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REVIEW

Dynamics of epigenetic phenomena: intergenerational and intragenerational phenotype 'washout'

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ABSTRACT

Epigenetic studies of both intragenerational and transgenerational epigenetic phenotypic modifications have proliferated in the last few decades. However, the strong reductionist focus on mechanism that prevails in many epigenetic studies to date has diverted attention away what might be called the 'dynamics' of epigenetics and its role in comparative biology. Epigenetic dynamics describes how both transgenerational and intragenerational epigenetic phenotypic modifications change in non-linear patterns over time. Importantly, a dynamic perspective suggests that epigenetic phenomena should not be regarded as 'digital' (on–off), in which a modified trait necessarily suddenly disappears between one generation and the next. Rather, dynamic epigenetic phenomena may be better depicted by graded, time-related changes that can potentially involve the 'washout' of modified phenotype both within and across generations. Conceivably, an epigenetic effect might also 'wash-in' over multiple generations, and there may be unexplored additive effects resulting from the pressures of environmental stressors that wax, wane and then wax again across multiple generations. Recognition of epigenetic dynamics is also highly dependent on the threshold for detection of the phenotypic modification of interest, especially when phenotypes wash out or wash in. Thus, studies of transgenerational epigenetic effects (and intragenerational effects, for that matter) that search for persistence of the phenomenon are best conducted with highly sensitive, precise quantitative methods. All of the scenarios in this review representing epigenetic dynamics are possible and some even likely. Focused investigations that concentrate on the time course will reveal much about both the impact and mechanisms of epigenetic phenomena.

KEY WORDS: Acclimation, DNA methylation, Epigenetics

Introduction: the focus and utility of contemporary epigenetic studies

Epigenetic phenomena have long been appreciated, but the last few decades have seen a veritable explosion of interest in epigenetic effects (for reviews see Burggren, 2014; Cantone and Fisher, 2013; Chahwan et al., 2011; Hauser et al., 2011; Ho and Burggren, 2010; Jablonka and Lamb, 1989; Jablonka and Lamb, 2002; Jablonka et al., 1998; Jablonka and Raz, 2009). Despite continuing advances in identification of the underlying molecular mechanisms, the scope of epigenetic effects in comparative biology is still both greatly underappreciated and poorly understood (Burggren and Crews, 2014). Historically, the original descriptions of epigenetics, beginning with Waddington in 1942 (Waddington, 1942) to Nanney in 1957 (Nanney, 1957) and Riggs and Holiday in the mid-seventies (see Holliday, 2005), were in the context of phenotype alteration without change(s) in the gene sequence. Sometimes – but not always – these phenotypic changes involved transgenerational phenotype transfer. An extensive literature now exists in medicine, where epigenetics is viewed in a pathological light (Jones and Sung, 2014; Mazzio and Soliman, 2014; Mill and Heijmans, 2013; Ogino et al., 2013); in biology, where epigenetics is of interest from an adaptive or evolutionary vantage rather than as a pathology (Burggren, 2014; Burggren and Crews, 2014; Cropley et al., 2012; Horowitz, 2014; Kuzawa and Thayer, 2011; Varriale, 2014); and in the psychological–behavioral field (Crews, 2008; Peña et al., 2014; Pishva et al., 2014; Rutten and Mill, 2009; Svrakic and Cloninger, 2010). Noteworthy is that, in recent years, terminology surrounding epigenetics has been rendered more complex by the increasingly common practice in medicine to refer to epigenetics as an intragenerational phenomenon that creates pathologies during an individual's lifetime – e.g. 'the epigenetics of cancer'.

Irrespective of how epigenetics is viewed or the field of study in which it is investigated, epigenetic effects can be classified in two categories (for a review, see Burggren and Crews, 2014). So-called 'context-dependent' epigenetic inheritance that affects phenotype results from direct and continuing exposure within or across generations to an environmental stressor. As long as the stressor is present, the phenotype remains modified. By contrast, so-called 'germline-dependent' inheritance results when the germline of an organism is directly affected, and phenotypic modifications consequently persist across generations in the absence of the original causative agent (i.e. the environmental stressor). This framework includes so-called maternal effects (or, perhaps, more expansively, parental effects to include paternal effects), as an example of context-dependent epigenetic inheritance (Burggren and Crews, 2014).

Epigenetics is of interest to more than clinicians or those focused on the mechanisms and impacts of transgenerational inheritance. Epigenetics can also be of great relevance to ecological and evolutionary studies focusing on mechanisms of survival in changing environmental conditions, such as the variable nature of environments subject to climate change. Unlike permanent phenotypic modifications that can result suddenly from gene mutation or more slowly through natural selection, epigenetic phenotypic modifications can be induced by a significant environmental perturbation, but can then be 'sunsetted' when and if the environmental stressor retreats or disappears. In this respect, epigenetic phenotypic modifications – either within or across generations – can be viewed as highly responsive mechanisms for responding to environmental change in a framework shorter than is provided for by 'conventional' mechanisms of evolution selection (Burggren and Crews, 2014; Cropley et al., 2012).

