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REVIEW

Epigenetics as a source of variation in comparative animal physiology – or – Lamarck is lookin' pretty good these days

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ABSTRACT

Considerable variation is inherent both within and between comparative physiological data sets. Known sources for such variation include diet, gender, time of day and season of experiment, among many other factors, but a meta-analysis of physiological studies shows that surprisingly few studies report controlling for these factors. In fact, less than 3% of comparative physiological papers mention epigenetics. However, our understanding of epigenetic influences on physiological processes is growing rapidly, and it is highly likely that epigenetic phenomena are an additional 'hidden' source of variation, particularly in wild-caught specimens. Recent studies have shown epigenetic inheritance of commonly studied traits such as metabolic rate (water fleas *Daphnia magna*; emu, *Dromaius novaellandiae*), hypoxic tolerance, cardiac performance (zebrafish, *Danio rerio*), as well as numerous morphological effects. The ecological and evolutionary significance of such epigenetic inheritance is discussed in a comparative physiological context. Finally, against this context of epigenetic inheritance of phenotype, this essay also provides a number of caveats and warnings regarding the interpretation of transgenerational phenotype modification as a true epigenetic phenomenon. Parental effects, sperm storage, multiple paternity and direct gamete exposure can all be confounding factors. Epigenetic inheritance may best be studied in animal models that can be maintained in the laboratory over multiple generations, to yield parental stock that themselves are free of epigenetic effects from the historical experiences of their parents.

KEY WORDS: Comparative physiology, Variation, Evolution, Epigenetics, Inheritance

Introduction: sources of variation in comparative physiological data

Since the inception of the field of animal physiology, experimentalists have been aware of significant, and often perplexing, variation within their data sets, either from study-to-study within the same laboratory or in the hands of different investigators in different laboratories performing the same experiment on different animal populations. Sometimes the source of variation can be identified, but sometimes it seems enigmatic and innate – just 'there'. Perhaps in response to our recognition of this variance, in our analysis of data sets, comparative physiologists (indeed, all physiologists) unintentionally divert attention away from the differences by pointing out the similarities, not unlike how a magician diverts the eyes of the audience with a distraction. Lest this be viewed as offering unfair criticism, consider a recent publication in which I was involved. Fig. 1 indicates two very different views of the same data set from Tazawa et al. (Tazawa et al., 2011). In the first approach (the graph that was published), a typical format is presented in which regressions are used to describe patterns in the data (and in so doing minimizing the visual appearance of variance). This presentation allowed the authors to make some reasonable conclusions as to hemoglobin and oxygen consumption changes during development in avian embryos. However, it also kept the authors (or at least this author) from realizing just how much variation actually existed in the data, which was revealed only with removal of the regression lines and examination of the data as cohorts.

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Managing physiological variation

Variation in our data is confounding, and a major consequence is that testable hypotheses are rejected because of excessive variation in the data. However, our inability to recognize and control for variation has, to some extent, contributed to an unfortunate disciplinary mindset of what might be called 'my data versus your data' rather than simply 'the data'. Frequently, animal physiologists publishing in similar areas will see a data set that differs – sometimes starkly – from their own: e.g. stimulation of the vagus nerve in a particular amphibian species variously stops the heart in one investigator's hands but has no effect in another's; larval freshwater fishes placed in salt-laden water variously struggle or thrive; oxygen consumption of social insects changes with density of population of members of their own species; to name just a few of the data enigmas that comparative physiologists might recognize. At some level, comparative physiologists realize that unrecognized or at least ill-defined variables will account for these sharp discrepancies (not to mention the more subtle differences in data). But the 'my data versus their data' mindset leads to an unintended view that The Other 'didn't have accurate enough instruments', or 'didn't calibrate their instruments' or 'didn't properly follow experimental protocols'. In other words, we fall into the trap of questioning the veracity of other people's data because of error introduced by the experimenter or his/her surroundings, rather than because of variables that we rarely openly acknowledge. One of the unintended consequences of the 'my data versus their data' mindset is that it leads to schools of thought based on investigators supporting various data sets that agree with their own scientific outcomes, rather than taking the approach of 'Our data sets are both accurate – what did we not understand about how we individually did the experiments?'. Even though, in my experience, comparative physiologists are among the most collaborative of all life scientists, the 'my data versus their data' mindset nonetheless stands as a barrier to even more collaboration – generating what might be termed 'collaborative opportunity costs' for which our discipline pays the price.

