos-neogaeus* taxon resulted from hybridization events between female finescale dace (*C. neogaeus*) and male northern redbelly dace (*C. eos*) [25]. The diploid hybrids reproduce clonally via gynogenesis [26, 27]. Sperm from one of the parental species is thus required but only to trigger embryogenesis: the resulting offspring are generally genetically identical to the mother [26]. In this complex, the paternal genome can be incorporated into the zygote [22, 26, 28] resulting in triploid or mosaic hybrids which differ in the proportion of diploid-triploid cell lineages [25].

Fish from seven lakes belonging to different watersheds of the St. Lawrence River, QC, Canada (Table 1; Figure 2(a)) were sampled in the reproduction season and over a short period of approximately two weeks. Total DNA from muscle tissue of parental species, three *C. eos* and three *C. neogaeus*, and 26 gynogenetic hybrids belonging to seven different lakes were extracted by proteinase K digestion followed by phenol-chloroform purification and ethanol precipitation [30]. The lakes sampled were each classified as one of the four different types of environment according to a characterization previously used to describe *C. eos-neogaeus* populations [29], water pH, and temperature were also measured. Total body length, total body weight, and gonads weight were measured for each individual in order to estimate the gonadosomatic index (GSI) and the Fulton's K condition factor index (K) [31]. The lakes sampled are known to contain either one

Table 1: Summary of ecological and molecular data. Lake environmental characteristics, individual morphometric characteristics, sampling size, genetic diversity (number of genotypes), and epigenetic diversity (number of epigenotypes).

Lakes	Geographic coordinates	Date of sampling	Habitat type*	Drainage	Altitude (m)	T (°C)	pH	Weight (g)	Length (cm)	K	GSI	Sampling size	Genotype	Epigenotype
Richer	45°50′35″ N 74°11′39″ W	2007-05-29	C	Nord	360	24	6.4	3.32 ± 0.94	6.93 ± 0.71	0.97 ± 0.05	7.33 ± 2.27	4	1	2
Merde	45°57′55.9″ N 74°1′41,8″ W	2007-05-28	B	L'Assomption	360	23	6.2	3.44 ± 1.36	6.78 ± 0.77	1.04 ± 0.07	11.37 ± 2.7	4	3	4
Barbotte	46°5′36″ N 73°52′7″ W	2007-05-30	C	L'Assomption	280	22	6.5	2.16 ± 0.4	6.04 ± 0.41	0.97 ± 0.05	7.19 ± 2.5	8	6	2
Jonction	45°46′37″ N 74°34′29″ W	2007-06-01	C	Rouge	340	24	7.1	2.38 ± 0.56	6.08 ± 0.54	1.05 ± 0.07	9.74 ± 0.71	4	1	1
Dépotoir	45°50′41.6″ N 74°33′20.9″ W	2007-05-31	B	Rouge	320	25	7.1	1.85 ± 0.38	5.65 ± 0.45	1.01 ± 0.03	10.73 ± 0.53	2	1	1
Saumons	45°59′38″ N 74°18′21″ W	2007-06-16	A	Nord	490	22	7.0	3.2 ± 0.26	6.8 ± 0	1.01 ± 0.08	6.51 ± 1.31	2	1	2
Saad	45°54′51.4″ N 74°1′41.3″ W	2007-06-16	D	L'Assomption	320	24	6.8	1.89 ± 0.18	5.9 ± 0.2	0.92 ± 0.008	4.48 ± 2.54	2	1	1

*Habitats characterization according to Schlosser et al. [29]: A: pond of moderate depth, B: a shallow beaver pond, C: a moderately deep area of open water upstream from a beaver dam, and D: pond of moderate depth with flooded standing and fallen tree.

