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Review

Epigenetics and transgenerational transfer: a physiological perspective

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Summary

Epigenetics, the transgenerational transfer of phenotypic characters without modification of gene sequence, is a burgeoning area of study in many disciplines of biology. However, the potential impact of this phenomenon on the physiology of animals is not yet broadly appreciated, in part because the phenomenon of epigenetics is not typically part of the design of physiological investigations. Still enigmatic and somewhat ill defined is the relationship between the overarching concept of epigenetics and interesting transgenerational phenomena (e.g. 'maternal/parental effects') that alter the physiological phenotype of subsequent generations. The lingering effect on subsequent generations of an initial environmental disturbance in parent animals can be profound, with genes continuing to be variously silenced or expressed without an associated change in gene sequence for many generations. Known epigenetic mechanisms involved in this phenomenon include chromatin remodeling (DNA methylation and histone modification), RNA-mediated modifications (non-coding RNA and microRNA), as well as other less well studied mechanisms such as self-sustaining loops and structural inheritance. In this review we: (1) discuss how the concepts of epigenetics and maternal effects both overlap with, and are distinct from, each other; (2) analyze examples of existing animal physiological studies based on these concepts; and (3) offer a construct by which to integrate these concepts into the design of future investigations in animal physiology.

Key words: epigenetics, inheritance, evolution, acclimation.

The emergence of epigenetics

The transfer to offspring of phenotypic traits acquired by the adult parent is an old notion in biology, espoused most famously by Lamarck (Lamarck, 1809) and infamously by Lysenko (Lysenko, 1948). Lamarck's thesis was of course quickly overwhelmed by the popularity and appeal of Mendelian genetics, which mechanistically described the inheritance of traits *via* the transmission of information encoded by gene sequence. However, since the time of Lamarck, phenomena not readily explained by Mendelian genetics have persisted. For example, biologists have long noted that the coiling pattern in the veliger of the snail, *Lymnaea peregra*, is influenced by factors other than straightforward Mendelian genetics (Boycott and Diver, 1923; Sturtevant, 1923). Despite Walter Garstang's delightful 1928 poetic interpretation of the coiling mechanism (see Hardy, 1951), biologists now hypothesize that maternal factors transferred in the yolk of the snail egg interact with conventional, genetically induced protein expression to influence veliger development (Freeman and Lundelius, 1982). This so-called 'maternal effect' is now a stalwart of the broader field variously described as 'epigenetics' or non-Mendelian 'transgenerational transfer'. Epigenetics has exploded to the forefront of biological research, and now figures prominently in almost all biological disciplines. At the time of writing, the United States National Library of Medicine's PubMed lists nearly 30,000 citations with the search term 'epigenetics'. Only ~3600 publications used this term up to 2000, but more than 5000 publications contained the term 'epigenetics' in 2008 alone!

Despite intense focus on epigenetics and the accelerated exploration of all varieties of transgenerational epigenetic transfer of phenotypic characters, precise definitions have remained both controversial and elusive. Numerous authors have offered up their own definitions, or an interpretation of others' definitions, often imparting a distinct disciplinary slant (Slack, 1998; Griesemer, 2002; Bird, 2007; Ptashne, 2007; Bossdorf et al., 2008; Youngson and Whitelaw, 2008; Jablonka and Raz, 2009). While tempting, we are not going to add yet another formal definition *per se* in this review. Rather, we will briefly consider the most robust definitions, interpretations and explanations and then, perhaps more importantly for our purposes, discuss how epigenetics and non-genomic transgenerational transfer of information play important roles in contemporary research in animal physiology. Simply put, we will focus less on what epigenetics is (specific mechanisms) and more on what epigenetics does (phenotypic alterations from a physiological perspective). More details on mechanism, evolutionary implications and the involvement of epigenetics in human diseases can be found elsewhere (see Rideout et al., 2001; Jones and Takai, 2002; Kiefer, 2007; Bossdorf et al., 2008; Feil, 2008; Youngson and Whitelaw, 2008; Champagne and Curley, 2009; Jablonka and Raz, 2009; Kuzawa and Sweet, 2009; Szyf, 2009; Zeisel, 2009).

What is epigenetics? A historical interpretation and definitions

Waddington (Waddington, 1942) coined the term epigenetics, derived from 'epigenesis', a general theory first articulated by Aristotle to describe the gradual and qualitative changes in development [Aristotle's '*On the Generation of Animals*', cited in Jablonka and Lamb (Jablonka and Lamb, 2002)]. Waddington (Waddington, 1942) offered a general definition of epigenetics (of course the mechanisms were largely unknown at that time), and

related the field of epigenetics to developmental biology, evolution, ecology and, of course, genetics. While many interesting historical views of epigenetics have supplanted Waddington's definition over the years, contemporary literature now uses the term epigenetics somewhat loosely, reflecting distinctive perspectives (Table 1). The broadest definitions take the word epigenetics literally ('above genes'), using this term to describe almost any non-genetic phenomena. Somewhat more restrictive definitions explicitly discuss stable inheritance of the epigenome and the lack of change in gene sequence as necessary components of epigenetics. Molecular biological studies often define 'epigenetics' in terms of underlying mechanisms (Jablonka and Lamb, 2002; Jablonka and Lamb, 2007a; Jablonka and Lamb, 2007b; Bird, 2007; Ptashne, 2007; Lemos et al., 2008). These studies focus on the known mechanisms, often referred to as 'epigenetic marks' or 'epigenetic inheritance systems' (EIS) when these mechanisms are transmitted from one generation to the next (Jablonka and Lamb, 2005). Two epigenetic processes involving chromatin remodeling have received much attention in the last decade. DNA methylation comprises the addition of a methyl group to nucleotides, which typically silences gene expression (Wachsman, 1997; Bird, 2002; Bender, 2004; Kucharski et al., 2008; Ng and Gurdon, 2005; Law and Jacobsen, 2009; Orta et al., 2009; Reinders et al., 2009; Teixeira et al., 2009). Histone modification is the acetylation and/or methylation of chromosome packaging proteins (Grendrel and Colot, 2005; Djupedal and Ekwall, 2009; Fidlerová et al., 2009; Fu et al., 2009; Gurrieri and Accadia, 2009; Shukla et al., 2009). Non-coding RNA activity, involving small RNAs, microRNAs and large RNAs, has also been shown to play an important role in modulating protein activity *via* regulation of translation, transcription or protein structure (Costa, 2008). Less well studied but equally important epigenetic mechanisms include self-sustaining loops and structural inheritance (Jablonka and Lamb, 2005). Self-sustaining loops refer to the auto-regulation of gene activity *via* their protein products, while structural inheritance includes the transmission of cell structures from cell to cell (i.e. membranes, mitochondria), or organism to organism (i.e. prions, cilia, egg factors). Together, these mechanisms (and mechanisms yet to be uncovered) contribute to 'cell memory' or 'epigenetic memory', general terms used to describe the stable inheritance of gene expression patterns from generation to generation. [Note that some biologists consider epigenetics as the stable inheritance of epigenetic marks (Bird, 2007), while others distinguish between epigenetics and epigenetic inheritance (Gluckman et al., 2007; Jablonka and Raz, 2009); see Table 1.] Developmental biologists often focus on epigenetic effects on development within a generation of organisms (e.g. Groenendijk et al., 2007; Ng et al., 2008; Gilbert and Epel, 2009; Krause et al., 2009), sometimes with less consideration as to whether components of the epigenome can be transferred between generations (transmission to gametes/germline). On the other hand, many evolutionary biologists focus on the outcome of epigenetic transgenerational transfer (i.e. phenotype) rather than on strict definitions bound by underlying mechanisms (Bernardo, 1996a; Mousseau and Fox, 1998; Wolf et al., 1998; Reinhold, 2002; Bossdorf et al., 2008). Entering this literature can be confusing, particularly when notions of 'hard' and 'soft' inheritance, 'gametic epigenetic inheritance', etc., are evoked (Youngson and Whitelaw, 2008). Moreover, a term fundamentally woven into the vocabulary of all biologists – namely 'inheritance' – is variously used in a colloquial or rigidly scientific sense. Depending on whether a cellular biologist, an evolutionary/ecological biologist or a behavioral biologist is consulted, 'inheritance' and 'heritable changes' may refer to processes at the cellular level (mitotic or meiotic processes) (Bird, 2007), populational level (Jablonka and