Another cost of unrecognized variation is that we can 'manage' data variation to our advantage. More than two decades ago, Al Bennett emphasized the 'tyranny of the golden mean' (Bennett,

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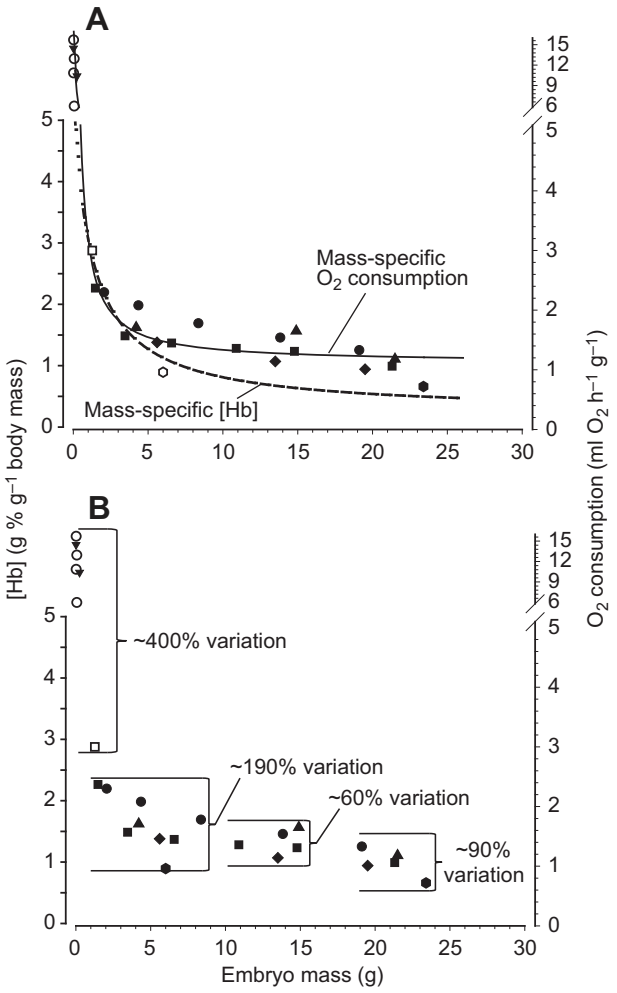

Fig. 1. An analysis of mass-specific embryonic hemoglobin concentration ([Hb]) and mass-specific O_2 consumption in chicken embryos. (A) The data as originally presented by Tazawa et al. (Tazawa et al., 2011), with a dashed line indicating the inverse second-order regression for [Hb] and a solid line indicating the inverse third-order regression of mass-specific oxygen consumption. (B) Regressions have been removed, and actual differences between data sets from distinct studies are expressed as percentages calculated for data cohorts. Note that data that looked closely grouped and falling in a clear pattern in A actually had very large percentage variation within cohorts as highlighted in B. See 'Introduction: sources of variation in comparative physiological data' for further discussion of implications. Original data, delineated by different symbols, are from nine different studies (see Tazawa et al., 2011).

1987), pointing out that in our typical rush to create single, averaged values to derive patterns, we were not fully mining the full extent of the data sets we generated. Variation is, after all, the stuff of evolution but if we can't recognize and control for the various sources of data, we can't move beyond the tyranny of the golden mean (Williams, 2008).

Recognizing – and then managing – variation in physiological data sets thus has considerable merit, but to do so, we must recognize the actual sources of physiological variation, as will now be discussed.

Sources of physiological variation – the 'usual suspects'

Many sources of potential or actual variation in physiological data sets have been recognized for decades, if not centuries. Diet and

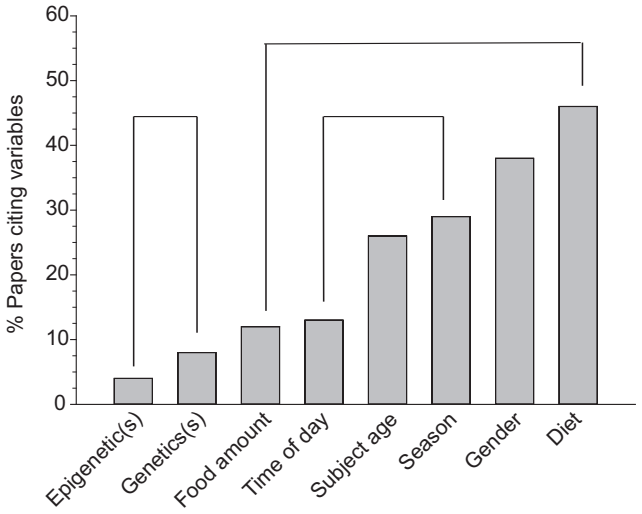

Fig. 2. Recognition of potential variation-inducing factors in comparative physiological studies. The graph shows the percentage of papers published in 83 randomly selected papers from *The Journal of Experimental Biology* in 2012 that contain specific key words relating to experimental conditions. Similar categories of variables are linked by brackets.

prandial state are widely recognized as influencing physiological processes (Wang et al., 1995; McCue, 2006; Secor, 2009). Gender, whether through sexual dimorphism or the hormonal differences between males and females, can have major influences on the physiology of experimental animals (Czerniak, 2001; Orlando and Guillette, 2007; Rogers et al., 2007; Shen et al., 2011). The enormous effect on metabolism in animals that undergo night-time torpor (Schleucher, 2004; Heldmaier et al., 2004; Swoap, 2008) or seasonal hibernation (Geiser, 2004; Drew et al., 2007; Storey and Storey, 2010; Jackson and Ultsch, 2010) also makes time of day and season key factors.