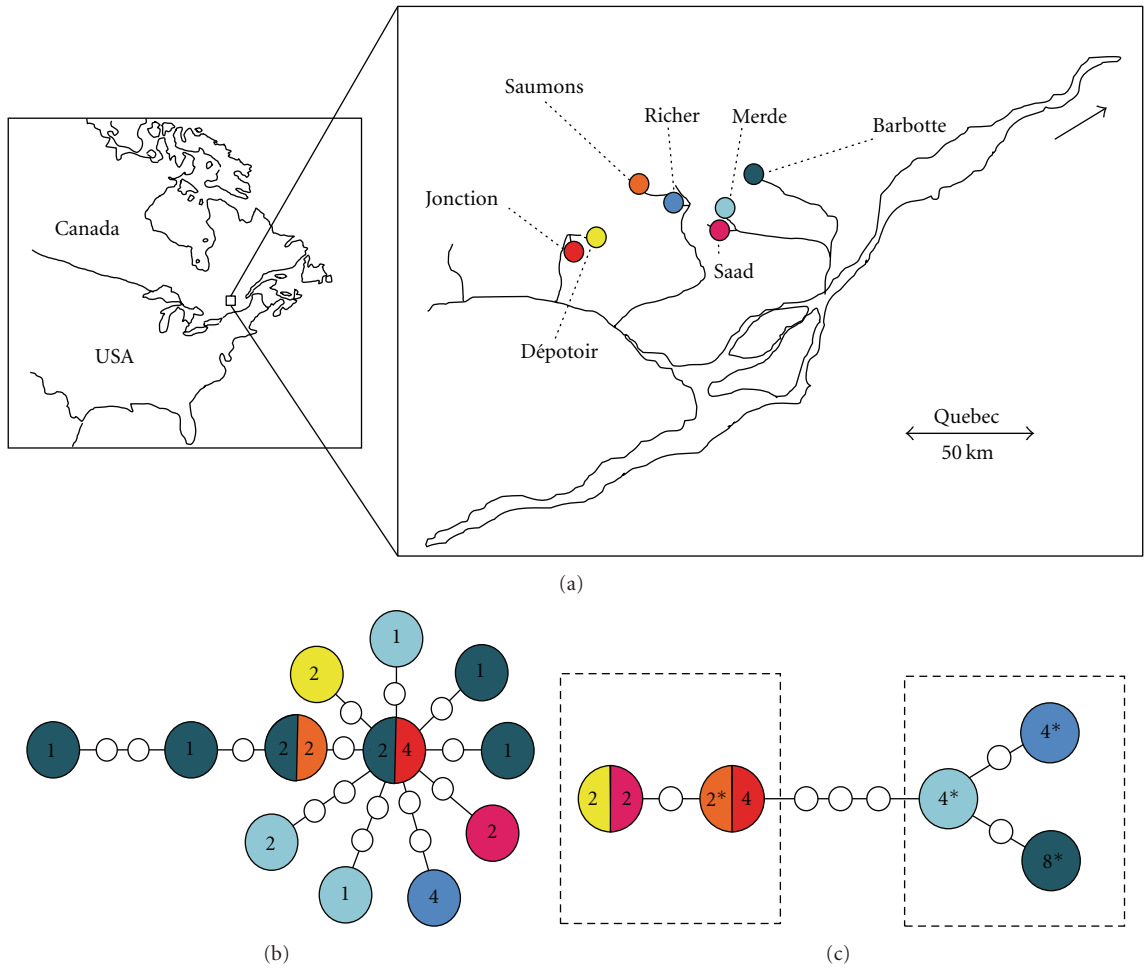

Figure 2: Details of sampling, genotypes, and epigenotypes diversity. (a) Sampled lakes in the Laurentian region, QC, Canada. (b) Minimum spanning network of the 12 genotypes identified by scoring nine microsatellite loci. The number of gynogenetic hybrids of each genotype per lake is indicated. (c) Minimum spanning network of the five main epigenotypes and two epigenetic groups (dash boxes) identified by the MSAP analysis. The number of gynogens of each epigenotype per lake is indicated. *refers to intrapopulation variation. The colour code of the sampled lakes from panel (a) is maintained throughout the rest of the figure.

or both parental species (*C. eos* and *C. neogaeus*) as well as gynogenetic and triploids hybrids [21, 28].

2.2. Genetic Identification. The gynogenetic hybrids were identified according to Binet and Angers [28]. Briefly, *C. eos-neogaeus* hybrids were identified using diagnostic markers designed on two genes. Primers of each marker were designed to provide PCR products of different sizes for *C. eos* and *C. neogaeus*, allowing chromosome identification. Individuals that displayed alleles of both parental species were classified as gynogenetic hybrids.

Gynogenetic hybrids (diploid) were then discriminated from triploid hybrids according to the ploidy level of the nuclear genome by using nine hypervariable microsatellites as detailed in Binet and Angers [28] and Angers and Schlosser [21]. Gynogens are expected to be hemizygous at every species-specific locus, while triploid hybrids (*C. eos-neogaeus* x *eos*) are expected to be heterozygous at loci specific for *C. eos* species. The microsatellites analysis also enabled the identification of the clonal lineage [21] and the discrimination of derived mutations. Only gynogenetic hybrids (diploid) were used for further analysis.

2.3. MSAP Analysis. We investigated epigenetic polymorphism at CCGG motif via an MSAP analysis [32] performed on parental species, three *C. eos* and three *C. neogaeus*, and the 26 *C. eos-neogaeus* gynogenetic hybrids identified in the procedure mentioned above. Each DNA sample was, respectively, digested with MseI/HpaII and MseI/MspI to allow the detection of differentially methylated sequences. Aliquots (4 μL) of each sample for each primer combinations were loaded on 6% polyacrylamide gels (19 : 1 acrylamide to bisacrylamide) containing 8 M urea and 1X TBE. Fragments that displayed methylation polymorphism among samples at restriction sites were identified by the presence/absence banding pattern between the two treatments. Full methylation of both cytosines and hemimethylation of the internal cytosines cannot be investigated by MSAP. As a consequence, it was impossible to distinguish these fragments from unmethylated sequences.