Table 1. A brief categorization of contemporary definitions of epigenetics

Epigenetics definition	Focus of definition	Interpretation	Representative reference
Regulation of gene expression	Mechanistic view of the epigenome	• Uses the literal etymology of 'above' or 'beyond' genetics • No particular focus on transgenerational transfer	Shukla et al., 2009
Stable changes in gene function without changes in DNA sequence	Gene function	• Narrows definition of epigenetics to consider modification of chromatin • No particular focus on transgenerational transfer	Griesemer, 2002; Bird, 2007
Non-genetic causes of a phenotype	Phenotype	• Focuses on linkage of mechanism to outcome (phenotype) • Transgenerational transfer is part of a larger suite of outcomes, including developmental plasticity	Wolf et al., 2008; Gilbert and Epel, 2009; Krause et al., 2009
Study of heritable changes in gene function that occur without a change in the DNA sequence	Transgenerational transfer of gene function	• Explicit focus on transgenerational transfer (inheritance) of gene function • Focuses on mechanism with lesser focus on phenotypic outcome or evolutionary implications	Kiefer, 2007; Lemos et al., 2008; Lopez et al., 2009
Study of heritable phenotype without a change in the DNA sequence	Transgenerational transfer of phenotype	• Explicit focus on transgenerational transfer (inheritance) • Focuses on phenotypic outcome and evolutionary implications, with minor focus on mechanism	Groothuis and Schwabl, 2008; Youngson and Whitelaw, 2008
Study of processes that give rise to developmental plasticity and canalization	Persistent phenotype as a result of events that occur during development	• Distinction among 'epigenetics', 'epigenetic inheritance' and 'cellular epigenetic inheritance' • Focuses on cellular phenotypic outcome and evolutionary implications, with major focus on mechanism • Focuses on transgenerational transfer *via* gametic transmission	Jablonka and Lamb, 2005; Jablonka and Raz, 2009
Alteration of gene expression by modification of chromatin	Strict inheritance of epigenetic marks such as imprinted genes	• Focuses on the overlap between transgenerational non-genomic transgenerational inheritance and epigenetic inheritance • Distinction between indirect and direct epigenetic inheritance	Gluckman et al., 2007

Raz, 2009), or cultural/behavioral level (Peedicayil, 2001; Jablonka and Lamb, 2005; Sinha, 2005). In this review we take the conceptually conservative position of restricting the use of inheritance to refer to genome- or epigenome-based transfer of phenotypic traits from one generation to the next. Finally, it is worth noting that animal physiologists, *per se*, have contributed little to the plethora of definitions (this could be a good thing!). This is more a reflection of animal physiologists not yet having paid a great deal of attention to epigenetics, rather than their reticence to enter the fray. Indeed, a search of the PubMed data base described in the previous section yielded only ~200 papers containing the twin search terms 'epigenetics' and 'physiology', or less than ~1% of all papers using the former term.

Before we leave definitions, it is important to note that if molecular mechanisms of action have not been identified, some biologists hesitate to consider the transfer of non-genomic factors such as physiologically relevant molecules and parentally provided environments across generations as epigenetic in nature (Gluckman et al., 2007). We take a less restricted stance by suggesting that the potential of transmitted non-genomic factors to change the phenotype of offspring *via* epigenetic marks qualifies it as epigenetic in nature. Continuing on the topic of semantics and definitions, some investigators distinguish between transgenerational epigenetic 'effects', where the observed phenotypic transfer is simply a consequence of the transfer of non-genetic information, and the more restrictive transgenerational or gametic epigenetic 'inheritance', where transgenerational transfer of phenotypes involves the transmission of epigenetic marks to the gametes (e.g. Youngson and Whitelaw, 2008; Jablonka and Raz, 2009). Often, the underlying mechanisms for physiological transgenerational transfer of traits are unknown; thus, in this review we take a more generalist view, using transgenerational transfer and transgenerational inheritance interchangeably to describe the transmission of traits, factors and/or information that induce phenotypic changes from one generation to the next.

We do not, in this review, wish to devolve into semantic arguments over definitions (which frequently occupy much of the question and answer period following our presentations describing comparative physiological studies involving epigenetics). Yet, we find utility in offering an operational description (we resist calling this a 'definition') that is both inclusive and flexible. Hence, in this review we will regard epigenetics as 'the transgenerational transfer of phenotypic characters without modification of gene sequence', which involves both mechanism and outcome in an integrative fashion, in line with the integrative nature of physiology. Importantly, we emphasize that this view of epigenetics requires the transfer of the actual phenotype, not necessarily evidence of the transfer of the mechanism of phenotypic adjustment. In offering this description of epigenetics, it is important to recognize that numerous other terms have been used, either with or instead of the term epigenetics, including 'parental transfer' (Malual, 2001; Lam and Wang, 2006; Boulinier and Staszewski, 2008), 'transgeneration memory' (Molinier et al., 2006), 'transgenerational plasticity' (Mondor et al., 2005; Galloway and Etterson, 2007; Marshall, 2008) and 'genetic imprinting' (Cheverud et al., 2008; Wolf et al., 2008); and, in medicine, 'parent-of-origin (POE) effects' (Klutz et al., 2002; Hager et al., 2008; Herrera et al., 2008; Zhou et al., 2009). Fundamental to all is that they refer to non-genetic phenomena. Indeed, 'non-genetic effects' is often substituted for 'epigenetics'.

Notably, the wealth of terms used to describe epigenetic phenomena and related terms do not imply an advantage to the organism – merely a response in the F1 generation that could be either advantageous or detrimental. Perhaps the term 'transgenerational acclimation' has some utility in implying a response that increases the fitness of the organism, in the same way we look at acclimation in adults. For example, an embryo, larva or fetus that is more resistant to temperature fluctuations or hypoxia because their parent(s) was acclimated to these conditions would be described as environmentally 'acclimated'. Transgenerational acclimation is not adaptation in the conventional sense in this context, because the trait may or may not involve a change in allele or epiallele (alleles which carry methylation sites and thus can have variable expressivity) frequency. Rather, the transgenerational phenotypic change may be due to the transmission of parentally derived, biologically relevant factors that confer to the offspring an advantage that is transient and/or reversible, contingent upon the presence of the affecting factors. Mechanistically, adaptive and non-adaptive phenotypes may share similar epigenetic modes of induction; however, the outcomes of these mechanisms are diverse and may have opposite ecological and evolutionary implications. For example, DNA methylation has been implicated as a key epigenetic mechanism of induction in epigenetic phenomena in the fields of medicine (applied) and basic research. The former focuses on the detrimental, non-adaptive outcome of transgenerational transfer of phenotypes (Gilbert and Epel, 2009; Youngson and Whitelaw, 2008), while the latter tends to address the potentially adaptive nature of transferred traits in overall evolutionary fitness. Because a relatively small percentage of random mutations in alleles or epialleles lead to an adaptive phenotype, the transgenerational epigenetic transmission of advantageous traits is likely to signal an active or predictive process by which populations of organisms prepare the next generation for environmental challenges present in the current generation (Mousseau and Fox, 1998; Jablonka and Lamb, 2005; Youngson and Whitelaw, 2008; Jablonka and Raz, 2009). Thus, although relatively uncommon, adaptive transgenerational transfer nonetheless is an important phenomenon.

Let us now consider the inter-relationships of epigenetics and genetics, and how they are influenced by environment before discussing specific examples of epigenetics in animal physiology.

