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Challenges and opportunities in developmental integrative physiology☆

C.A. Mueller[a,*], **J. Eme**[a], **W.W. Burggren**[b], **R.D. Roghair**[c], and **S.D. Rundle**[d]

C.A. Mueller: caseyamueller@gmail.com; J. Eme: johneme23@gmail.com; W.W. Burggren: burggren@unt.edu; R.D. Roghair: robert-roghair@uiowa.edu; S.D. Rundle: S.Rundle@plymouth.ac.uk

[a]Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON L8S 4K1, Canada

[b]Department of Biological Sciences, University of North Texas, 1155 Union Circle #305220, Denton, TX 76203, USA

[c]Stead Family Department of Pediatrics, University of Iowa, 1270 CBRB JPP, Iowa City, IA 52242, USA

[d]Marine Biology and Ecology Research Centre, Plymouth University, 611 Davy Building Drake Circus, Plymouth, Devon PL4 8AA, UK

Abstract

This review explores challenges and opportunities in developmental physiology outlined by a symposium at the 2014 American Physiological Society Intersociety Meeting: *Comparative Approaches to Grand Challenges in Physiology*. Across animal taxa, adverse embryonic/fetal environmental conditions can alter morphological and physiological phenotypes in juveniles or adults, and capacities for developmental plasticity are common phenomena. Human neonates with body sizes at the extremes of perinatal growth are at an increased risk of adult disease, particularly hypertension and cardiovascular disease. There are many rewarding areas of current and future research in comparative developmental physiology. We present key mechanisms, models, and experimental designs that can be used across taxa to investigate patterns in, and implications of, the development of animal phenotypes. Intraspecific variation in the timing of developmental events can be increased through developmental plasticity (heterokairy), and could provide the raw material for selection to produce heterochrony — an evolutionary change in the timing of developmental events. Epigenetics and critical windows research recognizes that *in ovo* or fetal development represent a vulnerable period in the life history of an animal, when the developing organism may be unable to actively mitigate environmental perturbations. ‘Critical windows’ are periods of susceptibility or vulnerability to environmental or maternal challenges, periods when recovery from challenge is possible, and periods when the phenotype or epigenome has been altered. Developmental plasticity may allow survival in an altered environment, but it also has possible long-term consequences for the animal. “Catch-up growth” in humans after the critical perinatal window has closed elicits adult obesity and exacerbates a programmed hypertensive

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*Corresponding author. Tel.: +1 905 525 9140x20075; fax: +1 905 522 6066.

phenotype (one of many examples of “fetal programing”). Grand challenges for developmental physiology include integrating variation in developmental timing within and across generations, applying multiple stressor dosages and stressor exposure at different developmental timepoints, assessment of epigenetic and parental influences, developing new animal models and techniques, and assessing and implementing these designs and models in human health and development.

Keywords

Critical windows; Development; Embryo; Epigenetics; Fetus; Heterochrony; Heterokairy; Leptin; Perinatal

1. Introduction

Comparative developmental physiology will be at the forefront of 21st century biological research, as it has long been recognized that maternal and environmental fluctuations shape the phenotype of developing organisms. In mammals, variation in maternal health and diet can influence the size and fitness of the fetus and newborn (Symonds et al., 2007; Vickers et al., 2008; Yu et al., 2013), and the eggs of egg-laying vertebrates and invertebrates are susceptible to different abiotic environmental conditions, including temperature, water availability, salinity, and respiratory gas levels (Dzialowski et al., 2002; Burggren and Reyna, 2011; Eme et al., 2015). Comparative developmental physiologists recognize that the maternal and developmental environment, as well as fluctuations in the timing of variations in environmental abiotic characteristics, can have permanent and possibly transgenerational effects on phenotype (Barker, 2004; West-Eberhard, 2005; Ho and Burggren, 2010; Forsman, 2014). Developmental phenotypic plasticity may allow for the physiology, morphology or biochemistry of the organism to adjust to fluctuations in maternal or environmental influences, but much less is understood about what length of time is necessary for a given environmental perturbation to have an effect, how long the effect persists, or whether the effect is passed on to subsequent generations.

