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SYMPOSIUM

Epigenetics in Comparative Biology: Why We Should Pay Attention

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Synopsis The past decade has seen an explosion of articles in scientific journals involving non-genetic influences on phenotype through modulation of gene function without changes in gene sequence. The excitement in modern molecular biology surrounding the impact exerted by the environment on development of the phenotype is focused largely on mechanism and has not incorporated questions asked (and answers provided) by early philosophers, biologists, and psychologists. As such, this emergence of epigenetic studies is somewhat "old wine in new bottles" and represents a reformulation of the old debate of preformationism versus epigenesis—one resolved in the 1800s. Indeed, this tendency to always look forward, with minimal concern or regard of what has gone before, has led to the present situation in which "true" epigenetic studies are believed to consist of one of two schools. The first is primarily medically based and views epigenetic mechanisms as pathways for disease (e.g., "the epigenetics of cancer"). The second is primarily from the basic sciences, particularly molecular genetics, and regards epigenetics as a potentially important mechanism for organisms exposed to variable environments across multiple generations. There is, however, a third, and separate, school based on the historical literature and debates and regards epigenetics as more of a perspective than a phenomenon. Against this backdrop, comparative integrative biologists are particularly well-suited to understand epigenetic phenomena as a way for organisms to respond rapidly with modified phenotypes (relative to natural selection) to changes in the environment. Using evolutionary principles, it is also possible to interpret "sunsetting" of modified phenotypes when environmental conditions result in a disappearance of the epigenetic modification of gene regulation. Comparative integrative biologists also recognize epigenetics as a potentially confounding source of variation in their data. Epigenetic modification of phenotype (molecular, cellular, morphological, physiological, and behavioral) can be highly variable depending upon ancestral environmental exposure and can contribute to apparent "random" noise in collected datasets. Thus, future research should go beyond the study of epigenetic mechanisms at the level of the gene and devote additional investigation of epigenetic outcomes at the level of both the individual organism and how it affects the evolution of populations. This review is the first of seven in this special issue of Integrative and Comparative Biology that addresses in detail these and other key topics in the study of epigenetics.

Introduction

The purpose of this review—the first in a series of seven review articles in this issue of Integrative and Comparative Biology—is to discuss epigenetics and epigenetic phenomena in the context of integrative, comparative biology. Specifically, we explore what actually comprises epigenetics, what comprises the underlying mechanisms and the emergent properties of these changes, and even more fundamentally why we should even care about this near-ubiquitous phenomenon. Reflective of the current state of the field of epigenetic research, this initial paper in the series does not intend to provide the "ultimate framework" for the papers that follow in this volume. We recognize that there is still disagreement about what comprises epigenetics, starting with the different and sometimes conflicting approaches taken by the various authors in this volume. Indeed, the two authors of this review retain some divergent

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views of the field. Our compromise has been to present as faithfully as possible the various schools of thought and accompanying definitions, and to let the readers come to their own conclusions without us being proscriptive (although we do have our opinions!).

Contemporary epigenetic research—multiple schools of thought

The past decade has seen an explosion of articles in scientific journals involving influences on phenotype through modulation of gene function without changes in gene sequence (Guerrero-Bosagna and Skinner 2012; Bohacek and Mansuy 2013; Youngson 2013; Burggren 2014), including a new peer-refereed journal on the subject. Indeed, an indication of the burgeoning of epigenetic studies in the scientific literature comes from a meta-analysis of the life-science literature in the NIH PubMed database, which reveals 12,000 papers published from 2010 to early 2013 that contain the words "epigenetic," "epigenetics," or "epigenome" (Burggren 2014). Indeed, this interest in epigenetics has spilled over into multiple lay reports in the media, commanding cover articles in magazines ranging from *Time* to *Der Spiegel* to *National Geographic*.

Unfortunately, a reading of the epigenetic scientific literature reveals that the use of the word "epigenetics" is currently relatively messy, with ambiguity apparent, especially over the past two decades. This ambiguity derives in part from the existence of divergent approaches—distinct "schools," as it were. The first of these schools might be called the "Intragenerational Epigenetic School." Exploiting the literal meaning of epigenetics—"*above genetics*"—there is little or no concern with transgenerational transfer—that is, inheritance by epigenetic mechanisms. This school is prominent in medicine and focuses on epigenetics as a pathological process. For example, there are currently nearly 3200 papers in PubMed that concurrently mention "epigenetics" and "cancer," usually in the context of the epigenetics OF cancer.

The second epigenetic school is what might be called the "Transgenerational Epigenetic School." Focused on transgenerational transfer, this school is prominent in the life sciences. A significant difference from the previously described school is that proponents of this school view epigenetic phenomena, and their underlying causes, as an adaptive mechanism, with ecological and evolutionary implications, as opposed to primarily a pathway to disease.

What is the relative size of these first two "schools"? Consider a meta-analysis of PubMed Database, using Boolean (and/or/not) searches involving 21 combinations of key terms such as "epigenetic," "transgenerational," and "inheritance." From 2010–2013, there were >17,000 papers that DO contain the root "epigene" (and thus potentially the words epigenes, epigenetic, and epigenetics) but DO NOT contain the roots "inherit" (and thus inheritance and inheriting) or "transgeneration" (and thus transgenerational). Over this same time period, there were <600 papers published that contained the root "epigene" AND contained the roots "inherit" and/or "transgeneration." In essence, then, there is very little overlap between the areas of intra-generational medicine and transgenerational medicine, and then very little overlap of these two clinical areas with the biological sciences (Fig. 1). Importantly, the much smaller amount of activity of epigenetics in the basic life sciences in absolute terms does not mean that consideration of epigenetics in the basic life sciences is unimportant, or that it is a marginal discipline of merely theoretical interest, as will be explored later in this essay.