Despite the fact that these factors are well known to influence physiological processes (and therefore the measurements we make of these processes), a meta-analysis reveals that surprisingly few studies report on these factors in their methodological descriptions. Fig. 2 shows the percentage of 83 randomly selected papers published during 2012 in *The Journal of Experimental Biology* (JEB) that reported on diet, amount of food provided, gender, season, age of experimental animals, time of day of the experiments (and lighting), etc. Evident from this analysis is that only a small proportion of papers actually report on these variability-producing factors, and from this we assume that in at least a large majority of these published experiments that did not report these factors, they were not actually controlled for. Why, then, should we be surprised by variation in otherwise comparable data sets emerging from different laboratories?

Especially notable from this meta-analysis of factors mentioned in JEB papers is how few papers mention the role of genetics in their findings – less than 10%. This paucity of discussion of genetics is surprising, given that the comparative physiological community increasingly appreciates the physiological variability that can be induced by genetic differences at race, population or individual levels (e.g. Yoneta et al., 2007; Kempenaers et al., 2008; Burton et al., 2011; Day and Bonduriansky, 2011; Andersen, 2012). Indeed, such differences are at the heart of the emerging field of pharmacogenetics (e.g. Howland, 2012; Johnson and Cavallari, 2013).

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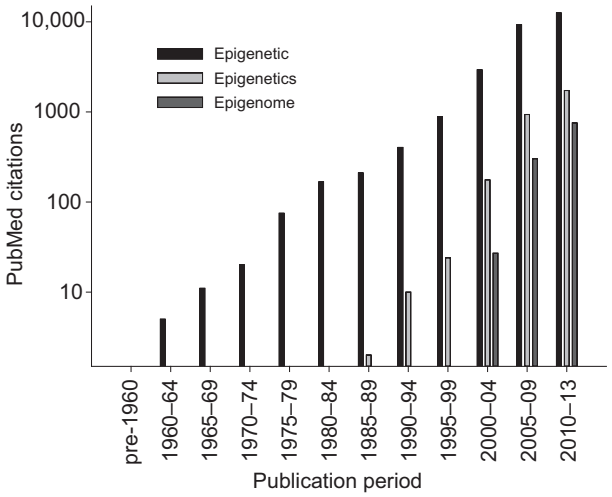

Fig. 3. A meta-analysis of the growth of publications on epigenetics. The term 'epigenetics' was coined by C. H. Waddington in 1942. The word 'epigenetics' first appears in the PubMed literature in the early 1960s. This figure represents a semi-log plot of the number of papers that contain the words 'epigenetic', 'epigenetics' or 'epigenome', as listed in PubMed, by 5 year intervals, published from 1960 to the present. Notice that the last interval comprises only 3.5 years, so the rate of growth is on a par with, or even accelerating above, the exponential growth of the previous decades.

Epigenetics as a source of physiological variation

If genetics is rarely recognized as a source of physiological variation, it is perhaps not surprising – though disappointing and increasingly of concern – to find an almost complete lack of recognition of epigenetics as a confounding factor in comparative physiology. Indeed, less than 5% of the surveyed papers published in JEB contain the words 'epigenetics' and/or 'epigenetic' (Fig. 2). Yet, there has been a virtual explosion of papers on this subject in biological research of all descriptions, including animal physiology. Fig. 3 is a semi-log plot of the number of papers indexed in PubMed, by 5 year intervals, published from 1960 to the present. Note that the last interval comprises only 3.5 years, so the rate of growth of papers mentioning epigenetics is on a par with, or even accelerating above, the exponential growth rate of the previous decades. Indeed, the notion that an organism can pass on phenotypic characteristics acquired during its lifetime to its offspring represents an important component of classic 'Lamarckism', inspired by the evolutionary theories of Jean Baptist Lamarck (1744–1829). After lying fallow (or perhaps, more accurately, being marginalized) for nearly two centuries, the idea of transgenerational transfer of acquired characteristics has experienced a rebirth in the form of epigenetics, as we will now consider.

Epigenetics as a form of non-genetically based transgenerational transfer

'We should altogether avoid, like the plague, discussing the meaning of words' (Popper, 1972). Notwithstanding the admonishment of this notable 20th century science philosopher, it is important to consider what we mean by epigenetics, or, putting it differently, what is the reach or scope of epigenetic research. The term 'epigenetics', coined by C. H. Waddington (Waddington, 1942) more than 70 years ago, was initially described as 'the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being'. Over the ensuing years, literally dozens of definitions of epigenetics have been offered up, and I am not without guilt in this respect, having offered up a definition in this very journal (Ho and Burggren, 2010). A key element of many of these definitions – both old and new – is that the phenotypic modifications brought about through gene–gene product interactions are heritable without gene sequence modification. Thus, most definitions of epigenetics emphasize non-genetic inheritance as a key component of epigenetics.