The field of developmental integrative physiology includes the study of invertebrates, vertebrates, model and emerging-model organisms, as well as humans. Classic experimental designs for examining effects of environmental changes on organismal phenotype include comparing control groups to groups chronically incubated in altered environments, shifting treatment groups between control and altered conditions, and acute studies of control and altered treatment groups in the control or altered environmental condition. These types of study, in which an organism is exposed to one or two environmental ‘extremes’ (*e.g.* in temperature or oxygen), can answer questions regarding the tolerance of organisms. Exposure of developing organisms to extreme environmental conditions, and the shift from descriptive to mechanistic studies, has undoubtedly improved our understanding of developmental processes. However, the classic experimental designs used for examining the interaction between environment and phenotype cannot comprehensively answer questions regarding the timing, variation, onset, magnitude, or permanence of developmental phenotypic plasticity. Hence, developmental physiologists must not only continue to form new experimental questions, concepts and hypotheses, but must also examine innovative experimental approaches and designs to answer the questions they pose. Collectively, these

approaches will ensure that comparative developmental physiology continues to provide an important interdisciplinary link between development, physiology and evolution (Somero, 2000; Burggren and Warburton, 2005; Warburton et al., 2006; Spicer and Rundle, 2007).

The importance of comparative and integrative developmental physiological research across taxa was discussed during a symposium at the 2014 American Physiological Society Intersociety Meeting titled *Challenges from the Very Beginning: Developmental Physiology, Epigenetics, and Critical Windows*. This review is a culmination of that discussion and includes highlights from the developmental physiological research presented during the symposium. Our symposium presented work on invertebrates, vertebrate emerging-model and model organisms, and discussion of experimental designs to answer fundamental questions in developmental physiology. The potential evolutionary importance of variation in the timing of developmental events (heterochrony) and of intraspecific developmental plasticity in event timing (heterokairy) are illustrated primarily using examples from freshwater and marine invertebrates. The concepts of developmental phenotypic plasticity and critical windows, and the experimental approaches used to assess them, are discussed with examples from both vertebrate and invertebrate models. The ever-growing recognition of the important link between intrauterine and neonatal conditions and adult onset cardiovascular disease in humans is demonstrated using the example of leptin administration. Finally, the significance of epigenetics in developmental physiology, and how its presence must be accounted for, is considered across a range of animal models. Thus, the aim of the symposium, and of this resultant review, is to highlight current, groundbreaking research while also generating discussion about the current challenges and opportunities that exist in the dynamic and growing field of developmental integrative physiology.

2. Evolutionary importance of variation in the timing of developmental events

2.1. Introduction

There is a growing view within biology that evolutionary theory needs to give greater prominence to processes such as developmental bias, niche construction and epigenetics (West-Eberhard, 2003; Arthur, 2004; Pigliucci and Müller, 2010; Laland et al., 2014; Noble et al., 2014). A key strand of this shift in emphasis centers around a reappraisal of the role of the environment from one where it only selects among phenotypes generated through genetic variation to one where it both selects and generates phenotypic variation (Pfenning et al., 2010). Hence, phenotypic and developmental plasticity are now gaining in prominence as potential drivers of evolutionary change (DeWitt and Scheiner, 2004; West-Eberhard, 2005). This section focuses on the potential evolutionary role of one specific form of developmental plasticity, environmentally induced variation in the timing of developmental events.

2.2. Heterochrony from heterokairy: a potential link between ontogeny and phylogeny?

Heterochrony, defined as altered developmental timing between ancestors and their descendants, has been proposed as a major driver of evolutionary change (Gould, 1977; Raff

and Wray, 1989). Such timing differences can take the form of evolutionary alterations to the rate of development of different structures (Gould, 1977; Alberch et al., 1979; Klingenberg, 1998). More recently, however, another strand of heterochrony research has focused on the relative timing of developmental events through ontogeny–sequence heterochrony (Fig. 1) (Smith, 2001; Jeffery et al., 2005; Smirthwaite et al., 2007). This approach allows a broad assessment of when variation in event timing occurs during ontogeny (Bininda-Emons et al., 2002), and allows the inclusion of physiological traits, including the onset and offset of regulatory processes (Adolph, 1968; Spicer and Rundle, 2003; Spicer, 2006).