Environment, epigenetics and genetics

Waddington's (Waddington, 1942) view of epigenetics created an intersection of ecology, development, evolution and genetics, a perspective not that different from some of the conceptual underpinnings of the resurgent field of 'evo-devo' (e.g. Cañestro et al., 2007; Raff, 2007; Carroll, 2008). Yet, now knowing both mechanisms and outcomes of epigenetics, we can offer a more contemporary consideration of how especially environment, epigenetics and genetics relate to each other (Fig. 1). Just as environmental factors will drive natural selection, leading to evolution and adaptation through an alteration in the genome, so too does environment drive epigenetic events through an alteration of the epigenome (the factors and mechanisms controlling gene function and expression). As evident in the left-hand side of Fig. 1, changes in an organism's environment can result in the modification of gene expression through the mechanisms of histone modification, DNA methylation and/or non-coding RNA as discussed above. Also depicted in the figure are other important modes of epigenetic transfer that involve the modification of proteins and cellular structures without a change in gene expression, such as self-sustaining loops and structural inheritance (Jablonka and Lamb, 2005). These epigenetic marks can result in phenotypic changes at the cellular and subsequently organismal

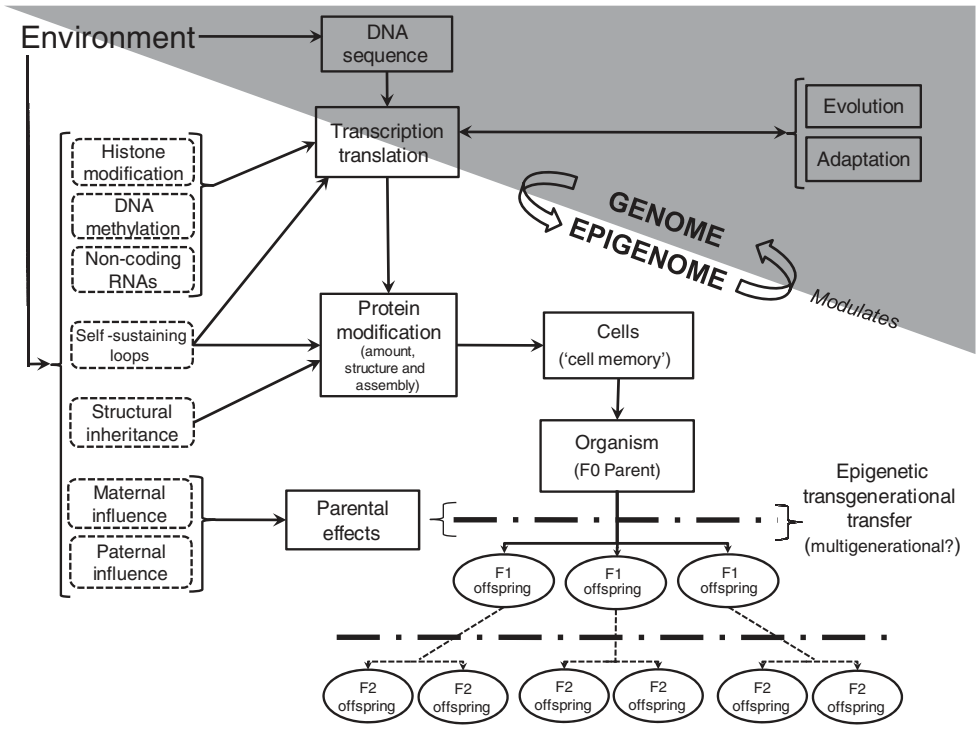

Fig. 1. Schematic diagram relating the genome, epigenome and environment with respect to transgenerational phenotypic characters in animal systems. See text for further explanation.

level of the parent. Changes in the parental condition that in turn affect the phenotype of their offspring create a transgenerational epigenetic (non-genetic) phenomenon that can persist over many generations, thus highlighting the impact of parents, grandparents and even great-grandparents (and beyond) on an organism's phenotype (moving across the bold dashed lines at the bottom of Fig. 1). Parental effects (most often termed 'maternal effects', more properly expanded to include effects from both parents) are sometimes viewed as a potential subset of epigenetics because these effects significantly alter offspring phenotype *via* transmission of non-genetic information (Badyaev and Uller, 2009). Recently, Wolf and Wade (Wolf and Wade, 2009) restricted the definition of maternal effects to include only 'causal influence of maternal genotype or phenotype on the offspring phenotype', thus preventing epigenetic mechanisms such as cytoplasmic inheritance and some cases of genomic imprinting from being considered maternal effects, as offspring phenotypic variation cannot be accounted for by maternal genotype in these cases. In the case of X chromosome inactivation due to genomic imprinting in mammals, the only 'influence' is whether the allele is maternally or paternally inherited. However, if imprinting of the offspring genome is under the active control of the maternal genotype (i.e. offspring imprinting due to maternal behavior), then this is a case of maternal effect [for a complete discussion of the topic see Wolf and Wade (Wolf and Wade, 2009)]. Here, we posit that essentially all environmentally induced transgenerational epigenetic effects at the multi-cellular organismal level result from a parental effect in which yolk, sperm and perhaps the parentally mediated environment in which the embryo, larva or fetus develops contribute to a modified F1 phenotype, which may then lead to the transmission of this phenotype to subsequent generations (F2, F3 and so on). Our reasoning falls in line with Wolf and Wade's conservative view of maternal effects because induced transgenerational epigenetic effects occur when the parent's (or parents') phenotype is altered by the environment, and this modification causes a change in the phenotype of the offspring, and potentially subsequent generations. Environmental induction of epigenetic marks differs from systems such as genomic imprinting, where all variation in offspring phenotype is accounted for by the offspring's imprinted genes, negating any maternal influence. Specific instances of maternal effects and their potential to induce epigenetic transfer of physiological traits will be further discussed below.

The relationship between environment, epigenetics and transgenerational phenomena has been a stalwart of genetics research in the last decade, and has also recently been considered in an ecological (Bossdorf et al., 2008) and evolutionary context (Pigliucci, 2007) (see also collection of articles in the theme issue 'Evolution of parental effects: conceptual issues and empirical patterns' of *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences*, 2009). Importantly, however, epigenetics has not been emphasized in studies of animal physiology – this is especially ironic for comparative animal physiology, where understanding environmental influences on physiological development has been a point of recent focus (see Warburton et al., 2006). Yet, as we will explore, there are numerous examples of epigenetic phenomena emerging in the physiological literature, and a better understanding of epigenetic phenomena can only enhance both our experimental design and our data interpretation.

We now move to a broad overview of some of the phenomena that have emerged in epigenetic studies and the associated experimental approaches, and, subsequently, narrow our discussion to studies which investigate the concept of transgenerational epigenetic transfer of physiological traits in a wide variety of animals. It is our intention to highlight not only transgenerational epigenetic transfer of physiological traits but also the complexity of the regulation of integrated physiological systems by epigenetic phenomena. Although we recognize that epigenetic phenomena in plants are formidable in depth and breadth, it is our intention to restrict our discussion to the findings that have focused on transgenerational transfer of physiological phenotypes in animals,

as differences in the timing of germline creation, reproductive physiology and behavior set animals and plants distinctly apart in terms of evolutionary implications of epigenetic effects (Jablonka and Raz, 2009). We do, however, encourage the reader to explore the rich literature in plant epigenetics (e.g. Martienssen and Colot, 2001; Berger and Gaudin, 2003; Steimer et al., 2004; Rapp and Wendel, 2005; Grant-Downton and Dickinson, 2006; Bossdorf et al., 2008; Boyko and Kovalchuk, 2008; Donohue, 2009; Reinders et al., 2009; Wang et al., 2009).