Epigenetics is also of great relevance because epigenetic phenomena may additionally be a source of considerable previously unidentified variation in comparative biological studies, with such

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variation previously attributed to 'experimental error' or simply regarded as inherent (and thus unavoidable) 'noise'. However, an as yet undetermined but potentially large component of this variation could result from epigenetic transgenerational effects that track back to either context-dependent or germline-dependent exposure (Burggren and Crews, 2014).

Epigenetic dynamics: a conceptual framework

What is 'phenotype' in a comparative biological context?

It may seem curious to have to discuss the meaning of 'phenotype', but it is critical to understanding this perspective on potential epigenetic dynamics. In many respects, phenotype is 'in the eye of the beholder'. Thus, an animal with a single gene knockout that fails to express the corresponding transcript is viewed by a molecular biologist as having a modified phenotype. Yet, following the 'buffering' of the knockout effect by the cellular and external environment (Noble, 2015), the absence of this transcript may have absolutely no effect on the overall phenotype at cellular, morphological, physiological or behavioral levels.

In the following discussion of epigenetic dynamics in comparative biology, I will be emphasizing 'complex' phenotypic changes that probably go beyond even pleiotropic gene expression to include major suites of genes whose collected expression may be suppressed to variable degrees by DNA methylation, histone modification, etc. Thus, rather than refer to a modified phenotype as, at a minimum, the presence or absence of a single expressed protein, I will be focusing on a more organismal level perspective, befitting of comparative physiology, that considers complex phenotypic changes, such as altered hypoxia resistance or body mass.

Analog versus digital views of transgenerational epigenetics

To date, there have been two general foci for transgenerational epigenetic studies: (1) what are the transgenerational phenotypic phenomena produced by epigenetic effects? (2) how are these epigenetic effects achieved – i.e. what is the mechanism? While the 'what' and 'how' of epigenetics are being intensely investigated, the 'why' and especially the 'when' – what might be referred to as the 'dynamics' of epigenetics – have not yet come to the fore. Indeed, when the time course of epigenetics is considered, investigators have typically treated transgenerational epigenetic phenotypic modifications as 'digital' – they are typically viewed as 'on' for one or more offspring generations, then abruptly 'off' in a subsequent generation. Put differently, investigators have primarily looked to see whether an epigenetic phenomenon is present or absent – not its extent or degree (hence the 'digital' description of this approach). Less frequently have studies considered whether the phenomenon is graded in some form within or across generations – what might be considered as an 'analog' view of a transgenerational epigenetic effect. Yet, without understanding the dynamics of epigenetics, we may misinterpret or miss altogether important implications of epigenetic phenomena, as we will now explore, beginning with transgenerational effects.

Transgenerational 'washout'

Can epigenetic phenotypic modifications fade or 'wash out' across generations in more of a graded or analog rather than on–off digital fashion and, if so, how would this be manifested? The left side of Fig. 1 shows a hypothetical epigenetic effect that slowly fades across generations, ultimately falling below detectable levels. This contrasts with the more typical view of an epigenetic effect that is fully present in the F_N generation before then suddenly disappearing in the F_{N+1} generation, as often assumed. How such washout phenomena might

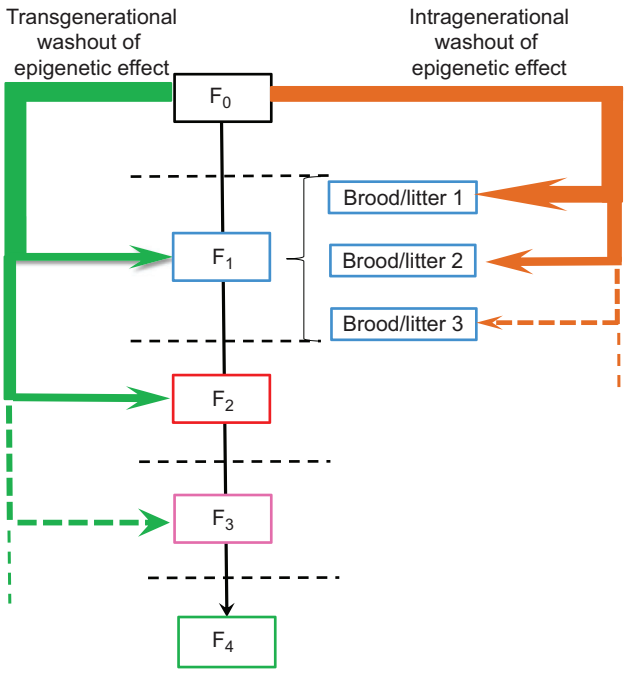

Fig. 1. Hypothesized transgenerational (left) and intragenerational (right) 'washout' of epigenetic effects. In this hypothetical scheme, phenotypic traits fade over multiple generations or within the F_1 over multiple broods.

reveal themselves is shown in Fig. 2A. In this scenario, the effect is not simply absent in the F_0, continuing unabated through the F_{0+N} generation, and then suddenly disappearing in the F_{N+1} generation. Rather, a context-dependent or germline-dependent epigenetic effect could progressively 'wash out' over subsequent generations (Fig. 2A). Once one begins to recognize the implications of an epigenetic dynamic framework and how such a perspective might affect transgenerational phenotype transfer, multiple scenarios emerge, some of which are outlined in Fig. 2B: the magnitude of a modified phenotypic trait could simply wash out (scenario A), as described in Fig. 2A; alternatively, such effects could slowly decline (scenario B), stay constant for some time (scenario C) or slowly increase (scenario D) during the F_1 generation.