A third school of thought—the most holistic of the three—is that epigenetics is a "perspective" rather than a collection of mechanisms within and across generations. As such, this is the most inclusive school (Fig. 1). The authors support this approach and expound upon this later in this essay. However, a key question is whether any, or all, of these schools "make sense" as independent entities, or whether they are simply different interpretations of the same over-arching phenomenon. To answer this question, we must understand where modern epigenetic research originated—that is, its rich history. However, despite a large and growing interest in epigenetics, it is our view that integrative comparative biologists are not broadly knowledgeable of the history of how the field first emerged, and then subsequently waned with the rise of the reductionist, genocentric view of life (Bolker 1995, 2012). Consequently, before a detailed discussion of what epigenetics is (and is not), we briefly introduce the storied history of epigenetic thought. Several comprehensive papers have recounted in depth both the origin of epigenetics in the life sciences, and the history of its ongoing development of this field (Jablonka and Lamb 1989, 2002; Jablonka et al. 1998; Crews 2008; Nijland et al. 2008; Jablonka and Raz 2009; Ho and Burggren 2010; Hauser et al. 2011; Burggren 2014). It is not the authors' intention to pass down this well-traveled road other than to draw attention to an expanded definition of the term and

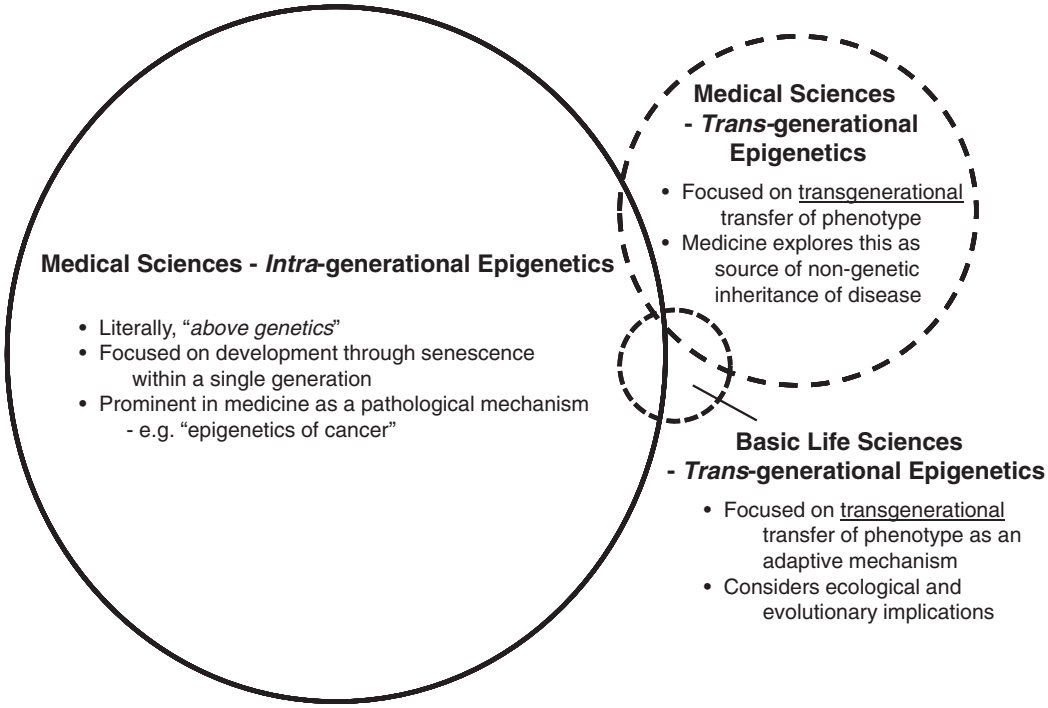

Fig. 1 A Venn diagram of the relationships between, and characteristics of, studies in intra-generational epigenetics common in the medical sciences, transgenerational epigenetics favored in the basic life sciences, and epigenetics as a "perspective." The sizes of the domains are not to scale. See text for further discussion.

provide a perspective that includes the full range of epigenetic studies.

Origin of epigenetics and epigenetic research

Aristotle believed that the environment sculpted the phenotype of individuals and that these effects were represented in their descendants. The first "scientific" hypothesis of inheritance can be traced at least as far back as Malpighi (1673), who proposed the theory that the embryo is pre-formed in the germ cell. Hartsoecker further extended this view when in 1694 he claimed to see the entire human form within a single sperm. Such ideas laid the groundwork for the great "Question" and framing of the debate around two schools of thought, "Preformationism" versus "Epigenesis." In the sixteenth–seventeenth centuries, the Question was how a fully integrated multicellular organism develops from a single cell (the fertilized egg). *Preformationism* believed that adult features were present fully formed in the egg and simply unfolded during growth. *Epigenesis*, not to be confused with the term "epigenetics" coined centuries later by C.H. Waddington (see below), held that traits emerge as a consequence of the progressive interaction of the constituent parts of the zygote and the context in which it developed (Stanford Encyclopedia of Philosophy 2005).

Jean-Baptiste Lamarck (1744–1829) plays an important historical milestone in evolutionary and epigenetic thought (Corsi 2011). In his classic work *Philosophie Zoologique ou Exposition des Considérations Relatives à L'histoire Naturelle des Animaux*, Lamarck laid out his views on inheritance of acquired characteristics, known as "soft inheritance", thus forming the first comprehensive framework for evolution (Lamarck 1809). As the subsequent flames of Darwinian evolution burned brightly over the ensuing decades and centuries, Lamarck was quickly relegated to the historical trash heap for his "obviously flawed" interpretations. However, Lamarck is coming back to haunt generations of his detractors as biologists increasingly appreciate the inheritance of acquired phenotypes through non-genetic inheritance (Jablonka and Lamb 1995; Jablonka et al. 1998; Crews and McLachlan 2006; Crews 2008; Honeywill 2008; Burggren 2014).