3. Results

3.1. Genetic Polymorphism: Microsatellite Loci Analysis. The analysis of nine highly variable microsatellite loci indicates that all samples belong to the same clonal lineage (lineage B6, [21]). Survey of microsatellite variation detected 14 mutations over nine loci and twelve multilocus mutant genotypes were identified within the clonal lineage (Figure 2(b)). These genotypes display very little divergence, since all but one genotype differ by only one or two mutations from the putative ancestral clone, with an average of 2.3 mutations among genotypes. The number of sublineages carrying derived mutations per lake varied from one to six (Table 1).

3.2. Epigenetic Polymorphism: MSAP Analysis. A total of 257 reproducible fragments detected between 150 and 600 bp were assessed with a set of six primer pairs. Over the 257 fragments detected in *C. eos-neogaeus* hybrids, 60 were exclusive to *C. neogaeus*, 67 to *C. eos*, and 114 were present in both parental species genomes. The remaining 16 fragments detected could not be associated to either of the parental species genomes. Eight fragments (3.11%) revealed informative methylation polymorphism among populations. Three fragments exclusive to *C. eos*, three fragments exclusive to *C. neogaeus*, and two fragments that were present in both parental species genome were differently methylated for some *C. eos-neogaeus* hybrids. The number of epigenotypes per lake varied from one to four (Table 1) and is not correlated with the number of samples ($R^2 = 0.07$, $P = 0.56$).

Two of the eight fragments are variable within populations, while the others are only variable among populations. For the 6 fragments that varied among populations, five main epigenotypes were detected. Although the sample size is low for some populations, individuals from a given population consistently shared the same methylation profile (Figure 2(c)). In most instances, individuals could be regrouped according to the lake of origin on the basis of their unique methylation profile.

Contrasting with genetic relationships among clones where variants are descendents of an ancestral genotype (Figure 2(b)), populations clustered in two distinct epigenetic groups separated by three epimutations (Figure 2(c)). No significant relationship was detected between genetic and epigenetic variation (Figure 3). For instance, individuals from two distinct lakes and harbouring the same genotype clustered in distinct epigenetic groups. Similarly, there is no relationship between genetic intrapopulation variability and epigenotypes. As an example, the six different genotypes from Barbotte Lake clustered into the same epigenetic group (Figure 2(c)).

There is no indication that epigenetic profile is related to geographic position, hydrologic network (Figure 2(a)), or date of sampling (Table 1). Also, no difference in individual body size length ($P = 0.26$), body weight ($P = 0.28$), Fulton's K ($P = 0.91$), and GSI ($P = 0.72$) were detected among populations. In addition, the shared epigenetic profiles among populations are not correlated with the habitats characterization of lakes (Table 1). While there is no important temperature fluctuation among lakes, we observed a significant pH variation that is consistent with the two epigenetic groups (Table 1). This is a particularly important result, since it correlates the clustering of populations in two epigenetic groups to the variation of a local environmental condition.

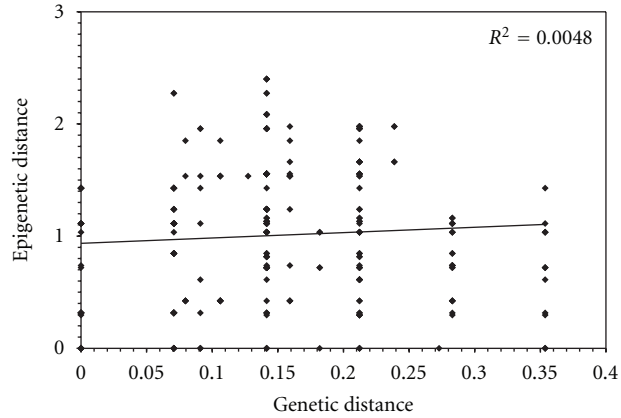

Figure 3: Relationship between the genotypes (genetic variation and microsatellite analysis) and the epigenotypes (methylation profiles difference and MSAP analysis).

4. Discussion

The present study report an effect of the local environmental conditions on the variation of the methylation profile among genetically identical individuals belonging to different natural populations. This is a particularly important result, considering that most studies investigating the influence of the integration of the extrinsic signal of the environment on epigenetic variation were performed under control conditions (e.g., [14, 16, 17]) (but see [18]). This indicates that the variation of natural environmental conditions can lead to DNA methylation polymorphism at the population level.