Evidence for transgenerational washout is limited, but there are several examples, a few of which will be recounted here. When the F_0 is exposed to dioxin in rats, the F_1 to F_3 generations experience multiple phenotypic modifications (Manikkam et al., 2012). Importantly, phenotypic modification including male pubertal abnormality, primordial follicle loss and male tumor development are reduced in the F_3 compared with the F_1 generation, but are not entirely eliminated, suggestive of transgenerational washout. Decreased sperm counts through altered methylation patterns across generations are caused by two endocrine disruptors (methoxychlor, vinclozolin) administered during gestation (Paoloni-Giacobino, 2014). Importantly, these effects gradually decline from the F_1 to F_3 generation, rather than showing an abrupt transition from the full-blown effects to their complete absence across a single generation.

Another poorly explored aspect of epigenetic dynamics is to what extent epigenetic phenomena can be additive in an environment that is changing in a complex manner with multiple modifications to stressor levels over multiple generations. Fig. 3A illustrates how an environmental stressor intermittently present during alternating generations could conceivably produce 'additive effects', in which the overall transgenerational phenotypic modification is actually

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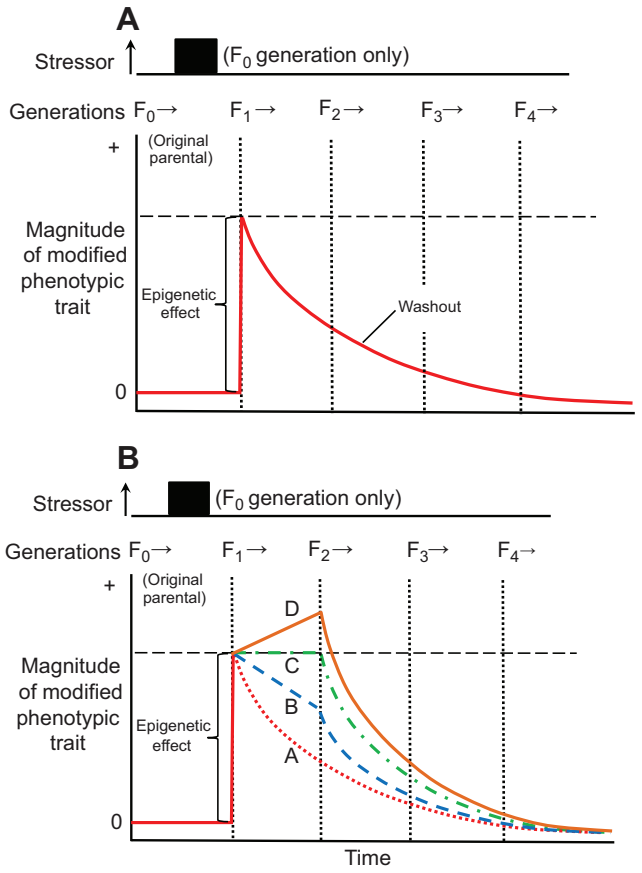

Fig. 2. Examples of epigenetic phenotype washout. (A) In this scenario the transgenerational epigenetic modification of phenotype caused by parental exposure to a stressor appears in the F_1 generation, but immediately begins to wash out or decay over time, progressively declining through and across generations. (B) Additional scenarios for possible dynamic patterns of transgenerational phenotype transfer and subsequent washout, based on variations of the scenario presented in A (reproduced here as Scenario A). In Scenario B, the modified phenotype slowly declines in the F_1 generation then begins to wash out in subsequent generations. In Scenario C, the phenotypic modification is static throughout the F_1 generation then subsequently washes out. Finally, in Scenario D, the phenotypic modification actually increases during the growth of the F_1 generation, then washes out. These scenarios are but a few of the many possible ways in which epigenetic dynamics can be expressed.