Experimental approaches and physiological studies of transgenerational epigenetics

Experiments involving transgenerational epigenetic inheritance are diverse in their methodology and approaches. Conceptually, transgenerational transfer studies can be grouped into four general categories based on the experimental approach employed, as depicted schematically in Fig. 2. The approaches range from observational (Fig. 2A) to experimental (Fig. 2B–D), with genetic crosses and heritability estimates being the cornerstone of observational studies of Mendelian and epigenetic inheritance (Fig. 2A). This approach capitalizes on parental genetic variation to estimate the heritability of traits using mathematical heritability constructs (Wilham, 1972; Bernardo, 1996a; McAdam and Boutin, 2003; Wilson et al., 2005), and to identify the mode of trait inheritance (i.e. epigenetic inheritance *versus* Mendelian inheritance) (Boycott and Diver, 1923). Because the two modes of inheritance (genetic and epigenetic) are necessary counterparts of phenotypic transmission, this first approach allows estimation of the extent to which genetics and epigenetics impact on offspring phenotype. In the case of the discovery of non-Mendelian (epigenetic) inheritance of directionality of veliger coiling in *Lymnaea*, phenotypic crosses based on the natural (inherent) shell coiling phenotypes allowed for tracking of the transmission pattern of this phenotype across many generations (Boycott and Diver, 1923). Likewise, an estimation of the proportion of variation in life history traits (i.e. reproductive age, lifespan) that can be accounted for by maternal effects in wild animal populations can be obtained from observation of naturally occurring phenotypes of each generation of a population (Pakdel et al., 2002; Baghbanzadeh and Decuypere, 2008). In contrast, large scale genetic screens used to identify maternal-effect/paternal-effect genes, such as *Bicoid* and *Nanos* in *Drosophila melanogaster* (Nüsslein-Volhard et al., 1987) (see also St Johnston, 2002; Luschnig et al., 2004), rely on the induction of genetic variation in the parental generation *via* mutagens such as ethyl methanesulfonate to assess the effect of parental genetic mutation on offspring phenotype (Fig. 2B). Essentially, mutagenesis of maternal-effect genes of the F0 generation will give rise to abnormal phenotype in the F1 generation even if the mutant allele was not inherited. The common feature of these two approaches is the emphasis placed on the role of the parental genome, *per se*, in transgenerational epigenetic transfer of offspring phenotype. Unfortunately, there is a paucity of physiological studies which utilize these approaches.

A third approach often employed in epigenetic studies is the modulation of the parental environment in a population of ideally genetically identical animals (Fig. 2C). By carefully controlling the conditions to which the parents are exposed, experimentalists can determine how the parental environment interacts with the parental genome to alter the offspring environment, and thus the phenotype of their offspring (Lacey, 1998; Henry and Harrison, 2004; Mondor et al., 2005; Ho, 2008). For example, the level of resistance of aquatic animals to a particular pollutant/toxicant has been largely

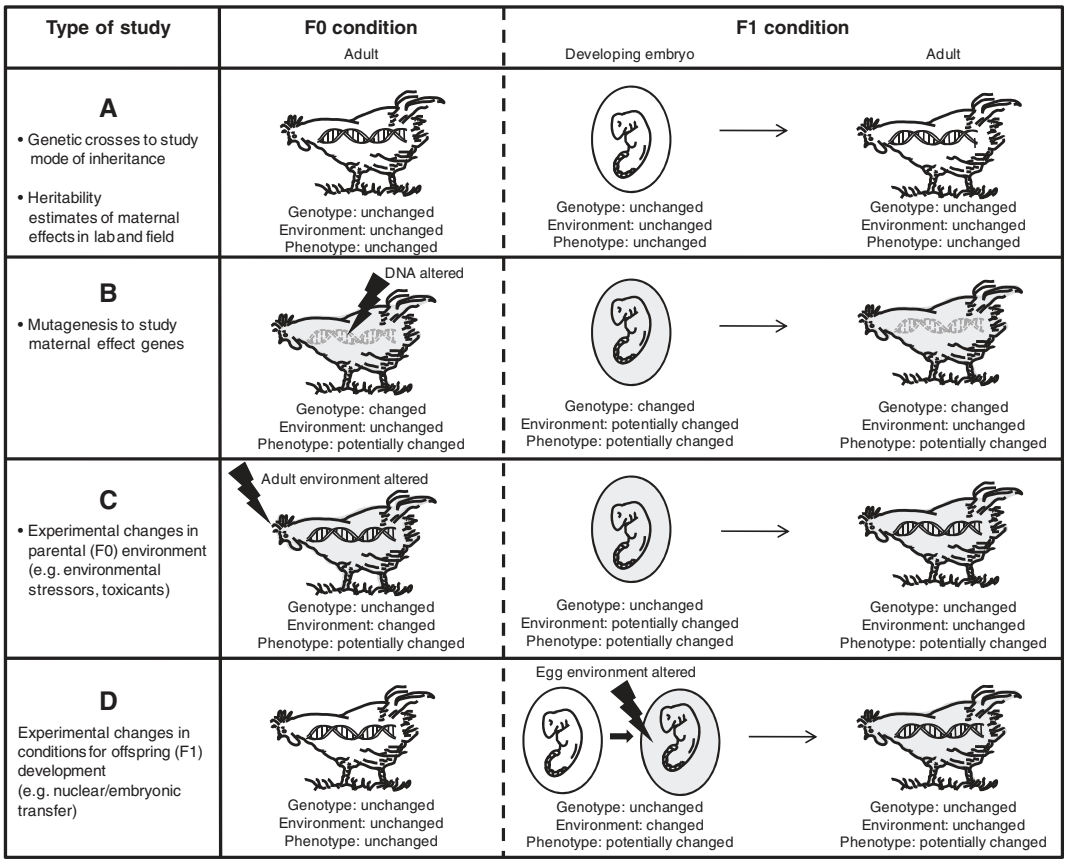

Fig. 2. Experimental designs for studies of parental effects. The top two rows depict experiments that change the allele frequency (A) or mutate genes (B) of the F0 generation to investigate the nature of the inheritance of offspring traits. The third experimental approach (C) manipulates the F0 environment, *per se*, to induce changes in parental condition. This change may cause subsequent alterations in the egg or intrauterine environment of the F1 generation, thus resulting in a change in F1 phenotype. The fourth approach (D) directly alters the parentally mediated offspring environment to induce changes in F1 phenotype. Here, the offspring environment is represented by the embryonic environment, but depending on animal species, this environment can represent a period of time extending beyond the embryonic stages into the juvenile periods of life. Lightning bolt symbol depicts experimenter-induced changes in DNA sequence (B), F0 environment (C), or F1 environment during development (D).

attributed to toxicant exposure in the parental generation (Munkittrick and Dixon, 1988; Meyer and Di Giulio, 2003).

A fourth approach capitalizes on the ability to mimic an animal's tendency to actively and/or passively regulate its offspring's environment (Fig. 2D). Experimental techniques that exemplify this approach include the direct manipulation of parentally regulated offspring environment, such as altering egg components (i.e. yolk, albumen, egg covering) (Finkler et al., 1998; Ho, 2008), cross-fostering (the artificial 'swapping' of individuals from natural parent–offspring units) (Badyaev et al., 2002; McAdam and Boutin, 2003; Biard et al., 2007), and modulation of postnatal nutritional source (Kucharski et al., 2008; Maleszka, 2008). An extension of this approach (and the previous experimental approaches) is then to directly induce mechanisms, such as DNA methylation, histone modification, and/or expression of non-coding RNAs, that were uncovered during the course of modulation of the offspring environment [e.g. drug-induced methylation of offspring DNA in honeybees to mimic the effects of royal jelly on developmental phenotype (Kucharski et al., 2008)], thus bridging the phenotypic phenomenon with potential mechanisms. A caveat of this approach pivots on the assumption that parents are capable of adjusting offspring environment in a particular manner. This assumption sharply differentiates studies that mimic parental control of the offspring environment from those that alter the abiotic environment of an organism without consideration of possible parental control.

As we subsequently discuss specific examples of transgenerational epigenetic transfer of physiological phenotypes, it will become clear that the above-mentioned experimental approaches have been commonly used, singly or in combination, to elucidate epigenetically transferred physiological traits in a variety of animal species (as illustrated in Table 2).