We now know numerous mechanisms of epigenetic inheritance – histone modification, DNA methylation and non-coding RNAs being the most common (for reviews, see Gluckman et al., 2009; Ho and Burggren, 2010; Martin-Subero, 2011; Moore et al., 2013; Canton and Fisher, 2013). More exotic mechanisms underlying epigenetic modification of phenotype across generations include self-sustaining loops and structural inheritance via proteins, which occurs in some invertebrates (Jablonka and Lamb, 2005; Beisson, 2008; Ho and Burggren, 2010). As a side note for the current audience, there also exists a rich investigation of epigenetics in plants (for an entry into that extensive plant literature, see Hauser et al., 2011; Sahu et al., 2013), and of epigenetic influences at the population level as well as at the level of the individual (Day and Bonduriansky, 2011; Geoghegan and Spencer, 2012). The discussion of these topics in epigenetics is beyond the scope of this paper, and the reader is directed to the references just cited for further information on epigenetic mechanisms.

Before leaving the topic of epigenetic inheritance (though we shall return to this at the end of this essay), it is critical to note that the very concept of epigenetics has been in flux almost since Waddington (Waddington, 1942) first proposed it (see Jablonka and Lamb, 2002; Ho and Burggren, 2010). Importantly, there has been a tendency in some of the literature to label a phenomenon as 'epigenetic' based on the presence of a modified phenotype coupled with a responsible mechanism (e.g. DNA methylation) – within a single generation and with no consideration of whether the phenotypic modification is heritable. This approach is very common in the arena of public health, ranging from cancer research (Kala et al., 2013) to drug addiction (Nestler, 2013) to heart disease (Tingare et al., 2013). Of concern is that, in the extreme, the simple presence of mechanisms known to be associated with epigenetic phenomena (DNA methylation, histone modification), without investigation of phenotypic inheritance or even phenotype modification during a single life span of an animal, now leads to the branding of a phenomenon as 'epigenetic' – as opposed to identifying an epigenetic phenomenon and its ramifications, followed by a search for the underlying mechanisms.

Reluctantly, then, given Popper's (Popper, 1972) admonishment regarding semantics, it appears prudent, or at least brings clarification to the topic, to not propose a new definition of epigenetics. Rather, I propose a demarcation between those phenomena dealing with the development and phenotypic modification of an organism within a single generation, which might be termed 'intragenerational epigenetics', and those phenomena that go back to the roots of epigenetic studies and investigate not just the phenomenon but also the non-genetic inheritance of the phenotype of interest. Such studies might be referred to as 'transgenerational epigenetics' or, as offered by Jablonka and Lamb (Jablonka and Lamb, 1995; Jablonka and Lam, 2002) and others, 'epigenetic inheritance'. Irrespective of the reader's view on possible demarcation of the field of epigenetics, for the purposes of this essay we will focus on those epigenetic phenomena that necessarily involve non-genetic transfer (inheritance) of phenotypic characteristics.

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Transgenerational epigenetics in comparative physiological studies

The epigenetic literature is now replete with evidence for epigenetic effects in everything ranging from longevity to behavior to morphology to molecular function (see references cited in the paragraphs above). Evidence for epigenetic influences on primary physiological processes (e.g. heart performance, kidney function, metabolic rate) that are transferred across generations is somewhat more difficult to quantify and illustrate, though such examples certainly exist. Schwerte et al. (Schwerte et al., 2005), for example, demonstrated in zebrafish that changes in food type (dry versus live) delivered to adult fish resulted in the non-genetic transgenerational transfer of cardiac parameters (stroke volume, heart rate, cardiac output) and hematology (e.g. red blood cell concentration) to their larvae. Mammals similarly show diet-related epigenetic inheritance. A low protein diet fed to female mice (*Mus musculus*) during oocyte maturation results in hypertension and modified responses to vasodilators such as acetylcholine and isoproterenol in the postnatal mice pups (Watkins et al., 2008). Epigenetic influences on heart rate also occur in chicken embryos. If one considers the egg yolk and albumin of avian embryos to comprise their 'diet', then xenobiotic experiments by Ho et al. (Ho et al., 2011) are instructive. Chicken embryos grown on the egg yolk of other bird species ranging from quail to ostrich at day 3–5 take on the chronotropic characteristics of the yolk they are grown on (heart rate is positively correlated with egg mass), rather than their 'genetic instructions' for heart rate at this age of development.