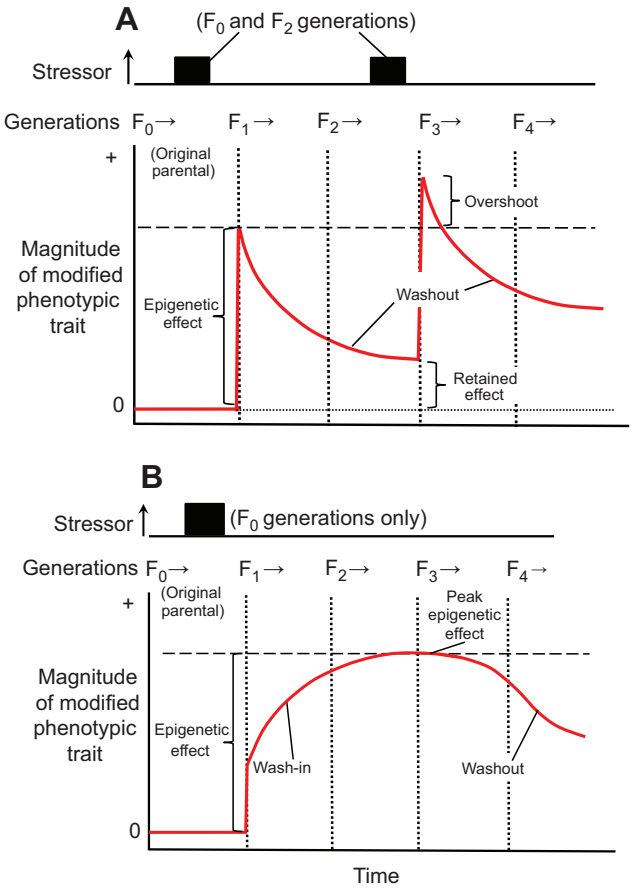

Fig. 3. Additional transgenerational epigenetic dynamics. (A) In this scenario portraying an additive effect, a slowly declining but still lingering epigenetic effect results in an even greater epigenetic modification (an 'overshoot') when a stressor is experienced a second time in the F_2 generation. (B) An epigenetically modified phenotypic trait could build over successive generations and then begin to wash out once it has reached its peak.

amplified by the lingering prior epigenetic modification of phenotype. In this scenario, the magnitude of the stressor-induced effect is the same, but the compounded (overall) effect is increased because of the elevated baseline expression of the modified phenotype.

Is washout of a phenotypic trait adaptive or maladaptive? Epigenetic effects, unlike phenotypic changes resulting from modification to the genotype, can be rapidly 'sunsetted' if the environmental stressor disappears and more favorable environmental conditions return (Burggren, 2014; Cropley et al., 2012). However, immediate sunsetting (i.e. not a washout, but rather an abrupt full return to pre-stressor phenotype) could represent a 'bet hedging' that represents a protection against the possible return of the environmental stressor. Thus, for example, an F_1 generation with a phenotype that includes increased hypoxia tolerance created by parental exposure to hypoxia might benefit from retaining that portion of its physiological repertoire in case there is a return of environmental hypoxia.

Transgenerational 'wash-in'

If epigenetic traits can wash out, it is also feasible that they could 'wash in' – that is, slowly build up over successive generations. Fig. 3B shows how such a phenomenon could manifest itself. A modified phenotypic trait could increase across a couple of generations, and then actually begin to washout after reaching its zenith. If one was simply looking for the presence or absence of an epigenetically modified phenotypic trait, the complex epigenetic dynamics indicated in Fig. 3B would simply be interpreted as a modified phenotypic trait that was present from the F_1 to F_4 generations.

Evidence for transgenerational wash-in, like that for washout, is sparse, but does exist. For example, in the aquatic larvae of the insect *Chironomus riparius*, exposure to the pollutant tributylitin (TBT) causes numerous phenotypic adjustments (e.g. development time, survival, fecundity, weight, hemocyte numbers and phenyloxidase activity) not just in the F_1 following parental exposure to TBT, but all the way through the F_5 generation (Lilley et al., 2012). Interestingly, at the highest F_0 exposure dose employed, the changes in survival, fecundity and hemocyte numbers were greater in the F_5 generation than in the F_1 generation, before disappearing in the F_6 generation. This suggests a wash-in of phenotypic modification, though whether it was progressive,

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exponential, stepwise, etc. could not be discerned from the study. Another example of transgenerational wash-in is found in the epigenetic inheritance of obesity in mice fed high-fat diets for three successive generations (Li et al., 2012). Obesity occurred earlier in development and was more severe across generations as follows, $F_2>F_1>F_0$, suggesting a phenotypic wash-in or, as the authors put it, a 'transgenerational accumulation of epigenetic modifications'. A final example comes from the epigenetic inheritance of adult-onset disease and sperm phenotypic modifications resulting from F_0 exposure to dioxin in rats (Manikkam et al., 2012). Female puberty abnormality and polycystic ovary disease were both greater in the F_2 generation than in the F_1 generation, again suggesting a wash-in of these pathological phenotypes.

Threshold/resolution effects in assessment of transgenerational phenotypic transfer

Many studies of epigenetic phenomena involve relatively straightforward and easily measured traits, e.g. stereotypic behaviors, anatomical structures and molecular markers. And why not? Many of these studies are exploring new territory in epigenetic phenomena and mechanisms. There are enough potentially uncontrolled sources of variation in, for example, comparative physiological studies (Burggren, 2014a), without extending our observations to transgenerational phenotypic effects that are difficult to measure. Unfortunately, failure to realize what might be regarded as a 'threshold' or necessary resolution for phenotype detection can have rather large implications when modified phenotypes wash out or decay, rather than suddenly disappear with a new generation. Fig. 4A shows the different interpretations of a transgenerational epigenetic effect that would be made depending upon the experimenter's threshold for phenotypic detection as the modified phenotype slowly washes out. Conventional detection thresholds (blue dashed line in Fig. 4A), even a relatively sensitive threshold, might nonetheless create a significant underestimation of modified phenotype when compared with even a slightly more sensitive threshold (brown dashed line in Fig. 4A).