Resolution of the Question was finally achieved in the formal debates before the Académie des Sciences between Georges Cuvier (who promoted a teleological functional approach to anatomy) and Etienne Geoffroy Saint-Hilaire (who held that form had

priority over function, promoting a more integrated morphological approach) (Appel 1987). Ultimately, Geoffroy Saint-Hilaire's arguments were vindicated and "Epigenesis" was accepted. The Question remerged when August Weismann (1893) proposed that the phenotype unfolds from the germ cells in a predetermined manner (in essence a restatement of Hartsoecker). Weismann's germ-plasm theory posited that mutation was random and only occurred in the gametes; any changes in somatic cells were destined to die with the demise of those cells. This view became central dogma in the Modern Synthesis. Thus, "hard" inheritance always trumped "soft" inheritance, and the idea that somatic components of the phenotype interacted with the environment, forming integrated adaptations as a type of inheritance, went into diapause for almost a century.

The equivalent of epigenesis was embodied by the faculty of the Biologische Versuchsanstalt (Institute of Experimental Biology) in Vienna. Founded and directed by Hans Przibram (Logan 2013; Södersten et al. 2014), the "Vivarium" had state-of-the-art facilities for experimental developmental biology, including the first constant temperature rooms. The faculties were some of the leading scientists of the day, including Paul Kammerer, Karl von Frisch, Eugen Steinach, and Paul Weiss. The primary focus of the Institute was to derive the laws (statistical regularities or patterns) governing the development of organism and their relationship to the environment and explore a "third way" between determinism and chance by capturing "... the complexity of the interaction between the organism and its environment"—that is, what we now know as a systems approach to biology and the concept of emergence. These beginnings of this systems approach to biology ("Systems Biology" is a somewhat contentious term in modern Biology. True to the meaning of its words, the phrase Systems Biology reflects a broad, holistic approach to the complex interactions that occur between biological systems (broadly defined). Recently, however, the term Systems Biology has been co-opted to describe a narrow set of network interactions or even a set of algorithms to predict molecular interactions. The reader can judge the merits of both approaches for themselves by considering some recent reviews on the topic: Mesarovic et al. (2004), Gatherer (2010), Joyner (2011), Joyner and Pedersen (2011), Margineanu (2012), Bizzarri et al. (2013), and Lapraz and Hedayat (2013).) were particularly evident in the PhD work of Weiss on butterfly flight patterns under the mentorship of Przibram. This emphasis on the idea of "plastic reactions," or the ability to change as a result of experience, continued in Weiss' work on cell differentiation and the transplanting and reforming of connections in the nerves of limbs using newts and frogs, after he moved to the United States in 1931 and published *Principles of Development* in 1939 (Weiss 1939). A still useful definition of emergence is that of Mayr (1988, 34) "... when two entities are combined at a higher level of integration, not all the properties of the new entity are necessarily a logical or predictable consequence of the properties of the components."

Arriving in the era of modern genetics and in heritance, modern constructs for epigenetics began in the middle of the twentieth century. Again, the lineage of the writers (molecular biologists, evolutionary and developmental biologists, and psychologists) sharply defines these histories. As Haig (2004) recounted, almost since the inception there has been controversy in what epigenetics is, and what it means for biologists. Conrad Waddington (1905–1975), a prominent twentieth century developmental biologist and knowing full well the history of the Question, coined the term epigenetics in his now-classic paper "The epigenotype" (Waddington 1942). In his thinking at about the time of the publication of this paper, Waddington promoted the study of "causal mechanisms" by which "the genes of the genotype bring about phenotypic effects." While transgenerational transfer (non-genetic inheritance) was implicit in his writings, his focus was primarily on canalization (another word Waddington coined), which refers to the tendency for a standard phenotypic outcome following development, regardless of minor variations in environment or phenotype. Another important concept was "assimilation," or the process by which acquired characteristics may become incorporated into the genome. Waddington thus made fundamental contributions to our thinking in modern developmental biology (Fig. 2).

Another milestone occurred with the emergence of D.L. Nanney, another key player in the story of epigenetics. In his 1957 paper entitled *The Role of the Cytoplasm in Heredity* (Nanney 1957) he created a very clear—elegant, even—differentiation between genetic and non-genetic inheritance. Particularly noteworthy was his view of genetics as "....concerned with the preservation and replication of information in structural form." This was contrasted with epigenetic mechanisms that are important in regulating the expression of genetic information. In particular, Nanney (1957) described epigenetic mechanisms as serving "....to translate structural symbols into phenotypic reality." Indeed, it is this very translation—the emergence of non-genetic "variance" in

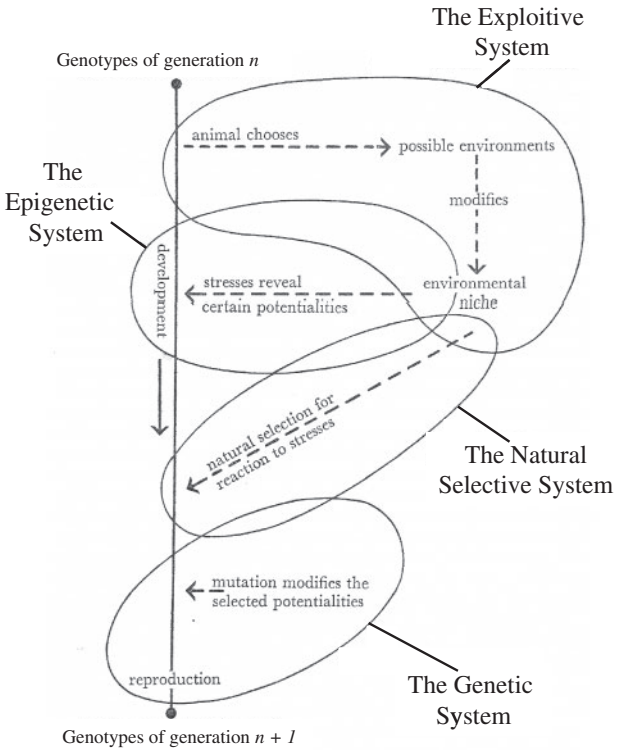

Fig. 2 A schematic interpretation of Waddington's (1959) conception of what he called "the logical structure of the evolutionary system." This figure, from his 1959 paper, illustrates how changes in gene frequency between successive generations involve interactions between what Waddington differentiated as four distinct subsystems—the exploitive, epigenetic, the natural selective, and the genetic.

phenotype—that has been a major focus of contemporary epigenetic studies in the biological sciences.