The zebrafish, chicken and mouse data described above are examples of so-called 'maternal effects', in which a stressor or other environmental cue experienced by the mother results in a non-genetic transfer of traits to her offspring. (Paternal effects have also been described, suggesting that 'parental' effects might be a more suitable all-encompassing term for this phenomenon.) There are multiple mechanisms by which this can occur, including egg (or potentially semen) 'provisioning', where hormonal or other controlling agents are inserted into the eggs/oocytes as they are being formed. These agents subsequently are involved in the regulation of development of the offspring, in extreme cases potentially modifying the normal environment for genetic instructions for development. The bird egg, for example, has long been recognized as a highly variable environment that can be heavily influenced by the mother's environment (for review, see Reed and Clark, 2011). The cleidoic eggs of reptiles (Lovern and Wade, 2003) and of insects (Geister et al., 2008) are similarly subject to hormonal provisioning, with subsequent maternal effects on the offspring growing within them.

Epigenetic inheritance of whole-animal metabolic characteristics also occurs. For example, in the emu (*Dromaius novaellandiae*), late embryonic oxygen consumption ($\dot{V}_{O_2}$) correlates positively with egg size (Dzialowski and Sotherland, 2004), in another example of egg provisioning. However, mere exposure to hypoxia as a parent can affect the subsequent offspring's $\dot{V}_{O_2}$. For example, the $\dot{V}_{O_2}$ of neonatal water fleas (*Daphnia magna*) was significantly influenced by multiple factors, including the extent of maternal hypoxic exposure conditions, the day of development and even the brood number (1st, 2nd or 3rd brood produced after maternal hypoxic exposure) (Andrewartha and Burggren, 2012). As a specific example, brood 1 neonates on day 0, whose mothers had been exposed to hypoxia (~4 kPa), had higher $\dot{V}_{O_2}$ at environmental $\dot{P}_{O_2}$ from 11.5 up to 21 kPa than did control neonates, i.e. those whose mothers had not been exposed to hypoxia. The body mass of experimental neonates was also depressed compared with that of controls over the first days of development. Interestingly, however, these effects 'washed out' with subsequent broods, a phenomenon that deserves far more attention.

Parental hypoxic exposure also affects hypoxic tolerance, which can be viewed as an amalgam of all of the processes beginning with O_2 acquisition from the environment and finishing with the delivery and utilization of O_2 in the mitochondria. For example, non-genetic inheritance of hypoxia tolerance has recently been demonstrated in the zebrafish, *Danio rerio* (Ho and Burggren, 2012). Adult zebrafish of both sexes were first raised in air-saturated water (normoxia, ~21 kPa) at 27±0.5°C in standard 12 h light/12 h dark conditions, and fed daily with tropical fish flakes. The fish were then randomly separated into four distinct populations and exposed to 1, 2, 3 or 4 weeks of moderate hypoxia (~13 kPa) (Fig. 4A). After hypoxic exposure, they were thereafter maintained as separate populations and returned to normoxia for recovery and conditioning for breeding. The larvae from each population were reared for the first 6 days in normoxia, then exposed to severe hypoxia (~4 kPa) as their first hypoxic exposure, which caused loss of equilibrium within 15–45 s. From these data, an index of hypoxic resistance was created for each larval population (Fig. 4B). Interestingly, just 1 week of parental hypoxic exposure resulted in a non-genetic transfer of hypoxic susceptibility to the larvae, while 2–4 weeks had the opposite effect, conveying heightened hypoxia resistance to the larvae!

Collectively, these data suggest that many, if not all, of the physiological parameters that comprise the domain of comparative physiology are susceptible to epigenetic influences. Thus, epigenetic inheritance is likely to be a significant factor in heretofore inexplicable physiological variation.

Phenotypic transgenerational modification by epigenetic inheritance, mutation and natural selection: which is 'better'?

Epigenetic phenomena result from epigenetic mechanisms that are themselves heritable, selected for and part of speciation and evolution (e.g. Jablonka and Ras, 2009; Skinner, 2011). For example, the ability of the DNA alpha helix to be modified by methylation is, in itself, a structural trait of the helix that is genetically coded for. Thus, epigenetics is not independent of evolution, but is, as Waddington (Waddington, 1942) intended by his coining of the term 'epigenetics', actually 'on top of genetics'. Thus, we should consider the transgenerational modification of physiological or other phenotypes to be an amalgam of epigenetic effects and genetic mechanisms of evolution (e.g. mutation, natural selection). Even so, the time course of onset or disappearance (i.e. duration) of a trait differs radically between these various mechanisms for transgenerational change.