Is the goal to achieve absolute detection of the modified phenotype across generations until it has fully washed out? This depends in part upon the goals of the experiment. If the goal is to assess the biological effects of transgenerational phenotype modification, then one could argue that a point (a generation) is reached at which traces of the modified phenotype are present, but they are far too small to have any biological effect. However, if one is trying to determine mechanism and quantify its persistence, there may be no threshold that is too sensitive to be relevant.

Having high resolution for detection of an epigenetically modified phenotype is also relevant to the wash-in phenomenon, as is apparent in Fig. 4B. In this scenario, a conventional, less-sensitive detection threshold for a modified phenotype may similarly results in an underestimation of the number of generations in which an effect is apparent. Even worse, a less-sensitive threshold could result in no detection of the phenotypic modification in the F_0 generation, leading the experimenter to erroneously conclude that there was no epigenetic effect at all, and to abandon any observations of subsequent generations when the effect would actually show up!

Intragenerational washout

Little attention has been paid to whether induced epigenetic phenotypic effects could fade or 'wash out' within a single offspring generation across successive broods produced by the F_1 offspring (Fig. 1, right-hand side). Certainly increases and decreases in the magnitude of known causative agents for intragenerational epigenetic

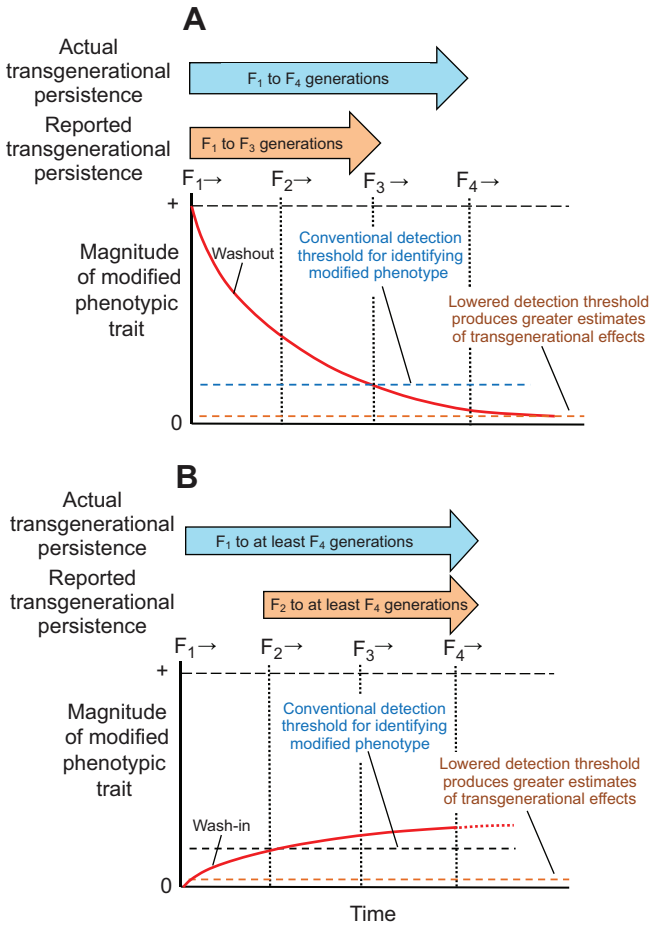

Fig. 4. Importance of thresholds for detection in epigenetic studies. (A) Depending upon the threshold for detection of the modified phenotype, the duration (measured in generations) of a transgenerational epigenetic effect that is progressively washing out can be considerably underestimated, particularly when the detection threshold is not highly sensitive (blue dashed line). (B) Just as intergenerational washout can be under-detected when a relatively low sensitivity detection threshold is in place for an epigenetically modified phenotype, so too can the wash-in of a modified phenotype. (See text for additional discussion.)

effects have been identified, e.g. intragenerational changes in DNA methylation with development, growth and senescence, as discussed below. Our laboratory has collected preliminary evidence for intragenerational washout of epigenetic phenotypic modifications evident in the body morphology of the water flea *Daphnia magna* (Andrewartha and Burggren, 2012), which is emerging as a promising model for epigenetic studies (Harris et al., 2012). We exposed adult female *D. magna* to hypoxia (4%) for 6 days (Fig. 5). Through an apparent germline-dependent epigenetic effect, this parental hypoxic exposure induced a reduced body mass in the subsequent F_1 generation for the first 4–6 days. Importantly, this reduction in mass was evident in the first and second broods comprising the F_1, but had disappeared or 'washed out' prior to the third brood, which exhibited identical body masses when compared with control F_1 generations whose parents had not been exposed to hypoxia (Fig. 2). Evidence for intragenerational washout at the molecular level also exists in the killifish (*Fundulus heteroclitus*), where a refractory CYP1a phenotype potentially acquired through germline-dependent epigenetic effects are progressively lost during larval development (Meyer and Di Giulio, 2002; Meyer and Giulio, 2003) and fade out over time within the F_1 generation (Meyer et al., 2002).