A key milestone in understanding the mechanisms of epigenetic processes was reached in the mid-seventies, when both Riggs (1975) and Holliday and Pugh (1975) proposed that DNA can be methylated in bacteria, and that their DNA is actually predominately in hemi-methylated substrates. Importantly, this methylation was determined to create stable differentiated states in the absence of genetic mutation and that the sequence-specific binding of these enzymes would have a role in the regulation of gene function. With this discovery thus emerged a putative mechanism for some epigenetic effects.

The most recent decade of molecular research has been highly reductionist in nature, with both attendant rewards and missed opportunities for synthesis (Mesarovic et al. 2004; Gatherer 2010; Joyner 2011; Joyner and Pedersen 2011; Margineanu 2012; Lapraz and Hedayat 2013; Lapraz et al. 2013). During this same period, epigenetic research was predominantly a reductionist exercise, drilling in on the non-genetic mechanisms by which phenotype is modified either intra-generationally or inter-generationally: methylation of DNA, modification of histone, microRNAs, positioning of the nucleosome and, apparently in a few invertebrates, so-called structural inheritance and self-sustaining loops (Jablonka and Lamb 2002; Ng and Gurdon 2005; Ho and Burggren 2010; Kovalchuk 2012). Again, it is not the purpose of this paper to fully review epigenetic mechanisms, as they have been reviewed numerous times, and several papers in this symposium focus on new findings both in animals and plants (Burggren and Randall 1978; Alvarado et al. 2014; Jones and Sung 2014; Mazio and Soliman 2014; Padilla 2014; Yi et al. 2014). Here we argue that, for the emerging field of modern epigenetic study, both reductionist approaches identifying mechanisms and systems-biology approaches providing broad ecological and evolutionary context are complementary and necessary, and advance our overall collective understanding of epigentics.

What most biologists leave out in their consideration of the history of epigenetics—whether from a reductionist or systems-biology approach—is the focus of psychology on the Question (Crews 2008). How the genotype interacts with the environment to produce this variation has been a major focus in psychology since its inception as a scientific discipline at the turn of the twentieth century. In this field, the interest is on the individual's interactions with the biotic and physical environment, usually from birth; this originally was termed "molar" epigenesis by William James (1950), but more recently has been termed "probabilistic epigenesis" (Gottlieb 2002). (We prefer the term "molar epigenetics," as William James used that term to connote the emergent properties of life process rather than the opposite view—a deterministic or reductionistic approach.)

It was during this period before the rediscovery of Mendel's work (and the consequent paradigm shift in most of biological and psychological sciences) that the controversy between neo-Darwinianism versus neo-Lamarckianism peaked (Simpson 1956). It was at this time that Baldwin, Lloyd Morgan, and Osborn independently and almost simultaneously hypothesized that "characters individually acquired by members of a group of organisms may eventually, under the influence of selection, be re-enforced or replaced by similar hereditary factors" (Simpson 1956, 110). Baldwin has been given primacy in the literature and so it is called the Baldwin effect.

Baldwin in essence reconciled Darwin (evolution by natural selection) and Lamarck (environment affecting phenotype of future generations) by positing selection on Norms of Reaction (in present terms

equivalent to phenotypic plasticity). Baldwin (1896a, b) proposed that the environment induces behavioral, physiological, or structural modifications in the individual that are not hereditary as such, but nonetheless are advantageous for survival, i.e., are adaptive for the individual having them. Thus, genetic factors (reaction range) produce hereditary characteristics similar to the individual modifications, or having the same sorts of adaptive advantages, in the population. These genetic factors are favored by natural selection and tend to spread in the population over the course of generations. Given enough time, mutations that result in this character without experience will appear. The net result is that an adaptation, originally individual and nonhereditary, becomes hereditary. This clearly presages Waddington's (1942) concept of assimilation. Indeed, there are many examples in the literature supporting this view, drawn from morphology ("crossvein" in *Drosophila*, callosities in man and ratite birds), physiology (heat shock and polyphenism), and behavior (host–plant preferences and speciation; sexual imprinting).

While the concept of epigenetics and epigenetic inheritance was becoming well established by the middle of the twentieth century, there was little or no appreciation of the underlying mechanisms outside of Psychology. As in Biology, there is a deep history of the Question in psychology, but in this case it is posed in various iterations of "instinct versus learning," "nature versus nurture," "heredity or environment," or "innate versus acquired." In this regard, it is important to keep in mind that genetic variation is not the same thing as developmental process. Interesting though it is, a review of this debate is outside of the scope of this paper. Suffice it to say that the resolution was in the form of ultimately recognizing that the Question itself is sterile and nonproductive. The major proponent of this view was Daniel S. Lehrman (1953, 1970) who pointed out that "the interaction out of which the organism develops is not one, as is so often said, between heredity and environment. It is between organism and environment! And the organism is different at each stage of its development." ((Lehrman 1953, 345); emphasis in original). Thus, behavior is influenced by the experiences that accumulate throughout life. Early experiences shape how individuals will respond to later experiences. However, later experiences can modify the effects of these earlier experiences. Finally, experiences can modify the genome without altering DNA structure and thus can be transmitted across generations—a perspective now embodied in the mechanistic view of epigenetics proposed in several of the papers in this symposium (Alvarado et al. 2014; Jones and Sung 2014; Mazio and Soliman 2014; Padilla 2014; Yi et al. 2014).