What, then, is the purpose/advantage of typically short-lived epigenetic phenomena? In many instances, epigenetic phenomena represent changes in phenotype, passed across a generation or more, that in some respects mimic those that might occur through mutation or natural selection. Fig. 5 schematically shows the different time courses of phenotypic modification through epigenetic effects, through mutation and through natural selection. A key difference is that epigenetic phenomena 'sunset' if the stressor that stimulated them in the first place subsequently disappears. Unlike a mutation selected for that stabilizes in the population, an epigenetic phenomenon is potentially short lived, affecting only a few generations. Consider as an example hypoxia tolerance in freshwater fishes that inhabit highly variable aquatic environments, with large year-to-year variations in water level associated with rainfall. Transgenerational transfer of enhanced hypoxic tolerance from parents to larvae may greatly

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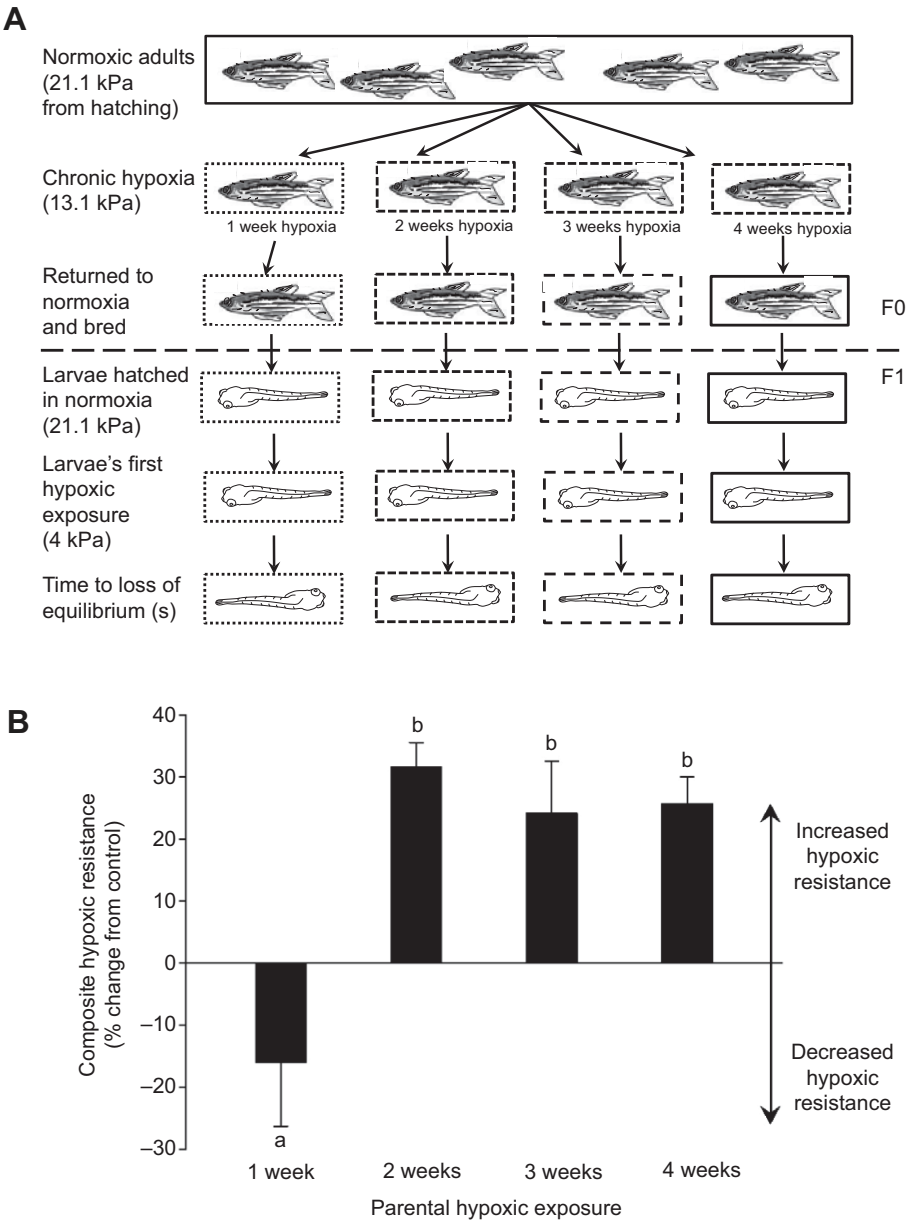

Fig. 4. Potential epigenetic inheritance of hypoxia tolerance in the zebrafish, *Danio rerio*. (A) Experimental protocol used to test hypoxia tolerance (determined by time to loss of equilibrium) of zebrafish larvae whose parents were exposed to different length bouts of chronic hypoxia. (B) Transgenerational transfer of hypoxia tolerance, with decreased and increased tolerance evident in larvae whose parents were exposed to 1 week or 2–4 weeks, respectively, of hypoxia. After Ho and Burggren (Ho and Burggren, 2012).

enhance fitness in the instance of a few years of successive low water levels and likely hypoxic water conditions. However, there is likely to be a 'cost' to this added hypoxic tolerance, or it would have been selected for and maintained as an adaptation in the population through conventional natural selection and genetic inheritance. When rains return after several years of drought, the environmental driver for the epigenetically based drought resistance – namely, aquatic hypoxia – disappears, as does the enhanced hypoxia resistance in the larvae. That is, the epigenetic phenomenon can be 'sunsetted' in a generation or two, only to arise again in later years if needed. This is quite different from genetically inherited phenotypes that the population is 'stuck with' until natural selection or mutation subsequently results in another phenotype.