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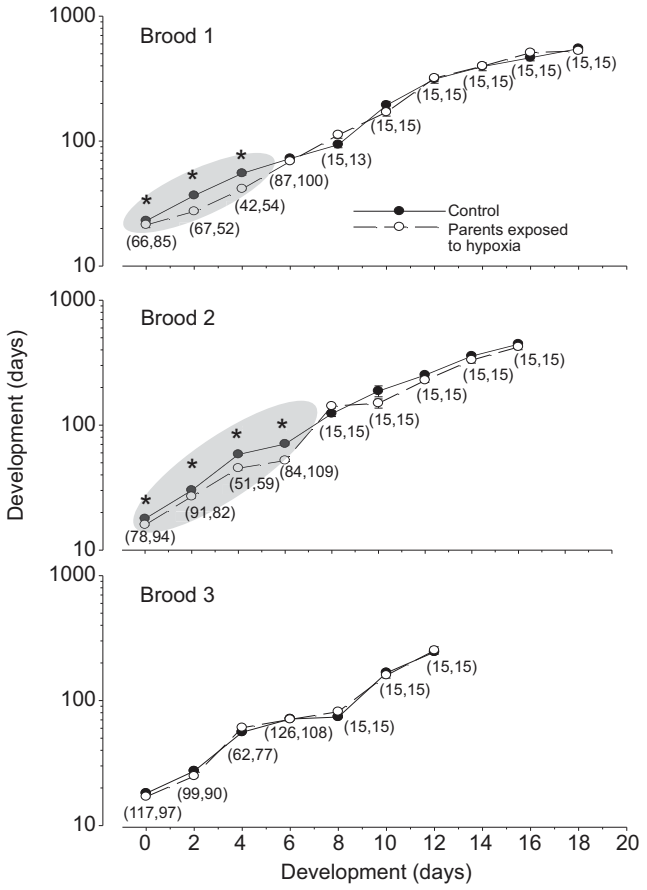

Fig. 5. Intra-generational washout of morphological epigenetic modifications in *Daphnia magna*. Chronic hypoxia exposure of adult parents created a significantly reduced body mass (shaded areas) in the offspring of the first and second – but not third – F_1 broods, as depicted in this semi-log plot. *N* values in parentheses. Graphs modified from Andrewartha (Andrewartha and Burggren, 2012).

Before leaving the topic of intragenerational epigenetic effects, consider the medically oriented view of the concept of intragenerational wash-in. If one views a disease that develops through non-genetic mechanisms as an intragenerational epigenetic effect then, almost by definition, as the disease progresses, the modified (diseased) phenotype is 'washing in'. In this respect, the concept of intragenerational wash-in is simultaneously validated and trivialized.

Phenotype normally changes during development, growth and maturation. Non-genetic modifications of phenotype result from environmental perturbations, of course, a phenomenon termed developmental plasticity (de Jong and Leyser, 2012; Kelly et al., 2012; Snell-Rood, 2012; West-Eberhard, 2003). Many of these changes result from mechanisms that have not been associated with the 'traditional' epigenetic mechanisms of DNA methylation, histone, nucleosome position, microRNAs, etc. This raises the interesting semantic question of whether the presence of a mechanism is associated with a particular phenomenon (e.g. DNA methylation causing epigenetic effects).

Epigenetic dynamics, gender and populations

Gender and epigenetic dynamics

To this point, we have considered 'epigenetic dynamics' in terms of temporal issues integrated into phenotypic modifications. Gender-specific responses might be considered another dynamic component of epigenetic responses. We know, for example, that there are gender-based differences in DNA methylation (Gallou-Kabani et al., 2010). Significantly, some X-linked genes can escape inactivation by methylation, and such genes are thus more expressed in females (Basu and Zhang, 2011). Y chromosome genes are typically widely expressed through life and in many tissues (Gabory et al., 2015). CpG methylation of the promoter regions for these regions could suppress male phenotypic traits. In the extreme, such gender-based trait suppression could alter mating success and thus affect the population beyond the individual (see below).

Gender differences could also manifest themselves through differential resilience of DNA methylation, histone modification or other mechanisms of epigenetic modification of phenotype. Thus, in a hypothetical example, males of a population could have a phenotypic modification that, across generations (or broods), outlasts the females of that same population. Obviously, specific epigenetic effects on either male or female gametes or on male or female tissues (e.g. prostate gland, mammary glands) will manifest themselves in gender-dependent patterns (Dada et al., 2012). Thus, the 'washout' (or potentially the 'wash-in') of a phenotypic trait could have quite different profiles based on gender. Again, reproductive success and thus overall fitness could come into play as a result of these gender-dependent effects.