Parental effects in epigenetics

Processes that drive transgenerational epigenetic transfer at the organismal level are 'parental effects', as previously noted (see Fig. 1). Parental effects generally can be defined as the change in offspring phenotype due solely to maternal and/or paternal influence without a concomitant change in offspring DNA sequence (Mousseau and Fox, 1998). Parental effects have a significant impact on behavioral, morphological and molecular aspects of an individual (Mousseau and Fox, 1998; Groothuis et al., 2005; Gilbert and Epel, 2009). Yet, few studies have directly assessed the impact of transgenerational transfer of non-detrimental physiological phenotypes. For example, Jablonka and Raz (Jablonka and Raz, 2009) offer an impressive (and nearly exhaustive) list of examples of transgenerational epigenetic inheritance ranging from *Caenorhabditis elegans* to *Homo sapiens*. The majority of traits reported are morphological in nature (i.e. dumpy phenotype in nematodes). Only about one-third of these phenomena represent the epigenetic inheritance of physiological traits (i.e. propensity for disease states in humans). Even fewer of these studies actually report non-detrimental phenotypic changes that represent acclimation. Nonetheless, insights gained from studies of transgenerational epigenetic effects on animal physiology are compelling, and elicit many new questions and provocative possibilities in the realm of physiology.

Maternal effects in epigenetics

Maternal effects can arise from modulation of egg components and composition (yolk, albumen, shell characteristics and overall egg mass) (Nüsslein-Volhard et al., 1987; Bernardo, 1996b; Sinervo and Huey, 1990; Mousseau and Fox, 1998; Finkler et al., 1998; Yan, 1998; Dzialowski and Sotherland, 2004; Groothuis et al., 2005; Groothuis and Schwabl, 2008; Ho, 2008; Dzialowski et al., 2009) or intrauterine environment of the mother (Vieau et al., 2007; Darnaudéry and Maccari, 2008; Chan et al., 2009; Briana and Malamitsi-Puchner, 2009; Mastorci et al., 2009). Postnatally, offspring phenotype can be further modulated by maternal milk (lactation) and maternal behavior (for reviews, see Weaver, 2007; Cameron et al., 2008) (see also Liu et al., 1997; Francis et al., 1999; Weaver et al., 2004; Weaver et al., 2005). We will now discuss specific examples of the main mechanisms/routes of maternal effects.

Egg components and intrauterine environment

In the field of transgenerational epigenetic transfer of physiological phenotypes, one of the most explored topics is the effect of maternal deposition of physiologically relevant factors during egg formation or pregnancy on offspring phenotype. Using a variety of approaches (as discussed above; see Fig. 2), physiologists have explored how maternal transfer of these physiologically active factors can influence the offspring physiological phenotype. In oviparous species such as reptiles, amphibians, birds and some fishes, development of the embryo occurs within a self-contained egg environment, the characteristics of which are a direct result of the maternal condition at the time of egg formation (McNabb, 1988; Price, 1998; Janzen et al., 2002; Poisbleau et al., 2009). Generally, egg composition and quality can be an accurate predictor of hatchling morphology (i.e. size and mass), but only a handful of studies have examined how the changes in morphological parameters may lead to, or correlate with, changes in physiological parameters (see Sinervo and Huey, 1990; Williams, 1994; Bernardo, 1996b; Calta, 2001; Finkler et al., 1998; Dzialowski and Sotherland, 2004; Wallace et al., 2006; Dzialowski et al., 2009). Sinervo and Huey (Sinervo and Huey, 1990) reduced the body size of a southern population of lizards (*Scleoporus occidentalis)* by yolk removal prior to incubation to mimic the phenotype of the smaller, northern population. This treatment affected juvenile morphology (hindlimb span) and physiology (sprint speed and stamina) differently. Irrespective of population identity, burst speed was highly correlated with artificial modulation of hindlimb span, while stamina remained population specific despite artificial shortening of hindlimb span. Similar to this 'allometric engineering' in lizards, changes in the volume of chicken (*Gallus gallus domesticus*) egg albumen caused changes in the morphology of near-term precocial embryos, but no changes in metabolic rate (Finkler et al., 1998). In contrast, the metabolic rate of both the precocial emu (*Dromaius novaellandiae*) and the altricial double-crested cormorant (*Phalacrocorax auritus*) embryo was significantly correlated with natural inter-individual variation in egg size (Dzialowski and Sotherland, 2004; Dzialowski et al., 2009). These bird studies strongly suggest that, despite the inter-specific difference in developmental maturity at the time of hatching (altricial *versus* precocial), egg size-dependent transfer of some physiological phenotypes is universal across species. The inter-specific difference seen between chicken embryos, and emu and cormorant embryos may be due to the difference in experimental approach used (artificial modulation of egg components *versus* natural variation in egg size), or may be a true effect of inter-species difference among birds.

Maternally derived factors contained in the egg or passed from mother to offspring *via* the placenta or milk, such as hormones, antibodies, antioxidants (carotenoids), and levels of other biologically active factors show considerable natural variation

Table 2. Representative studies of transgenerational transfer in animal comparative physiology

Species	Experimental approach	Transferred physiological or related traits	Reference
Invertebrates			
American dog tick (*Dermacentor variabilis*)	Alteration of maternal humidity level	Dehydration tolerance limit Humidity tolerance	Yoder et al., 2006
Daphnia (*Daphnia magna*)	F0 exposure to pesticides (diazonin) and herbicides (molinate)	Decreased tolerance to diazonin Increased tolerance to molinate	Sánchez et al., 2000; Sánchez et al., 2004
Fish			
Zebrafish (*Danio rerio*)	Alteration of food type in F0 (live food *versus* dry food)	Cardiac parameters (stroke volume, heart rate, cardiac output) Red blood cell concentration	Schwerte et al., 2005
Zebrafish (*Danio rerio*)	F0 exposure to low oxygen environment	Hypoxia resistance	Ho, 2008
Feral white sucker (*Catostomus commersoni*)	Maternal copper exposure	Growth Survival Copper resistance	Munkittrick and Dixon, 1988
Fathead minnow (*Pimephales promelas*)	Maternal copper exposure	Larval copper resistance	Sellin and Kolok, 2006
Reptiles			
Lizard (*Scleoperus occidentalis*)	Yolk removal	Burst speed Exercise stamina	Sinervo and Huey, 1990
Viviparous lizard (*Lacerta vivipara*)	Maternal exposure to variable rainfall	Survivability	Marquis et al., 2008
Birds			
Finch (*Carpodacus mexicanus*)	Cross-fostering Laying order	Sex-dependent growth Sex-dependent survivability	Badyaev et al., 2002
Blue tit (*Parus caeruleus*)	Egg yolk carotenoid manipulation Cross-fostering	Cell-mediated immune response Growth rate	Biard et al., 2007
Chicken (*Gallus gallus domesticus*)	Breed-specific alteration of egg yolk environment	Heart rate Growth rate	Ho, 2008
Broiler chicken (*Gallus gallus domesticus*)	Heritability estimate of traits using mathematical models	Ascites-related traits	Pakdel et al., 2002; Baghbanzadeh and Decuypere, 2008
Quail (*Coturnix coturnix*); chicken (*Gallus gallus domesticus*); duck (*Anas* sp.); turkey (*Meleagris gallopavo*); goose (*Anser cygnoides*); emu (*Dromaius novaellandiae*); ostrich (*Struthio camelus*)	Species-specific alteration of egg yolk environment	Heart rate	Ho, 2008
Emu (*Dromaius novaellandiae*)	Inherent variations in egg size	Metabolic rate	Dzialowski and Sotherland, 2004
Double-crested cormorant (*Phalacrocorax auritus*)	Inherent variations in egg size	Metabolic rate	Dzialowski et al., 2009
European starling (*Sturnus vulgaris*)	Experimental elevation of yolk corticosterone	Flight muscle physiology Flight performance	Chin et al., 2009
Pied flycatcher (*Ficedula hypoleuca*)	Alteration of maternal pre-laying nutrition Antioxidant supplementation	Growth rate Resistance to mite infestation	Moreno et al., 2008
Mammals			
Mouse (*Mus musculus*)	Heritability estimates	Response to glucose challenge	Jarvis et al., 2005
Mouse (*Mus musculus*)	Maternal low protein diet during oocyte maturation	Postnatal hypertension Attenuated arterial responsiveness to vasodilators (acetylcholine and isoprenaline)	Watkins et al., 2008
Mouse (*Mus musculus*)	Maternal nutritional alteration prior to pregnancy and during pregnancy	Obesity Leptin resistance Insulin resistance	Howie et al., 2009
Rat (*Rattus norvegicus*)	Alterations in maternal licking and grooming behavior (i.e. cross-fostering)	Stress response	Liu et al., 1997; Francis et al., 1999; Weaver et al., 2004; Weaver et al., 2005
Rat (*Rattus norvegicus*)	Maternal glucocorticoid exposure	Glucose metabolism	Drake et al., 2005
North American red squirrel (*Tamiasciurus hudsonicus*)	Heritability estimate of traits using models Cross-fostering	Growth rate	McAdam and Boutin, 2003
Human (*Homo sapien*)	Observed nutritional status of parents	Risk of syndromes and disease states	Reviewed in Gluckman et al., 2007