Caveats, warnings and other inconvenient truths in epigenetics

Reaching out from the grave – extreme parental effects

Parental effects can be pernicious and confounding (to studies of non-genetic transfer of phenotype) and can have effects on multiple generations. For example, the males of some arthropods, such as crickets, produce very large spermatophores (Vahed et al., 2011). In some instances, the spermatophore of the male can represent up to 20% of its body mass. When deposited within the female's reproductive tract, this can represent a source for subsequent chemical signals to be incorporated in existing eggs awaiting fertilization. Indeed, when the food of male crickets is laced with radioactive proteins, the radioactive label passed to the female in the male's spermatophore can still be identified in the F2 generation (S. Kaulenas, personal communication). This suggests that for some species, consideration of parental history can extend back generations, but does not represent a classic case of epigenetic inheritance produced by epigenetic mechanisms of DNA methylation, histone modification or non-coding RNAs.

Who is the father, and when?

Epigenetic studies can be additionally confounded in those many species where the male's sperm is stored in the female for long periods of time. The phenomenon of sperm storage is common in

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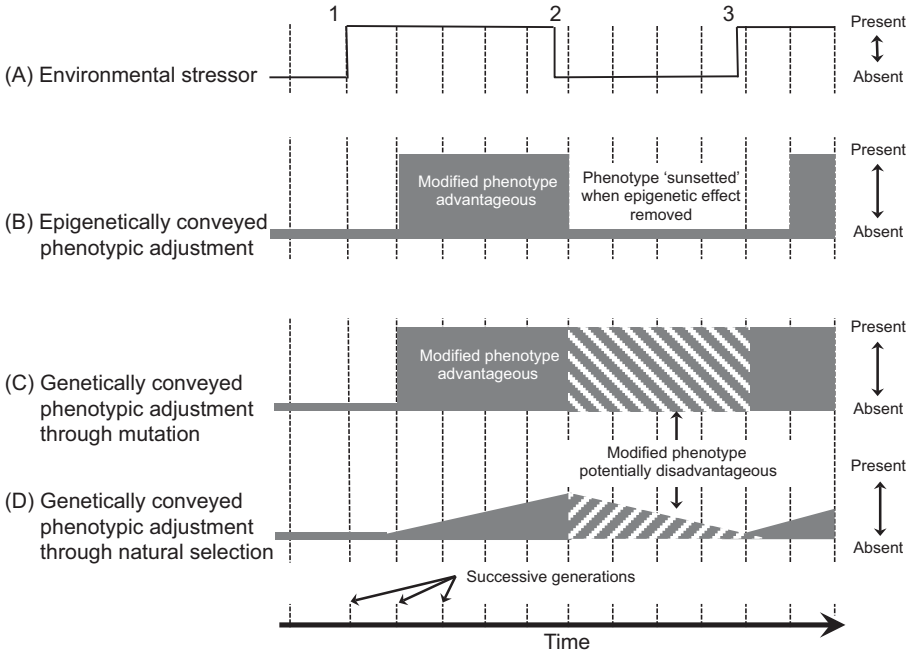

Fig. 5. Conceptual diagram of the various time courses for development and/or loss of phenotypic characteristics in response to environmental stressors. (A) In this scheme, which is over-simplified by mainly depicting responses as 'on–off' rather than graded, an environmental stressor intermittently appears in a non-graded fashion over multiple successive generations (indicated by dashed vertical lines). (B) Epigenetically conveyed phenotypic adjustment appears within a generation of the onset of the environmental stressor (at 1), and conveys additional fitness upon the animal. However, when the environmental stressor declines or disappears (2), the epigenetically maintained phenotype (with its associated advantages but also its costs) disappears, to return once again when the environmental stressor returns (3). In contrast, a phenotypic modification arising by mutation (C) or by natural selection (D) persists in the population even with the disappearance of the environmental stressor at 2.

numerous insects and other invertebrates; the fruit fly *Drosophila melanogaster* is an extensively studied model in this respect (Schnakenberg et al., 2012). However, sperm storage is also common in many vertebrates, where sperm can remain viable for up to 7 years in some reptiles and more than a year in some fish (Holt and Lloyd, 2010). Sperm storage often occurs in animals that have copulations with multiple males ('multiple paternity'), where the combination can create selective advantages (e.g. Vonhof et al., 2006; Fedina and Lewis, 2008; Griffiths et al., 2012). Indeed, in some species, fertile eggs of different ages fathered by different males can coexist in a female's oviduct.