Individual versus population-level responses

Epigenetic dynamics – for that matter, most epigenetic phenomena – are typically considered at the level of the individual. That is, investigators try to identify phenotypic changes and underlying mechanisms in individuals, and then most often express these differences as means drawn from the sampled population. This time-honored statistical approach has some significant implications to the field of epigenetics. Most directly, the practice of using mean values to express a collection of individual responses de-emphasizes the often interesting data from individuals. Rather than viewing outliers as representative of confounding (and annoying) variance, comparative biologists have increasingly realized the importance of celebrating outliers and what these individual responses can tell us (e.g. Bennett, 1987; Feder et al., 2000; Williams, 2008). It is ironic then, that the field of epigenetics, with its framework of how individuals can be affected outside the mechanism of conventional genetic inheritance, has not similarly embraced the significance of individual outliers, although these are just starting to be considered in areas ranging from epigenetic inheritance in athletes (Ehlert et al., 2013) to the interpretation of twin studies (Ollikainen and Craig, 2011).

Paralleling the ongoing discussion about individual versus population-level approaches in quantitative genetics, it is also of interest to contemplate how epigenetic dynamics might manifest at the population level as well. Consider the scenario portrayed in Fig. 6. Even if all individuals in a population actually showed a 'digital' epigenetic response to a stressor, with the modified phenotype disappearing completely with a new generation, variation in the persistence of the response between individuals, with some individuals perpetuating the response through more generations than others, could generate an exponential washout at the population level.

Mechanism(s) for epigenetic dynamics

A key question is 'what are the potential mechanisms for epigenetic dynamics'? To begin to answer this question, let us briefly consider the dynamics of known epigenetic modulators of gene expression.

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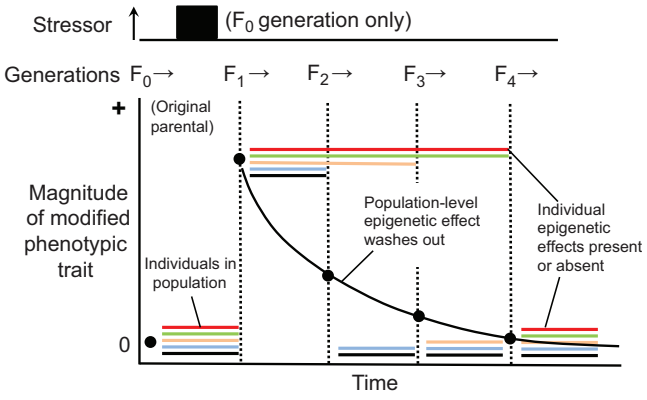

Fig. 6. Epigenetic dynamics can be considered at the population level as well as the individual level. In this scenario, individuals may actually be showing 'digital' responses in which the epigenetic effect is present or absent, rather than analog responses. However, the statistical mean of the population may show an analog washout.

The dynamics of DNA methylation and other epigenetic modulators

DNA methylation has been extensively reviewed (and several papers in this special issue on epigenetics contribute in this area), and it is not my intent to revisit this extensive literature. DNA methylations can variously be silent in terms of influence on phenotype, or they can be essential. Methylation patterns are also highly regional and tissue specific, with even different tissues within an organ (e.g. placenta) showing quite different levels of DNA methylation. DNA methylation also varies greatly among taxa. In mammals, some 60–80% of the DNA is methylated in adulthood (Gallou-Kabani et al., 2010; Smith and Meissner, 2013), but in many invertebrates the degree of methylation falls to <3% and may even be difficult to detect, as in the fruit fly *Drosophila* (Hunt et al., 2013; Schoofs et al., 2015; Weiner and Toth, 2012). Within an individual, DNA methylation changes during embryonic development, maturation and aging/senescence (Ficz, 2015; Gabory et al., 2015; Rodríguez-Rodero et al., 2010; Smith and Meissner, 2013; van Otterdijk et al., 2013). Indeed, in some species, the relationship between CpG methylation and age is so tightly correlated as to lead to the concept of an 'epigenetic clock' based on 'DNA methylation age' (Gršković et al., 2013; Horvath, 2013; Teschendorff et al., 2013). Yet, these developmental changes do not follow universal patterns or even general trends. Both increases in DNA methylation during the progression of an individual organism's life span as well as decreases have been reported (Jaenisch, 1997; Wu and Zhang, 2010). Furthermore, at least in mammalian germ cells and zygotes there is a period of 'methylation erasure' for DNA, followed by *de novo* remethylation after the blastocyst stage and beyond in the embryo, thought to be dictated in part by the environment (Ficz, 2015; Horvath, 2013; Ooi and Bestor, 2008; Wu and Zhang, 2010). In addition to inter-individual differences in DNA methylation, specific regions of DNA are more methylated than others (Schär and Fritsch, 2011; Smith and Meissner, 2013), which leads to large differences in DNA methylation of genes responsible for specific tissue types within an organ, such as the placenta (Gabory et al., 2015). It is not at all clear what the drivers are for such intra-individual and inter-organ changes in DNA methylation. However, given that DNA methylation is far from fixed in organisms, a progressive decline in such methylation could underlie a time-dependent decay of a modified phenotype within a single generation. Confounding the situation, however, is that demethylation of promoter regions does not automatically lead to re-expression of previously suppressed genes (Ficz, 2015).