The investigation of sequence heterochrony required the development of computational approaches for assessing changes in the timing sequences of developmental events within phylogenetic contexts (Jeffery et al., 2002; Colbert and Rowe, 2008; Laurin and Germain, 2011). These techniques demonstrated the widespread occurrence of sequence heterochronies in both vertebrate and invertebrate phylogenies (Bininda-Emonds et al., 2003, 2007; Smirthwaite et al., 2007). These analyses also provide the potential for generating hypotheses to explore the mechanistic basis for evolutionary changes in event timing (Richardson et al., 2009; Spicer et al., 2011). However, research in this area is still in its infancy, and hard evidence for the mechanistic basis to heterochrony is lacking.

An obvious candidate mechanism for heterochrony is for selection to act on intraspecific variation in the timing of developmental events (Spicer et al., 2011), and recent studies have shown that considerable standing variation in event timing exists (de Jong et al., 2009; Rundle et al., 2011; Tills et al., 2013b,c). Such variation may or may not have a genetic basis and could be enhanced through an interaction of the organism with its environment (Tills et al., 2011). Spicer and Burggren (2003) named such intraspecific, environmentally-induced altered timing heterokairy. Their formal definition "…plasticity in the timing of onset of physiological regulatory systems or their components" placed heterokairy within a physiological context, and examples of physiological heterokairy were reviewed by Spicer and Rundle (2007) and Spicer (2006). There is, however, no reason why heterokairy should not be thought of within a more general framework, as a specific type of developmental plasticity (Fig. 2). Indeed, its inclusion would seem essential within the context of the proposed significance of heterochrony in evolution.

2.3. Heterochrony and heterokairy in adaptive contexts

One form of evidence for gauging the potential role of developmental plasticity in evolution is to use phylogenetically-controlled analyses to test for correlations between plasticity and environmental tolerance among species — the adaptive plasticity hypothesis (Van Buskirk, 2002). Alternatively, the flexible stem hypothesis predicts that plasticity in ancestral species acts as a source for speciation (West-Eberhard, 2003) and can be measured as a correlation between variation in phenotypes produced by plasticity in descendant ecotypes (Gomez-Mestre and Buchholz, 2006; Wund et al., 2008). While these hypotheses have not been tested in relation to plasticity in developmental timing, there are examples of links between heterochrony and heterokairy (although not named as such) within adaptive contexts. These inferences tend to center on exposure of taxa to new environments. In marine animals, for

example, historical changes in resource levels may have led to evolutionary change through sequence heterochrony. Example heterochronies here include advanced development of the left caecum in sea urchins in response to evolutionary increases in maternal provisioning (Snoke Smith et al., 2007) and earlier development of the feeding, veliger stage in gastropods to facilitate exploitation of increased levels of oceanic productivity (van den Biggelar and Hazprunar, 1996; Lindberg and Guralnick, 2003). Strathman et al. (1992) looked for parallels between the hypothesized heterochrony in sea urchins and showed advanced timing of juvenile structures within the species *Paracentrotus lividus* following exposure to high food rations. Further, Heyland and Hodin (2004) demonstrated that advanced timing of juvenile traits in larval sand dollars (*Dendraster excentricus*) was due to an increased supply of thyroid hormone in the algal food source. Evolutionary shifts towards a shorter larval period in spadefoot toads (Scaphiopodidae) following their expansion into more ephemeral habitats has also been shown to parallel a correlation between larval period and limb and snout lengths within species (Gomez-Mestre and Buchholz, 2006). Finally, kairomone-induced heterokairy in physiological traits (*e.g.* mantle muscle flexing, first heartbeat, crawling) for two freshwater snails (*Radix balthica*, *Radix auricularia*) (Rundle et al., 2011) matched sequence heterochronies associated with the same traits in the phylogeny from which the snails were derived (12 species from 3 families; Lymnaeidae, Planorbidae, and Physidae) (Smirthwaite et al., 2007).