and can also be modified experimentally. These maternally derived factors can have a significant impact on offspring physiological phenotype (i.e. immunological capacity, growth and development, cardiovascular function and toxicant resistance) in a wide range of animals (Munkittrick and Dixon, 1988; Meyer et al., 2003; Weaver et al., 2004; Groothuis et al., 2005; Sellin and Kolok, 2006; Groothuis and Schwabl, 2008; Ho, 2008; Moreno et al., 2008; Romano et al., 2008; Hasselquist and Nilsson, 2009). In chickens, growth rate and heart rate of early-stage embryos were significantly altered when the embryos were removed from their native yolks and explanted to continue development on the yolk of either other bird species [quail (*Coturnix coturnix*), chicken (*Gallus gallus domesticus*), duck (*Anas* sp.), turkey (*Meleagris gallopavo*), goose (*Anser cygnoides*), emu (*Dromaius novaellandiae*) and ostrich (*Struthio camelus*)] or other chicken breeds (Cornish Rock Broilers and a variety of Bantam breeds) (Ho, 2008). Metabolic rate, however, was not affected (Ho, 2008). Interestingly, the effects of yolk environment were both breed and species specific. This indicates that the onset and rate of change of some early physiological processes are largely governed by phylogenetically determined maternal factors contained in the egg yolk. Indeed, analysis of the yolks revealed that thyroid hormone concentration, a chronotropic and growth-enhancing hormone, varied significantly both among and within bird species (Ho, 2008), as had been indicated previously by McNabb (McNabb, 1988).

Experiments that alter levels of maternally derived factors through manipulation of maternal conditions (i.e. maternal supplementation or stress-induced modulation), or that artificially alter yolk factor concentrations report dose-dependent changes in physiological parameters such as stress response, immune capacity, flight performance and growth rate of the offspring. In the pied flycatcher (*Ficedula hypoleuca*), increased yolk immunoglobulin concentration *via* maternal nutritional supplementation increased nestling plasma immunoglobulin Y levels and resistance to mite infestation (Moreno et al., 2008), while the transfer of carotenoids (antioxidants) from the female to her offspring in the blue tit (*Parus caeruleus*) and the grey partridge (*Perdix perdix*) positively modulates the immune response of nestlings (Biard et al., 2007; Cucco et al., 2008). In contrast, maternal testosterone negatively affects hatchling immune function while enhancing growth in birds and lizards (Groothuis et al., 2005; Cucco et al., 2008). Moreover, in European starlings (*Sturnus vulgaris*), increased yolk corticosterone concentration, indicative of maternal stress levels, enhances juvenile flight performance by increasing the mass and maturity of flight muscles (Chin et al., 2009). In placental animals, maternal conditions such as nutritional stress affect growth rate, immune capacity, survival and breeding performance of offspring (Festa-Bianchet and Jorgenson, 1998; Lummaa and Clutton-Brock, 2002; Lummaa, 2003; Jones et al., 2005). Interestingly, exposure to a low protein diet during oocyte maturation in the mouse (*Mus musculus*) resulted in abnormal postnatal cardiovascular function in the offspring (Watkins et al., 2008). This indicates that in placental animals, maternal effects on offspring physiology can be a consequence of processes involved in oocyte component deposition in addition to consequences of placental transfer. In the case of maternal effects in mammalian (human) disease states, a large amount of literature on rodent research reveals that nutritional perturbations in the F0 generation will result in disease phenotypes (i.e. diabetes mellitus, endocrine disruption, impaired glucose metabolism) that persist beyond the second generation (for review, see Gluckman et al., 2007).

The influence of maternal exposure to environmental toxicants on offspring physiology has been documented in mammalian, bird and fish species (Munkittrick and Dixon, 1988; Golub et al., 1998; Sánchez et al., 2000; Sánchez et al., 2004; Meyer and Di Guilio, 2003; Rogers et al., 2005; Sellin and Kolok, 2006; Chen et al., 2008; Liu et al., 2008). In birds and mammals, the physiological parameters assessed have largely been detrimental in nature; however, in some fish species, maternal toxicant exposure confers offspring resistance to that toxicant, with decreased survival when exposed to a variety of other stressors such as ultraviolet radiation (Munkittrick and Dixon, 1988; Meyer and Di Guilio, 2003; Sellin and Kolok, 2006). These findings suggest that the environmentally induced transfer of physiological traits to offspring is highly complex. There seems to be a trade-off of advantageous and detrimental physiological traits which may not necessarily increase the overall fitness of the individual.

Maternal behavior as an epigenetic factor

Neonatal care-taking behavior of parents has a great influence on offspring physiological phenotype. Experimentally, the effects of care-taking behavior on offspring phenotype can be addressed by removing the parental behavior or inducing variation in parental behavior by cross-fostering. Probably the most convincing evidence of the transgenerational transfer of physiological traits is the maternal transfer of the stress response and immune capacity in rodents. These physiological traits are well correlated with maternal behaviors such as grooming and pup licking in the rat (Francis et al., 1999; Liu et al., 1997; Weaver et al., 2004; Weaver et al., 2005). In oviparous species, it appears that cross-fostering (maternal care-taking behavior) may not be as influential on offspring physiology as in mammals (Badyaev et al., 2002) (but see Biard et al., 2007), but further examination of the effects of parental behavior on offspring physiological traits must be carried out to substantiate these claims.

Clearly, this brief synthesis of maternal-effect studies reveals the complexity and disparity of physiological changes that can occur within one individual and among populations and species.

Paternal effects in epigenetics

Paternal effects play a large role in the development of widely different animals such as fruit flies (Fitch et al., 1998), mice (Chong et al., 2007), non-human primates (Charpentier et al., 2008) and humans (Tesarik et al., 2002), and thus are crucial to the developmental trajectory of an organism. Paternal factors, such as mRNAs contained in spermatozoa, have been implicated in the regulation of cleavage during embryogenesis. This regulation is likely to be a result of mRNA-induced histone modification and DNA methylation (Fitch et al., 1998; Nanassy and Carrell, 2008). Moreover, the results of phenotypic crosses (Fig. 2A) attribute offspring morphology and defensive behavior phenotypes to paternal transgenerational epigenetic transfer (Guzman-Novoa et al., 2005; Yamamoto and Reinhardt, 2003). Despite evidence of paternal effects in offspring phenotype, paternal effects have often been discounted as major forces in epigenetic inheritance because the origin of paternal effects has not been as clearly defined as that of maternal effects. This is an ill-conceived assumption because, as discussed below, there is strong evidence of spermathecal fluids playing an important role in oviposition and perhaps even in offspring development. Additionally, the level of paternal care largely influences offspring body size and horn size in the dung beetle (Hunt and Simmons, 2000).