In studies of epigenetics, the goal is to identify a phenotypic outcome and the mode of transmission. The problem in this case, as in all aspects of science, is which phenotype to study? Evolution selects for outcomes, with the mechanisms producing those outcomes being carried forward as well, and the predicate is heritable transmission both in time and space. Further, we are beginning to re-appreciate that the "unit of selection" is not the gene, but the integrated organism. The term "phenotype" is not meant to convey a unitary physical feature (e.g., behavioral, physiological, or morphological feature) but, in most instances, a consolidation of multiple traits at multiple levels of organization. Traditionally, a trait is defined as any measurable aspect of the individual. In general, a deeper understanding of a particular phenotype increases proportionally with the number of traits that are measured in the same individual. The choice of particular genetic, morphological, physiological, and behavioral traits chosen by the researcher should be predicated on the pertinent literature and be demonstrated to be important for testing the hypothesis at hand. Thus, it is important to keep in mind that a gene has no greater meaning than, for example, body mass or circulating concentration of a hormone. Indeed, expression of individual genes only has meaning in the context of other genes within and outside their functional categories. Because higher-order traits are compounded and transformed from lower levels (e.g., emergent properties of the combination of traits at lower levels of biological organization), the expression of any "particular" gene has relatively little importance due to epistasis and redundancy.

Integrating inheritance and epigenetics

We now turn in this (somewhat optimistically) titled section to clarifying our view of epigenetics, and epigenetic inheritance. There is considerable variability and even confusion in the terminology used in the field of epigenetics, broadly defined (Ho and Burggren 2010; Salinas et al. 2013). A meta-analysis of publications in PubMed indicated >300 papers with both the words "epigenetics" and "definition" in them (Burggren 2014). While this does not mean that each of these papers offers up definitions of epigenetics, many of them do repeat, interpret, or re-invent the meanings of these terms. Thus, at the risk of further contributing to this semantic noise, we offer our interpretations of epigenetics in the context of inheritance. We do this not to try to

convince the readers, whose opinions may (likely) differ, but because we feel it important for every research in epigenetics to indicate the position or "school" from which they come.

What is "inheritance"?

One of the necessities of clarifying meanings derives from situations in which a word in general use in lay language is also used in highly specific ways in scientific discourse. In this regard "Inheritance" is a poster-child for loose language. There are multiple definitions and Fig. 3 summarizes several related definitions and their interrelationships. From these definitions, it is possible to define different types of inheritance, all of which are valid and may, or may not, have a molecular basis. "Cultural" Inheritance refers to the process through which organisms (animals and humans) learn behaviors by watching and imitating others. This involves the storage and transmission of information by such means as communication, imitation, teaching, and learning. "Mendelian" or Genetic Inheritance refers to the transmission of traits from parents to offspring through meiosis and recombination. According to the Modern Evolutionary Synthesis, DNA is regarded as the sole unit of heredity.

What, then, is "epigenetics"?

An important lesson this paper can provide, especially against the backdrop of some of the relatively mechanistic symposium papers in this volume, is that epigenetics is not simply a set of techniques or a new advance in molecular biology or a set of mechanisms (familiar territory for the intra-generational and transgenerational schools of thought, outlined above). Rather, a more comprehensive view is that epigenetics is a "perspective." To biologists it may seem that most contemporary research activity deals with the interface between the environment and gene regulation. This, in turn, appears to have led to the expectation among some that all answers will come from defining the underlying molecular mechanisms. It also has spawned the often untested assumption that if the mechanism is found, then the phenomenon must exist. Specific to the field of epigenetics, a common assumption is that if DNA methylation is present, then "it" must be epigenetics. We simply don't know enough about the full ramifications of DNA methylation and other mechanisms for gene regulation to make this assumption.

Beyond the study of epigenetic mechanisms at the level of the gene, more investigation is needed of epigenetic outcomes at the level both of the individual organism and of the population. The life-history approach to the study of behavioral development emphasizes both the continuity and interplay between the internal and external environmental characteristic of the specific life stages. In this regard, an excellent example is to think of the development of brain and behavior, which involves at least two distinct epigenetic programming mechanisms—context-dependent and germline-dependent.

"Epigenetic inheritance" refers to the manner in which the environment affects the expression of the

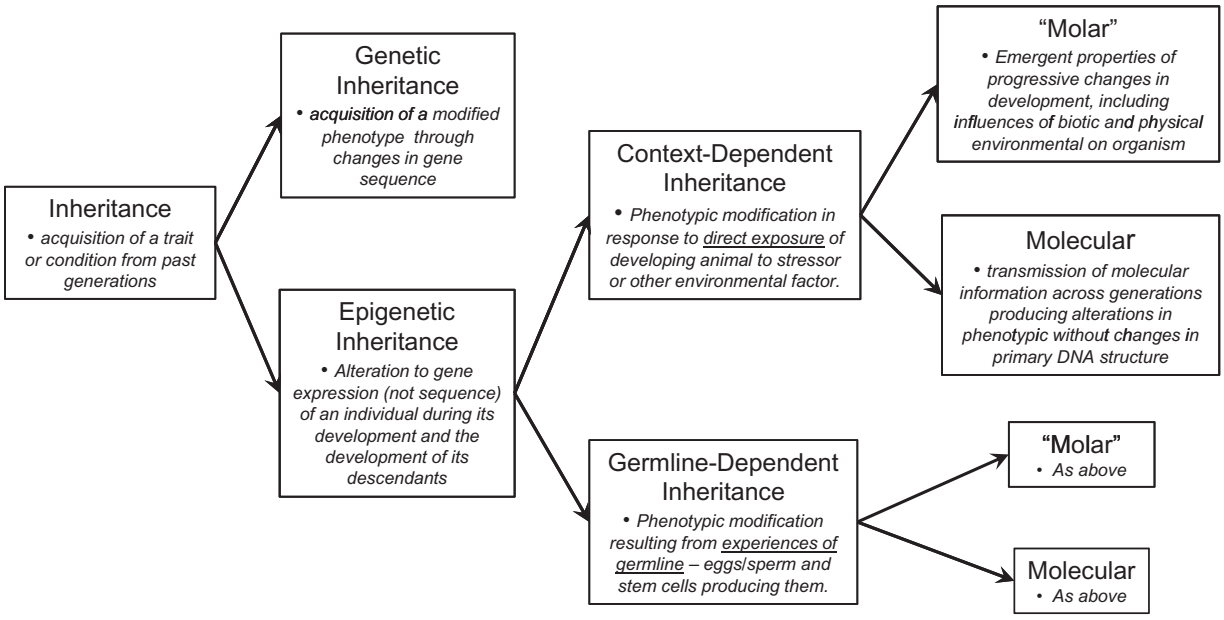

Fig. 3 The relationships between, and definitions of, various forms of epigenetic inheritance.