While phylogenetic instances of heterochrony can be used as a basis for testing models for the evolutionary role of heterokairy, the study of heterokairy *per se* could also be used as a starting point for investigating the microevolutionary importance of intraspecific variation in event timing. Hence, the importance of heterokairy in processes such as genetic accommodation, genetic assimilation (Pigliucci et al., 2006; Suzuki and Nijhout, 2006) and costs of adaptive plasticity (Auld et al., 2010), including those of physiological traits, could make a significant contribution to our understanding of the importance of developmental plasticity in evolution. A powerful approach in this regard is where species are exposed to novel environments and can persist under the new conditions through plasticity, allowing a comparison of ancestral and derived populations (*e.g.* Scoville and Pfrender, 2010). There are currently a large number of ecosystems that are undergoing significant changes to their environmental conditions through biotic (*e.g.* introduced species) or abiotic drivers (*e.g.* increasing air and sea temperatures and occurrences of hypoxia in the sea). Several of the examples of physiological heterokairy outlined in Spicer and Rundle (2007) concerned the effects of such drivers associated with anthropogenic activities, and suggested species' ability to respond to environmental change may relate to their plasticity in timing of developmental events. Again, this strengthens the case for the inclusion of heterokairy within a developmental plasticity framework.

2.4. Alternative approaches for investigating developmental event timing

Much of the phylogenetically based work on heterochrony in vertebrates has involved anatomical data from embryos sacrificed at different stages of their development (*e.g.* Bininda-Emons et al., 2007). Testing models for the importance of heterokairy is difficult for such species, given the limitations of working with preserved specimens or making observations from live embryos within opaque eggs. Recent advances in bio-imaging

technology, however, allow the simultaneous *in vivo* observation and videoing of multiple, individual embryos, including analytical techniques that can be used to quantify physiological responses to stress in species such as *R. balthica*, *Danio rerio* and *Xenopus laevis* (Tills et al., 2013a). Video footage at high temporal and spatial resolution enables an integrative approach whereby physiological, behavioral and morphological traits and their time of onset and offset can be measured. This technology has recently been used in studies of event timing in gastropod (*R. balthica*) embryos from which it was possible to measure the timing of events such as the first heartbeat, mantle muscle flexing, crawling and shell and radula formation (Tills et al., 2013b). It revealed high levels of intraspecific variation (Tills et al., 2013c) that appeared to have a genetic basis (Tills et al., 2011). A parent–offspring comparison also revealed heritability in the timing of crawling within the egg capsule (Tills et al., 2013b) — current work is investigating whether such variation acts as the raw material for selection using a developmental plasticity approach. This demonstrates that using an event sequence-based approach to select a functional trait shows potential for investigating the evolutionary role of heterokairy. The substantial knowledge of the adaptive importance of crawling post-hatching in this clade of freshwater snails, including local adaptation in this trait (Dalesman et al., 2009, 2015), alongside its importance in heterochrony (Smirthwaite et al., 2007) and heterokairy (Rundle et al., 2011) add further to the utility of this group of invertebrates for studying event timing.