Epigenetic effects are, of course, also mediated by other molecular mechanisms, including histone modification, nucleosome positioning, non-coding RNAs, etc. (Hughes and Rando, 2014; Rose and Klose, 2014; Zhang and Pradhan, 2014). Far less is known about the life-cycle dynamics of these mechanisms, but they are sure to be implicated with additional studies. For example, Ashe and co-workers noted stressor-induced changes in piRNAs across generations (Ashe et al., 2012). Interestingly, in this multi-generational study, the focus was on the presence or absence of transgene silencing of GFP. However, a careful examination of their data suggests that the extent of transgene silencing was reduced by 10–15% over the course of four generations of a phenotypic modification lasting at least 10 generations. Moreover, in considering these mechanisms, we should recognize that most investigators focus on DNA methylation, histone medication, nucleosome repositioning or microRNAs. The real world being what it is, we are likely to find that it is suites of mechanisms acting in concert – rather than individual mechanisms – that underlie phenotypic trait modifications. Returning to the original question of 'what are the potential mechanisms for epigenetic dynamics?', there are multiple possibilities, two of which will now be explored.

'Active' and 'passive' washout of epigenetic modulators

Across generations, there could be an active 'washout' or decline of DNA methylation, histone modification, and/or nucleosome positioning, for example. Just as epigenetically modified phenotypes are variable across generations, so too is DNA methylation, the extent of which can occur across a continuum. In fact, with each subsequent generation, DNA methylation patterns are created, maintained, cleared (erased) and then re-established during the life cycle and subsequent inheritance of a phenotype (Alvarado et al., 2014a; Alvarado et al., 2014b; Golbabapour et al., 2011; Ohno et al., 2013). Thus, patterns of decline in DNA methylation or other epigenetic molecular agent could be the causal agent behind a decline in the epigenetically modified phenotype, until the effect had either disappeared or fallen below a threshold for detection of the modified phenotype.

An alternative or additional explanation for epigenetic washout could involve a time-related 'passive' decay of the causal agent. That is, just like a radio-isotope decays over time, there may be a diminishment of the causal mechanism over time. 'Spontaneous' loss of histone modification over time has been suggested (Przybilla et al., 2012). Consider again our experiments with *Daphnia magna* (Fig. 5). The third brood, which has recovered from the epigenetic effect that reduced body mass, was not only the third of three broods produced, but obviously was produced several weeks later than the first or second broods. Perhaps, then, DNA methylation (or other epigenetic mechanisms) simply may not persist across time in gametes, and so passage of time results in the observed washout of the epigenetic effect. This decay may also explain loss of morphological modifications as developmental time progresses for each brood. Certainly, progressive intragenerational changes in DNA methylation have been quantified in ants, mice, ground squirrels, cichlid fishes and many other organisms including humans (Alvarado et al., 2014a; Alvarado et al., 2014b; Bollati et al., 2009; Gabory et al., 2015). This hypothesis of a simple time-dependent decline in DNA methylation (or other mechanism) can be readily tested in an animal producing multiple broods over time, such as *Daphnia*, by delaying the production of the first brood until the normal timing of the third brood, and determining whether the

phenotypic modification is present, or has not stood the passage of time.

Conclusions and future directions

The reductionist focus on mechanism that prevails in many of the epigenetic studies to date (the 'what' and the 'how') has diverted attention away from what might be called the 'dynamics' of epigenetics (the 'when'). Epigenetic dynamics describes the extent to which transgenerational and intergenerational epigenetic phenotypic modifications change in non-linear fashions over time – potentially across many generations. Central to this view is that epigenetic dynamics is not a suite of digital (on-off) phenomena. Rather, a more nuanced view frames epigenetic dynamics as a graded series of changes that can involve 'washout', 'wash-in' and additive effects in the individual, both within and across generations.

Studies on body mass in the water flea *Daphnia magna*, molecular markers in the killifish *Fundulus heteroclitus* and other examples provide evidence for graded trans- and intragenerational (respectively) epigenetic effects. Yet, many of the scenarios for epigenetic dynamics presented in this paper – while possible, and even likely – are not yet supported by experimental data. Future studies, then, should be directed towards validating the various possible forms of epigenetic dynamics and then identifying the underlying mechanisms. The accuracy of assertions that a specific transgenerational epigenetic effect lasts through *n* generations and then disappears is highly dependent on the threshold for detection of the phenotypic modification of interest. Thus, studies of transgenerational epigenetic effects (and intragenerational effects, for that matter) that search for persistence of the phenomenon are best conducted with highly sensitive precise analytical methods. Almost all epigenetic effects are measured on the individual, but reported as averages for the experimental population. Although comparative biologists have for years recognized the power of examining outliers, this increasingly time-honored practice for determining everything from mechanism to fitness to natural selection has yet to be exploited by the broad epigenetic community.

Finally, a key unanswered question involves the mechanism by which epigenetic dynamics such as phenotype washout might occur. Is washout, for example, an active process in which, for example, DNA methylation is progressively diminished by dilution across generations or across broods, or is it a passive process that 'simply' occurs with the passage of chronological time?

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Competing interests

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