genome of the individual during its development AND the development of its descendants. Epigenetic inheritance can, in turn, be classified as "molecular" or "molar" (Fig. 4). Molecular epigenetic inheritance refers to transmission of molecular information across generations, thereby producing alterations in phenotype without changes in primary DNA structure; this would include, but is not limited to, mechanisms of DNA methylation and histone modification. In contrast, molar epigenetic inheritance would refer to the emergent properties of progressive changes in development and how the biotic (including social) and physical environment influences the organism. For example, although many song-birds have characteristic species-typical calls, geographical dialects are a product of what the individual hears as it develops into an adult. Similarly, the context in which the bird develops will dictate who it perceives and will display sexual behavior towards, as a mate.) Thus, as pointed out by Haldane (1946), the genetic constitution and its phenotypic expression under one environment may have a completely different phenotypic expression under another environment, just as a particular environmental change may have a great influence on the phenotypic development of one genome but may have no effect on that of another genome.

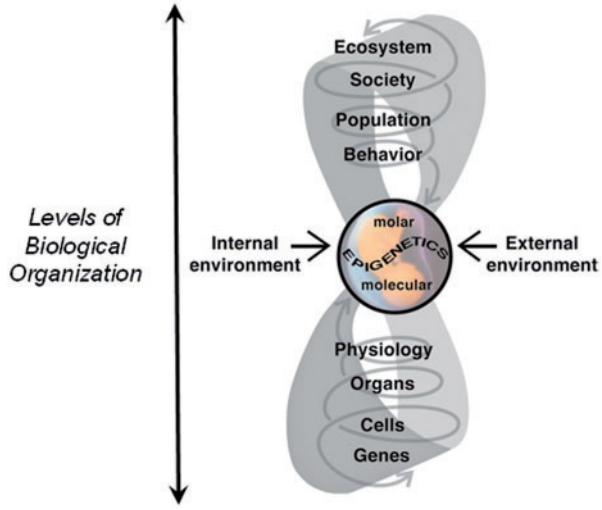

Fig. 4 The external environment interacts with the internal environment to influence fetal development with both immediate and life-long consequences. Such environmentally-induced changes can occur at all levels of biological organization. Ultimately, these influences may be epigenetic in nature, inducing mitotically heritable alterations in gene expression without changing the DNA. Epigenetics can be studied in a reductionist manner (Molecular) to understand the manner in which gene expression is altered. Alternatively, epigenetic modifications can be examined as consequences (Molar) amplifying through higher levels of biological organization. For example, these alterations can bring about functional differences in brain and behavior that result in changes in the phenotype. Behavior is the product of brain activity and is an emergent property. Behavior becomes an externalized signal that changes the social environment; in essence the individual's behavior creates its own niche space and modifies how individuals respond to conspecifics and their environment. The evolutionary impact of such questions is still an open question. What is known is that human society has changed the ecosystem in a manner that has had demonstrable impact on the health of humans and wildlife. Figure modified from Crews (2008).

A number of phenomena have variously been defined in the past as "inside" or "outside" of epigenetic effects—namely, maternal effects and the effects of direct exposure of the gametes to environmental stressors while within their parents (Ho and Burggren 2010). To reconcile this, we find it useful to have an additional set of definitions, with examples that "classify how epigenetic inheritance is created."

Context-dependent epigenetic inheritance—maternal effects and transgenerational cytoplasmic transfer

"Context-dependent epigenetic inheritance" occurs in response to direct exposure of the developing animal to a stressor or other environmental factor. So long as the environmental factors bringing about the epigenetic modification persist, the epigenetic modification will be "manifest in each generation." When referring to a mother–infant transmission, this may also be referred to as "multigenerational inheritance." The effects of this type of epigenetic inheritance can be reversed in a developing animal by removal of the factor or by addition of a therapeutic environmental factor. This is equivalent to the "Intragenerational Epigenetic School" (see below).

Common forms of context-dependent epigenetic inheritance are so-called maternal effects. Perhaps more accurately termed "parental" effects (see below), maternal effects are a confounding and variably interpreted phenomenon in the overall field of epigenetics. A maternal effect is "... the causal influence of the maternal genotype or phenotype on the offspring phenotype." (Wolf and Wade 2009). In other words, a maternal effect occurs when an organism's phenotype results from not only from its own genotype and environmental experiences, but also from its mother's environment and genotype. The mechanism(s) by which the F_1 phenotype is modified in a maternal effect do not involve regulation of gene function through any of the now-traditional mechanisms of epigenetic modification,

including DNA methylation and histone modification. Instead, cell signaling in maternal effects results directly from either cytoplasmic effects on cell function or modification of the process of gene expression through nuclear receptor mechanisms—in either case typically involving mRNA or proteins that the mother passes on to the offspring through the eggs she produces (Wolf and Wade 2009). Classic maternal effects abound in birds, in which both cellular (yolk cell) and extracellular (albumin) signals loaded into the egg upon its formation directly influence gene expression of the embryo that subsequently develops in that egg. Such maternal effects in birds have been shown to modify both morphological and physiological phenotypes (Ho et al. 2011; Ho 2014). Another recent example involves sex determination in reptiles through the actions of maternal hormones (Matsumoto et al. 2013). Indeed, maternal effects are widely observed both in plants (Roach and Wulff 1987) and animals (Bernardo 1996).

Before leaving maternal effects, note that this category of phenomenon could more aptly be labeled as the more inclusive "parental effects," as paternal effects resulting from influence of the father have been identified in plants, animals, and unicellular organisms (Fitch et al. 1998; Lacey 1998; Lacey and Herr 2005; Allan et al. 2014).