3. Critical windows in developmental physiology

3.1. What are critical windows?

The concept of phenotypic plasticity is well established, but the recognition that animals may show greater levels of phenotypic plasticity during certain periods, or ‘critical windows’, of development is a more recent area of physiological study (Burggren and Warburton, 2005; Burggren and Reyna, 2011; Burggren and Mueller, 2015). Critical windows are recognized in the medical field as time periods during which the developmental environment must be appropriate for phenotypic changes to occur that ensure normal development of, for example, motor and language skills, higher cognition and other neurological activities (Rice and Barone, 2000; Hensch, 2004; Hensch and Bilimoria, 2012). In physiology literature, however, critical windows are defined as developmental periods when an animal is particularly susceptible to internal or external stressors, resulting in changes away from the normal phenotype. For example, exposure to chronic hypoxia or hyperoxia throughout embryonic/fetal development or during specific developmental windows results in modifications to morphological and respiratory phenotypes in bird, mammal, and reptile embryos (Dzialowski et al., 2002; Bavis, 2005; Chan and Burggren, 2005; Crossley and Altimiras, 2005; Bavis and Mitchell, 2008; Ferner and Mortola, 2009; Owerkowicz et al., 2009; Eme et al., 2011a,b, 2013a,b, 2014; Bavis et al., 2013; Marks et al., 2013; Tate et al., 2015). Likewise, exposure to chemical stressors such as endocrine disruptors, ethanol and retinoids in fish and amphibian ecotoxicology studies often demonstrate the negative changes in morphology and physiology following exposure during critical windows (Degitz et al., 2000; van Aerle et al., 2002; Ankley and Johnson, 2004; Maack and Segner, 2004; Ali et al., 2011). Changes in the phenotype may be transient or permanent in nature, having potential consequences during later ontogenetic stages of an

animal. It is apparent that the recognition of critical windows as a key principle in comparative developmental physiology is burgeoning, and the way in which we approach their study is of growing importance.

3.2. Current approaches in critical window research

In their simplest form, critical windows are viewed as one dimensional, and raise questions such as “When during development or time is there a critical window?” and, “How long is the critical window?” During the normal developmental trajectory of a species, a supposed ‘normal’ phenotype is produced (Fig. 3A). Along this trajectory, there may be a critical window in which developing systems are particularly plastic. Therefore, if an embryo is exposed to an environmental stressor before or after this critical window there is no change in the phenotype. However, if we apply an environmental stressor during the critical window, then we can alter the developing phenotype, which indicates the presence of a sensitive period. In some instances, once the stressor is removed an animal may show self-repair capabilities, so that at some later stage they have returned to the normal phenotype (Burggren and Reyna, 2011).

The one-dimensional concept of the critical window is most prevalent, as most studies have the straightforward aim to detect if a critical window is present or not. The response of Lake whitefish (*Coregonus clupeaformis*) embryonic metabolism, heart rate and survival to a temperature change at particular developmental milestones is one such example of this type of critical window study (Eme et al., 2015; Mueller et al., 2015). Lake whitefish were incubated in either constant temperatures (2, 5 or 8 °C) throughout embryonic development or underwent at permanent temperature shift to one of the other temperatures either at the end of gastrulation or the end of organogenesis (see Table 1 in Eme et al., 2015 for complete experimental design). Lake whitefish embryos that experienced a temperature shift at the end of gastrulation showed a general trend for reduced oxygen consumption rate, heart rate, and survival compared to embryos in constant temperatures. In comparison, embryos that experienced a temperature shift at the end of organogenesis did not show any change in metabolism or heart rate at pre-hatch, which suggests embryos show greater plasticity during organogenesis than during the late growth period.

Studies by Crossley and colleagues have sought to understand effects of hypoxia (10% O_2) on development of the cardiovascular system in reptile embryos, including the American alligator (*Alligator mississippiensis*) and Common snapping turtle (*Chelydra serpentina*) (Crossley and Altimiras, 2005; Eme et al., 2011a,b, 2013a,b, 2014; Marks et al., 2013; Tate et al., 2015). These studies have demonstrated that chronic hypoxia throughout the majority of embryonic incubation (20% to 90% of incubation) causes cardiac enlargement, chronic hypotension, reduced mRNA expression of adrenergic and growth factor receptors, and reduced embryo mass. Most recently, it was demonstrated that hypoxia between 50% and 70% of incubation only in *C. serpentina* embryos caused cardiac enlargement, while exposure to hypoxia before or after this critical window had no detectable effect on heart mass (Tate et al., 2015). In addition, a window for hypoxia to cause hypotension was identified that was much broader, 20 to 70% of incubation, and embryonic body mass was dependent on the duration of time spent in hypoxia without a specific critical window (Tate

et al., 2015). The study from Tate et al. (2015) is the first to specifically reference a 'critical window' in reptile cardiovascular development, and this area of research is a promising avenue for isolating important time periods in development during variable embryonic oxygen levels.