Unfortunately, the majority of paternal-effect studies have focused on early embryonic mitotic division, cell number, general morphology and behavior, and very few have assessed potential influences on physiological parameters. Honest attempts have been made to assess the influence of paternal effects on physiological traits; however, morphology (i.e. otolith size) is often used as a corollary measure for a physiological trait (i.e. metabolic rate) (Yamamoto and Reinhardt, 2003). Further investigation of paternally induced epigenetic processes is highly warranted and should prove useful to understanding this component of epigenetics.

Exploring the unknowns of physiological epigenetic effects

Some epigenetic investigations examine the proportion of the variation in offspring traits that is explained by direct genetic effects and parental effects. These include experimental approaches of genetic crosses and heritability estimates where a statistical model is utilized to calculate the percentage of variability accounted for by parental effects (Boycott and Diver, 1923; Bernard, 1996b; McAdam and Boutin, 2003; Wilson et al., 2005). In these cases, the provenance of the transgenerational epigenetic effect often remains unknown (or uninvestigated). Also, studies that assess offspring physiology after manipulating parental conditions/environment (see Fig. 2B) often do not directly identify the potential parental factors responsible for the physiological change (Schwerte et al., 2005; Yoder et al., 2006; Ho, 2008; Watkins et al., 2008). In zebrafish, for example, modulation of parental abiotic environment and nutrition confers increased resistance to the environmental stressor and alterations in cardiovascular parameters (stroke volume, heart rate, cardiac output and red blood cell concentration) to the subsequent generation (Schwerte et al., 2005; Ho, 2008). However, it remains unknown whether maternal or paternal effects, or some combination of the two, are responsible for the transferred physiological traits. Moreover, the specific factor/signal responsible for the changes observed in offspring physiology has yet to be determined.

Other studies have modulated maternal environment, *per se*, to investigate the effects it has on the subsequent generation, but have not identified the particular maternally derived factor responsible for the observed effects. For example, humidity resistance in dog ticks (*Dermacentor variabilis*) can be attributed to the environmental humidity that the female parent was exposed to during egg laying (Yoder et al., 2006), yet the particular mechanism of transfer remains elusive. Even the studies discussed above (see 'Maternal effects in epigenetics'), which have narrowed the origin of the effect to maternally derived egg factors, have yet to lead to identification of specific signals/mechanisms of physiological change (Munkittrick and Dixon, 1998; Badyaev et al., 2002; Sellin and Kolok, 2006; Ho, 2008; Watkins et al., 2008). For example, although maternal transfer of toxicants in oviparous species has been well documented (Donaldson et al., 1999; Elliot et al., 2005; Jaspers et al., 2005; Chen et al., 2008; Van den Steen et al., 2009), it has not been determined whether maternal transfer of copper (in fishes) or diazinon (in invertebrates) is responsible for the maternal effects on toxicant resistance observed (Munkittrick and Dixon, 1998; Sánchez et al., 2000; Sánchez et al., 2004; Sellin and Kolok, 2006).

Some studies have begun to provide exciting connections between mechanism and physiological phenotype. There are conclusive studies that identify histone methylation in offspring *via* maternal nutritional state or behavior as modes of transgenerational transfer of physiological traits such as glucose metabolism and stress responsivity in mice (Liu et al., 1997; Weaver et al., 2004; Weaver et al., 2005; Weaver et al., 2007) (see also Table 2). Other studies have indirectly established a connection between nutritionally induced DNA methylation *via* DNA cytosine-5-methyltransferases and honeybee reproductive physiology and social behavior by assessing sexual/reproductive morphology (Kucharski et al., 2008). Furthermore, the genomic and non-genomic actions of maternally derived molecules such as hormones and carotenoids in offspring development have been implicated in long-lived maternal effects; most notably in birds (see 'Maternal effects in epigenetics'). However, given the considerable number of studies showing transgenerational transfer of physiological characteristics without a description of mechanism (and, conversely, those showing epigenetic mechanism without directly addressing its impact on physiological traits), we encourage the consideration of experimental approaches which bridge the gap between biochemical and molecular mechanism, physiological changes observed in the offspring, and the ecological and evolutionary implications of these changes. For example, chromatin remodeling *via* methylation and acetylation of histones and DNA is commonly hypothesized to be the likely candidate for epigenetic transfer of traits, but these and other modes of action previously discussed (i.e. self-sustaining feedback loop, structural inheritance, small RNAs) have not been as rigorously explored in animal models as they have in plant models (Johannes et al., 2008; Johannes et al., 2009; Reinders et al., 2009; Teixeira et al., 2009). Thus expansion of potential epigenetic mechanisms underlying transgenerational transfer of physiological traits would be of great value to the integration of physiological and epigenetic phenomena. Additionally, although maternal effects make up a large portion of documented epigenetic phenomena, the exact physiological impacts of maternally transmitted molecules on offspring are still under intense investigation. Overall, there is a tendency for scientists to use morphology as a predictor of physiology, thus curtailing the potential advance in direct physiological assessment of epigenetic effects. Another point of concern is the lack of formal consideration of ecological and evolutionary implications (or adaptive nature) in the experimental design of transgenerational transfer studies. This is largely due to the lack of experimental data on the underlying mechanisms of epigenetic phenomena, thus further emphasizing the need for a deeper understanding of mechanisms of epigenetic transfer of physiological traits.

How pervasive are epigenetics and non-genetic transgenerational transfer of phenotype in animal physiology?

As evident from the preceding discussion of physiological epigenetics, there are numerous studies that either have been designed to test epigenetic physiological effects or, during their execution, have revealed physiological epigenetic influences. Much like some optical illusions that at first have to be pointed out but then are difficult to ignore, it is increasingly easy to see potential epigenetic influences in many aspects of animal physiology. If epigenetic effects are indeed pervasive, then this makes it all the more important that comparative physiologists have a thorough knowledge of the provenance of animals coming from animal suppliers, or of environmental fluctuations experienced by wild-caught animals. Even the use of genetically pure stocks may not guard against the induction of new phenotypes in their offspring through epigenetic effects, which both increases variation in the data and confounds its analysis. Additionally, developmentally related phenomena such as fetal programming of physiological and

biochemical phenotype, and physiological developmental plasticity could in some instances actually be attributed to epigenetic effects, or at the very least involve them [an introduction to the extensive literature can be found elsewhere (Gluckman and Hanson, 2005; Bezek et al., 2008; Gilbert and Epel, 2009)].

Epigenetics can be broadly pervasive not just in the single transgenenerational scenario but also through multiple generations – i.e. beyond the F1 generation as indicated in Fig. 1. Anway and colleagues (Anway et al., 2005) report that a reduction in sperm count in rats induced by endocrine disruptors in the F0 male is still evident in the F4 generation. Epigenetic effects lasting 3–4 generations in mammals are not uncommon (Gluckman et al., 2007; Jablonka and Raz, 2009), but physiological effects into generations beyond this are rare or as yet unsubstantiated. However, epigenetic effects lasting 10–15 generations have been reported in insects and even up to 10–40 generations in the nematode *Caenorhabditis elegans* (see Jablonka and Raz, 2009). It will be very interesting to see whether physiological effects can persist over as many generations as morphological, biochemical or molecular effects, for example.