4.1. A Successful Generalist Lineage. The *C. eos-neogaeus* hybrid lineage studied here (lineage B6) is widespread in the south-western part of Quebec and is abundant in many populations from numerous watersheds [21]. The seven lakes under investigation are thought to be characterized by different environmental conditions of a variety of abiotic and biotic conditions (e.g., the oxygen concentration, the diets, the predation level, and the presence of competitors) [29]. Accordingly, each of the different lakes can be thought of as a different ecological niche. As a result, clonal lineage B6 can be characterized as a generalist lineage that is able to adjust in order to persist among many ecological niches. This situation has already been reported in northern Minnesota lakes (USA) and Algonquin Park lakes (Ontario) [23, 29]. Interestingly, *C. eos-neogaeus* hybrids from a single clonal lineage have been shown to present a high level of phenotypic variation [22]. Such variation of the phenotype in the absence of genetic variation has also been observed among *C. eos-neogaeus* hybrids from Quebec populations (B. Angers, unpublished data).

4.2. Environmentally Induced Epigenotypes. First, we did not detect any relationship between genotype and epigenotype. This is in accordance with a previous study that demonstrated pure (or facilitated) epigenetic variation in natural populations of *C. eos-neogaeus* hybrids [19]. More importantly, the genomic mutations detected are restricted to highly variable microsatellites loci, there is no mutation at mtDNA [21], and very few mutations were detected on AFLP loci [19]. This supports that the fragment variation detected with the MSAP analysis is due to difference in methylation not to DNA mutation.

Interestingly, the epigenetic polymorphism observed is shared among individuals of the same population in most instances. This suggests an influence of common environmental factors on the resulting epigenetic profiles or a long-term inheritance of epigenetic variation (modifications that could have been acquired before postglacial colonization). Considering the low probability of the inheritance of epigenetic variation across generations [33] and the absence of correlation between genetic and epigenetic polymorphism, the long-term heritability hypothesis can be ruled out. Accordingly, the observation of among lakes epigenetic variation suggests that current environmental conditions have an influence on the DNA methylation profiles among genetically identical individuals from different populations as opposed to hard-wired or germline dependent [34, 35]. In contrast with previous observations, the detection of the same epigenotype in different lakes indicates that the epigenetic polymorphisms observed are not the result of random variation [19]. More importantly, the correlation between the two epigenetic groups and the pH variation strongly support an effect of the local environmental conditions on the variation of methylation profile. Such pH variation may be caused by and/or will result in the variation of many other environmental factors potentially having respective or conjoint effects on the methylation polymorphism.

4.3. Revisiting the Importance of Heritability for Epigenetic Variation. Previous reports in the literature suggests that in order to be of importance in evolution, epigenetic changes must be heritable across generations [36–38]. In the situation for which an epimutation leading to a beneficial phenotypic modification appears in one generation and that the environmental conditions do not change in subsequent generations, the heritability of the new epigenetic mark may represent a transient step leading to genetic assimilation [39]. Although epimutations potentially represent a fast pathway toward adaptation [38], we do not believe that the main interest of epigenetic mechanisms is to mimic what is occurring at the adaptive genomic level. If heritable, both genetic and epigenetic polymorphisms are frozen. In temporally unstable or heterogeneous environments, such canalization of the phenotype does not seem beneficial [40]. Furthermore, heritability of epigenetic changes in vertebrates is not expected to be frequent considering the two phases of erasure prior to the initiation of zygote development [33]. Angers and coauthors [9] have recently identified some of the beneficial aspects of epigenetic mechanisms in that these processes may enable rapid and reversible changes in response to environmental perturbations. For instance, such is observed for the influence of the maternal behaviour on a glucocorticoid receptor gene promoter in the rat hippocampus [14]. Rather than passing on to the next-generation epimutations that may not be adaptive under new environmental regimes, selection might favour individuals with a plastic genome that easily adjusts epigenetically to environmental variables. Thus, the hard-wired genetic variation and the flexible epigenetic variation may be complementing each other by, respectively, leading to long-term and short-term adaptation.

5. Conclusion

While preliminary, these results appear to confirm that response of the genome when under variable environmental conditions leads to the formation of different epigenotypes. Each population presenting different epigenetic profiles can be seen as an acclimated epigenotype from a single flexible genetic lineage. It thus seems that this lineage has the potential to respond via epigenetic modifications such as DNA methylation when under variable environmental conditions. Even more importantly, this lineage potentially has the capacity to colonize different environments and/or the ability to adjust following a perturbation in the environment as expected from the long-term maintenance of multiple populations in this lineage. Thus, epigenetic processes may represent a molecular mechanism sustaining the GPG model.

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