Sperm storage and multiple paternity potentially result in differential provisioning and development of future eggs. This can seriously complicate any study of genetic as well as non-genetic transgenerational transfer unless carefully controlled for across generations.

Is it really epigenetic? The phenomenon of direct gamete exposure

The burden of proof for non-genetic transgenerational transfer of phenotype is relatively high, and many studies claiming to describe epigenetic phenomena likely fall short (including some of my past studies). Consider for example, a mature female mammal exposed to an environmental stressor (e.g. physicochemical, a toxicant). Not only is the mature female exposed but so too are the gametes she carries. Even when there is apparent transgenerational epigenetic inheritance, the real issue is whether there was any transgenerational transfer at all or simply a direct effect on the germ cells that would ultimately form the F1 generation (Ho and Burggren, 2010). And, to add to the complexity, if the mature mammal is already pregnant, then the fetus' gametes are also directly exposed to the stressor. This means that a phenotypic modification that is suspected to be of epigenetic origin would have to persist through the F1 and F2 generation into the F3 generation to rule out direct gamete exposure and allow an accurate claim of epigenetic transgenerational transfer. Yet, many if not most alleged epigenetic effects do not persist for more than one generation (or, to be accurate, have not been followed beyond one generation) – not sufficient longevity to rule out direct gamete exposure as the true underlying cause for the phenomenon.

How then, does one 'prove' a phenotypic modification in an offspring is of epigenetic origin, and not a direct effect? Certainly, coupling a transgenerational phenotypic modification with a known epigenetic mechanism (DNA methylation, histone modification, non-coding RNAs) is a step in the right direction. Another approach is to confine studies of epigenetic inheritance to those species with constant turnover of gametes in both males and females.

Limitations of wild-caught experimental animals in transgenerational epigenetic studies (and beyond)

Comparative physiological studies have traditionally relied heavily upon wild-caught animals as experimental subjects. Indeed, the strength of our discipline rests upon experimentation to reveal patterns of adaptation to environmental conditions in which animals have evolved (e.g. Burggren, 1997), and this often evolves studying wild-caught animals in their natural environment. Nothing epitomizes this approach better than the August Krogh principle, which is gospel to comparative physiologists (e.g. Krebs, 1975; Burggren, 2000; Bennett, 2003) and often leads us to exotic locales to study exotic animals in their native environment (see other articles in this issue). Yet, as we have discussed above, there is a subtle yet persistent influence of epigenetic phenomena (including but not limited to diet, physicochemical environment, timing of experiments) that can extend to physiological data. Additional potentially confounding phenomena include sperm storage, multiple paternity and direct stressor effects on gametes. The often-considerable variability inherent in comparative physiological data sets using wild-caught animals (or animals of unknown provenance supplied by animal suppliers) is thus not so very surprising.

I do not advocate an outright shift to traditional animal models raised generation after generation under standard, documented laboratory conditions. Indeed, such populations have their own shortcomings, with accumulation of genetic suppressors in inbreeding resulting from bottlenecks in the laboratory-maintained populations. Yet, it is important to recognize that minimizing variation in physiological data sets also means minimizing uncertainty as to the origin and prior experiences of the experimental

animals used. And, if one's focus is on the influence of epigenetic inheritance on physiological processes and teasing apart the complex ways in which prior experience can influence offspring phenotype through non-genetic mechanisms, then one has to minimize the uncertainty as to the origin and prior experiences of the parents and even grandparents of the experimental animals used! I thus conclude, somewhat reluctantly, that the most rapid progress will be made with either (1) conventional laboratory animal models (e.g. *Caenorhabditis elegans*, *Drosophila* sp., the zebrafish, *Xenopus laevis*, the chicken, the mouse and rat) or (2) non-conventional models that can effectively be reared for multiple generations under carefully controlled conditions.

Conclusions

Every animal has a developmental history, and every animal has had parents with their own set of unique environmental experiences that have potentially modified their offspring's phenotype through epigenetic inheritance. Increasingly, we are learning that the longstanding Lamarkian view of transgenerational transfer of acquired characteristics has some new-found validity. Moreover, epigenetic effects have likely contributed in previously unrecognized ways to the variation in comparative physiological data that has characterized (plagued) our data sets. We can ignore, at our peril, such effects, which can often be simultaneously subtle yet influential. Alternatively, I would urge, we can begin to recognize the major influence that 'historical' events in the lives of our experimental animals have in the physiological data we collect. Such considerations should influence our choice of the species and/or populations of animals we work on. This approach will only reduce the variation in our data, and consequently strengthen our hypothesis testing in comparative physiology.

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Competing interests

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