Sorting out non-genetic transmission of phenotype beyond the F1 generation is not as straightforward as one might imagine. Consider that an egg- or fetus-bearing adult female holds not only the F1 generation but also within that egg or fetus the germ-line of the F2 generation (Skinner, 2008; Jablonka and Raz, 2009). Similarly, an exposed male contains the germ line, if not the actual sperm, subsequently used in fertilization. Thus, for example, exposure to a stressor as an adult could influence the stressor responses of that adult's grandchildren without evoking elaborate, indirect epigenetic mechanisms, because the germ line from which they arose was also directly exposed to hypoxia (Fig. 3). Jablonka and Raz (Jablonka and Raz, 2009) refer to this type of induced transmission as 'direct induction', a case in which a stimulus directly modifies the germ line epigenome but not the parental soma. To add to the complexity of epigenetic transmission, there are three other routes by which the induction of an inherited effect can occur from one generation to the next [for detailed discussion see Jablonka and Raz (Jablonka and Raz, 2009)]. Skinner (Skinner, 2008) has argued that transgenerational transfer of a phenotype can only be confirmed by the appearance of a transferred phenotype in the F3 generation where neither the embryo nor its germ line was directly exposed to the stressor. Unfortunately, many studies of transgenerational transfer of physiological traits do not assess phenotypes beyond the F1 generation, leaving open only the potential of the transfer of epigenetic mechanisms as the inducing factor. Germ line exposure in animals has been implicated in multi-generational transfer of some human diseases, including cancers with a familial component (Gilbert and Epel, 2009; Jablonka and Raz, 2009; Fleming, 2008) and even the condition of obesity and some related forms of diabetes (Rampersaud et al., 2008). Notably, germ line exposure in the F0 generation is not an issue in plants where, unlike in animals, reproductive structures and their gametes do not form in the F1 generation until plant development is well underway.

Additional mechanisms for transgenerational transfer?

While DNA methylation, histone modification and, more recently, the regulatory role of non-coding RNAs are widely acknowledged mechanisms for non-genetically induced transgenerational transfer, the fact that detailed study of epigenetic phenomena is a fairly recent activity suggests that additional mechanisms are likely to be discovered. Indeed, the study of mammals that are able to alternate between two extremely different physiological states (i.e. hibernation) has shed some light on a wide array of potential epigenetic controls of metabolic rate suppression (Morin and Storey, 2009). Also consider, for example, that biologists generally assume that any paternal contribution to epigenetic effects is strictly through the germ line, because in many animals little other than the sperm and some modest amounts of supporting fluids are transferred at mating. Yet, in some insects the male packages his sperm into a large spermatheca that is transferred to the female at the time of mating. A variety of proteins, hormones and other bioactive materials produced by the male reproductive accessory glands of insects form an integral part of the spermathecal contents. Using radioactive labels, these materials have been tracked in the female hemolymph and beyond to the ovary, where among other actions they can stimulate oviposition in the female (Kaulenas, 1992; Gillott, 2003). We speculate that it is also possible that these

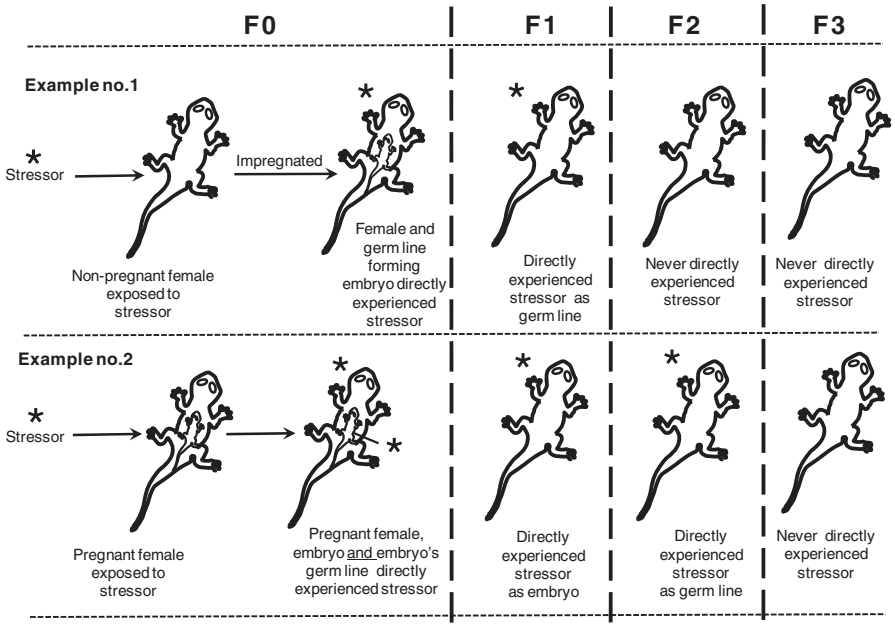

Fig. 3. Consideration of epigenetics in the context of embryonic and germ line exposure to a stressor in animals. In Example no. 1, offspring beginning with the F1 generation have never been exposed to the stressor. In Example no. 2, however, the fact that the female was pregnant when exposed to the stressor means that her embryos and the embryos' germ lines all experienced the stressor, a direct exposure that only disappears with the F3 generation.

bioactive molecules could be incorporated into the embryo and persist into subsequent generations. There, they could have direct epigenetic influences on phenotype (e.g. an endocrine effect rather than a DNA methylation effect), eventually 'diluting out' and falling below the effective concentration. A large transfer of fluid at mating is characteristic of some insects but not, for example, of vertebrates, highlighting the need for animal physiologists to be alert for novel mechanisms of transgenerational transfer.

The 'genetics' of physiological epigenetics

Epigenetic phenomena are typically portrayed separately from direct genetic phenomena, and we continue this trend in Fig. 1. Yet, while the field of epigenetics distinguishes itself from that of genetics, there are potential interactions between the genome and the epigenome that have important implications for evolutionary biology in general [first extensively discussed by Jablonka and Lamb (Jablonka and Lamb, 1995) and later expanded upon by them (Jablonka and Lamb, 2005) and by Pigliucci (Pigliucci, 2007)], and human disease mechanisms specifically (e.g. Esteller, 2007). Two interesting components of the interaction between epigenetics and genetics are the potential of epigenetic marks (i.e. change in chromatin structure) to influence the probability of mutation, transposition and/or recombination of DNA sequence (see Jablonka and Lamb, 1995), and the predisposition of a gene to be selected due to environmentally induced epigenetic marks imposed upon it (see Jablonka and Lamb, 2005). Additionally, damage to DNA potentially leads to heritable epigenetic marks (Jablonka and Raz, 2009). Now, also consider that, at the most basic level, all of the mechanisms that enable epigenetic effects are already incorporated into an organism's genome and are themselves subject to evolution (Mohn and Schübeler, 2009). That is, differences in the extent to which different species show epigenetic phenomena are themselves potential adaptations resulting directly from natural selection. For example, consider Species A producing an inherently larger transgenerational effect than Species B as a result of the same environmental stimulus. If that response is beneficial (i.e. results in better offspring survival in the face of that stimulus), then the ability to show a larger epigenetic transgenerational transfer of phenotype is, itself, an inherited characteristic. To clarify, it is not the effect that evolves but the ability to show the effect, as evident in the linkage between epigenetics/epigenome and genetics/genome in Fig. 1. Whether there are true 'epigenetic genes' (as distinct from genes that code for components of the epigenetic mechanisms) remains to be determined. Future studies exploring the interplay of epigenome and genome should prove interesting (if complex!).

Conclusions

Epigenetics is at a crossroads, with a rapidly growing literature that currently co-mingles multiple mechanisms, a variety of definitions, and different levels of effect (molecular, cellular, organismal). Against this complex backdrop, epigenetics and transgenerational transfer are beginning to be a focus in physiological studies, a trend we anticipate will keep growing. As more and more physiological studies begin to look at the transgenerational transfer of physiological traits, it will become increasingly important to distinguish transgenerational epigenetic transfer from simpler non-genomic effects, and both of these from genetic effects. This will require the actual incorporation of epigenetics into the interpretation and design of future experiments.

The true reach of transgenerational transfer of traits through epigenetic mechanisms will likely be demonstrated in the near future. In the meantime, we should guard against unrecognized epigenetic effects introducing additional variation into physiological studies. Simply controlling for, or at least verifying, the generational history of the animal populations being studied can help reduce undesirable variation. This will ensure that physiological effects are not emerging from the expression of epigenetic phenomena. More than just guarding against epigenetic influences, however, we can extend the number of studies that actually explore epigenetics, especially when considering issues of adaptation and fitness.

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