Another example of context-dependent epigenetic inheritance occurs in transgenerational cytoplasmic transfer. For example, in the act of breeding, some invertebrate males may transfer surprisingly large amounts of their own cytoplasm (and all that it contains) to the female upon copulation. As an extreme case, the spermatophores that male crickets transfer to their female counterparts during copulation can comprise as much as 20% of their total body mass! Male crickets fed food containing radioisotopes will incorporate these labels into their sperm packets, and then directly pass these labels on to the female during copulation. Amazingly, that radioactive label can still be detected in the F_2 generation (S. Kaulenas, submitted for publication)! The persistence of possible cytoplasmic signals and their effects on the phenotype that they create across multiple, as opposed to a single, generation, represents an often unappreciated example of context-dependent epigenetic effect.

Germline-dependent epigenetic inheritance

"Germline-dependent epigenetic inheritance" differs from context-dependent epigenetic inheritance because it is mediated through experiences of the germline—the eggs and sperm and the stem cells that produce them (Fig. 3).

"Germline-dependent epigenetic inheritance" in the form of phenotypic modification will manifest in one or more subsequent generations "in the absence of the initial causative agent." At this point, there is no known therapeutic amelioration. This is equivalent to the "transgenerational epigenetic school" (see below). It is important to note that while both forms of epigenetics have been attributed with "generational" properties, only "germline-dependent" epigenetic modification is truly transgenerational inheritance.

"Germline-dependent epigenetic inheritance" often occurs in mammalian embryos or fetuses, for example, while still *in utero*. Many toxicants/pharmaceuticals experienced early in development will influence normal development in profound ways, especially when such exposure occurs during critical developmental periods (Burggren and Reyna 2011; Burggren and Mueller 2014). Unfortunately, examples abound in human development. One classic example from the 1960s involves direct exposure of human fetuses in their first trimester to the drug thalidomide (α-(*N*-phthalimido) glutarimide), an effective sedative that reduced morning sickness but which was subsequently found to often result in severe limb abnormalities (Knobloch and Rüther 2008). Fetal Alcohol Syndrome, in which direct exposure of the fetus *in utero* to even single episodes of high maternal ethanol levels, can result in craniofacial and cognitive disorders (Behnke and Smith 2013; Memo et al. 2013; Ungerer et al. 2013). Other examples of inheritance of phenotypic modification through exposure to environmental toxicants abound in the literature. (Noteworthy is that some researchers do not view direct embryo/fetal exposure to environmental stressors as an example of epigenetic inheritance. As supporting evidence for this perspective, consider that the direct exposure to alcohol that affects mammals *in utero* also affects development of vertebrates that develop in free-living eggs exposed directly to the environment: Carvan et al. (2004) and Ali et al. (2011). In such situations, the F_0 experiences are irrelevant, as there is no "inheritance" *per se*.)

The effects of "germline-dependent epigenetic inheritance" may be quite insidious when evaluating potential transgenerational effects. Consider, for example, the situation of a pregnant mammal carrying female offspring who herself is directly exposed to an environmental stressor. Not only is the F_1 directly exposed to the stressor *in utero*, but so too is this F_1's entire germline (one component of the future

F_2). Following this reasoning, it would take following a lineage through to the F_3 generation to ensure that any lingering phenotypic modification is an actual epigenetic effect, and not simply resulting from direct exposure of the female germline. Most studies to date in mammals have only been followed for a single or at most two generations ($F_0 \rightarrow F_2$); thus, the hypothesis that any "downstream" phenotypic modifications are simply the result of direct exposure of the germline cannot be unequivocally rejected. However, there are now studies that go to the F_2 and up to the F_5 generation (Anway et al. 2005; Crews et al. 2007; Skinner et al. 2008; Crews et al. 2012; Wolstenholme et al. 2012; Manikkam et al. 2013; Skinner et al. 2013).

Epigenetics and developmental plasticity

A common question in discussions of epigenetics is "What is the relationship between "epigenetics" and "developmental plasticity"? Developmental plasticity is generally regarded as "...a single genotype's ability to alter its developmental processes and phenotypic outcomes in response to different environmental conditions" (Moczek et al. 2011). As pointed out above, this is a restatement of a concept >100 years old known as the reaction norm (Hertwig 1894; Woltereck 1909; Fuller et al. 2005). It could be argued, perhaps pedantically, that epigenetic manipulation of phenotype within or across generations represents developmental plasticity, with an environmental stressor leading to methylation of DNA, for example, representing an "environmental condition." However, the early leaders in the field acknowledged that the "genome learns from its experience" (Jaenisch and Bird 2003). As Holliday (2006) stated:

> Genetic changes are stable and rarely reversed, whereas epigenetic changes are often reversed. A good example of that is genomic imprinting, where the changes imposed on DNA sequences may be lost during development, or if they persist, are erased and re-set during gametogenesis. Environmental influences do not change the genotype (leaving aside mutagens), and there is no inheritance of acquired characteristics. Epigenetics is quite different, because normal development depends on communication between cells. Thus, a hormone, morphogen or growth factor may induce an epigenetic change that may be heritable. This means that the environment of a cell may be all important in determining its properties or its fate in the developing organism. In this sense, epigenetics encompasses Lamarckian inheritance.

Why should comparative biologists care about epigenetics?

The answer to the question "Why should comparative biologists care about epigenetics" should not be "Because many other life scientists are interested in epigenetics." While we have presented impressive numbers on the burgeoning interest in epigenetics in the life sciences, there are far more compelling reasons than just getting on the bandwagon.

Epigenetics as a method for heritable transmission without changes in DNA

Epigenetics encompasses the mechanisms by which "experience"—broadly defined—can be transmitted to future generations without involving mutation. An epigenetic framework helps to define how environmental experiences (whether internal or external, biotic, or abiotic) modify the molecular factors and processes around DNA to regulate genomic activity "independent of the DNA sequence," essentially establishing an "imprint" that provides temporal and spatial control of genomic activity. The functional consequences are that the organism responds differently to its environment—and in a way not predicted from a structural analysis of the genome.

There are particular times in development when the individual is particularly sensitive to fluctuations in the environment—that is, so-called "critical windows" or "sensitive periods" for development (Burggren and Reyna 2011; Burggren and Mueller 2014). The earliest stages of life, beginning well before birth/hatching and immediately following, are generally the time of maximal plasticity. Another sensitive period is the adrenarchy and puberty (adolescence in humans). It is this latter period when the individual graduates from environmental dependence to independence. Obviously, suites of genes underlie the fundamental plasticity of an organism, particularly during development or during transitions in life history, but how these gene networks interact with the cumulative experiences of an individual's life history is the stuff of epigenetics. Thus, epigenetics provides to integrative, comparative biologists an additional set of tools and perspectives for understanding their findings in a systems-biology context.

Epigenetics as a mechanism for survival

Epigenetic responses provide for rapid multilevel (molecular/cellular/morphological/physiological/behavioral) acclimation to changing environments (Salinas et al. 2013; Burggren 2014). Of key importance, however, is that epigenetic responses do not

represent a permanent redirection of phenotype in the same way as does mutation or natural selection. Indeed, epigenetically induced phenotypic modifications of downstream generations are "sunsetted" when the environmental stressor that created them in the first place diminishes or disappears (Burggren 2014). Such a rapid "on-off" nature of an epigenetically modified phenotype can allow for optimization of phenotype if the environment changes, or if the animal migrates and reproduces. Consider, for example, the epigenetic transgenerational responses of fishes to hypoxia. The hypoxic tolerance of zebrafish larvae, as measured by the time to loss of equilibrium in severely hypoxic water, is enhanced by weeks-long exposure of their parents to hypoxia (Ho and Burggren 2010). While the underlying mechanism has not been elucidated, this enhanced tolerance (whether from increased cardiorespiratory rates, modified gill structures, altered metabolism, or other attributes) must ultimately come at some cost to the animal. Enhanced tolerance to hypoxia thus serves larvae well when they result from reproduction of their parents residing in potentially chronically hypoxic water. However, the cost of the epigenetic modification of phenotype may exceed the modest or non-existent benefit when parents reproduce in environments rarely experiencing hypoxia. In this sense, the phenotype of the animal can be finely tuned to the environment in a far more rapid fashion than could occur by mutation or natural selection.

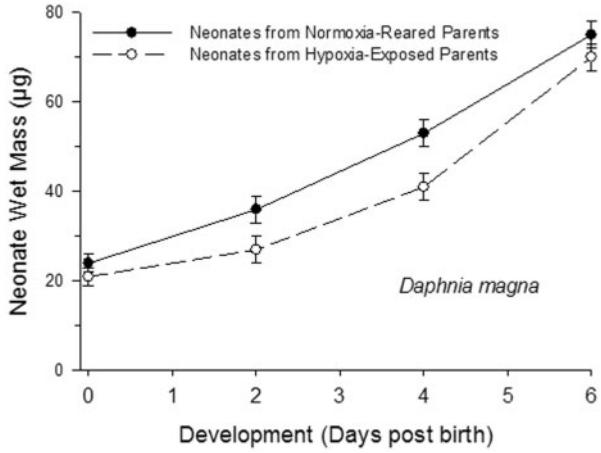

Fig. 5 Body mass changes as a function of development in neonatal water fleas, *Daphnia magna*. Neonates from mothers exposed to hypoxia for 6 days produced offspring that were significantly smaller in body mass for the first 6 days of their own development. See text for additional discussion. Modified from Andrewartha and Burggren (2012).

Epigenetics as source of variation in data

Another compelling reason to appreciate the potential for transgenerational epigenetic modification of phenotype derives from the variation that such phenotypic modification can inject into our comparative biological datasets (Burggren 2014). As example, consider the effects of a chronic, but limited, exposure to hypoxia by the F_0 generation on the morphology of the F_1 generation in the water flea, *Daphnia magna*. As evident in Fig. 5, larvae in the first brood are far smaller during the first few days of development in normoxia if their mother had been exposed to 6 days of hypoxia (4 kpa). If a comparative biologist was assessing morphological changes during growth and did not control for parental exposure to hypoxia, body mass, as just one example, would appear to be highly (and randomly) variable between individuals or populations.

Such radical phenotypic variation could presumably be imposed by inter-individual or inter-population differences in parental exposure to temperature, light, nutrition, pH, or other conditions. Yet, in a meta-analysis of the reported potential sources of variation in experimental biological studies, <5% mentioned any potential epigenetic effect, while ~45% mentioned (and controlled) nutritional aspects of the study (Burggren 2014). Clearly, we ignore at our own peril potential epigenetic influences on the animals and plants we study, brought about by uncontrolled and undocumented stressors of the "parents" of the animals and plants we study.

Conclusions and future directions

Epigenetics is an exciting, yet still somewhat enigmatic and highly immature, field of biology. The literature on epigenetics is growing at an almost unprecedented rate, and the full reach of epigenetics in the intra-generational and transgenerational manifestations of human disease is only beginning to be exposed. Additionally, epigenetics is emerging not just as a pathway for disease, but also as a highly reactive mechanism for short-term adaptation to changing environmental conditions, and as such is likely to be a key ingredient in the deeper understanding of gene–environment interactions in environmental biology.

Epigenetics plays a prominent role in understanding acclimation, adaptation, and evolution. Beyond that, however, our unraveling of epigenetic functions serves as a cautionary lesson in the ongoing study of DNA's role in inheritance. Our former "certainties" about DNA's function and its role in inheritance—such as the non-functional nature of non-protein

coding (junk) DNA, and the sole role of genes in inheritance—have been eroded over the years by discovery after discovery that overturns previous assumptions and adds new layers of complexity. That gene function can be regulated across generations though epigenetic mechanisms is very likely just the latest chapter in a large and detailed book on inheritance that remains to be completed.

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