Indeed, perinatal exposure to hyperoxia and hypoxia in mammals causes changes to respiratory control that can persist through early life (see Bavis, 2005 for review). Hyperoxia and hypoxia both alter the hypoxic ventilatory response (HVR; the increase in ventilation caused by hypoxia); the majority of studies demonstrate that perinatal hyperoxia causes blunted adult HVR responses, and humans raised at high altitude (hypoxia) also exhibit a blunted HVR (Moore, 2000; Gamboa et al., 2003). Postnatal, but not adult, exposure to hyperoxia reduces the size of carotid bodies, demonstrating that the period immediately following birth is a critical window for normal development of the chemosensing carotid bodies (Dmitrieff et al., 2012). Therefore, for cardiorespiratory control in mammalian models, including humans, the perinatal and postnatal periods can represent critical windows for phenotypic plasticity in response to altered oxygen levels.

3.3. Interaction between exposure window, stressor dose and phenotypic modification

The studies detailed above demonstrate the usefulness of the one-dimensional critical window approach in providing a "first pass" for detecting periods of particular susceptibility or plasticity. However, this approach may overlook potentially important aspects of phenotypic responses within a critical window that may be revealed with a more detailed experimental design. A simple improvement to critical window studies is the use of multiple doses of a stressor. The Lake whitefish study used three temperatures, which is a very basic multi-dose design (Eme et al., 2015; Mueller et al., 2015). However, many studies that aim to detect critical windows do so using just a single dose of a stressor (*e.g.* Koger et al., 2000; van Aerle et al., 2002; Hogan et al., 2008; Hanlon and Parris, 2014), and this may influence the detection of a critical window. Moreover, there is potentially a strong reliance on how successfully a critical window is detected based on the doses used. For example, a low dose of a stressor may produce no phenotypic change, but a slightly higher dose may reveal modification, and hence a critical window (Fig. 3B). If an even higher dose is used, the critical window may be revealed as being wider than indicated by the lower dose, as the stronger dose is able to induce an effect slightly earlier in development and persist slightly longer at the end of the critical window. Hence, the use of multiple doses adds an extra dimension to how critical windows are examined. This approach contributes to the detection and determination of the *size* of a critical window, but it is limited in how we view phenotypic modifications occurring *within* a critical window.

To truly understand a phenotypic change that may occur within a critical window, we must examine its magnitude, not just its presence or absence. Fig. 3C demonstrates a three-dimensional critical window in which effect size, or phenotypic change, varies based on when exposure occurs and the dose of the stressor applied. This three-dimensional approach, therefore, analyses critical windows in more detail by examining how the interaction between time of exposure and stressor dose alters the magnitude of the phenotypic change, not just if a phenotypic change is produced (Burggren and Mueller, 2015). An example of

the application of the three-dimensional approach is the effect of salinity during different developmental exposure windows on survival of brine shrimp (*Artemia franciscana*). Survival was shown to be highest following exposure to low salinity (10 and 20 ppt) compared to high salinity (40 and 50 ppt) early in development, but this difference disappeared when animals were exposed later in development (Burggren and Mueller, 2015). This example illustrates how the magnitude of phenotypic change (in this instance, survival) can be examined, and visualized, in terms of the interaction of time of exposure and stressor dose. Such an approach can be applied across development or only with a period of known susceptibility as a means to examine the nuances of phenotypic modification.

3.4. Considerations for critical window experimental designs

The search for critical windows during development often requires a large number of treatments, replicates and animals. Each new exposure window or stressor dose necessitates the addition of another treatment, and levels of replication need to increase with treatment number to maintain statistical power. Thus, the animals and space required for comprehensive critical window studies are much greater than traditional chronic exposure studies, and are important considerations when designing this type of research. Besides the benefits of critical window studies in detecting and understanding developmental phenotypic plasticity, the experimental approaches outlined above can be used across a broad range of organisms, systems and stressors. However, for any physiological experiment the choice of organism is partly based on the attributes that make it ideal for the study ("Krogh Principle"; Krogh, 1929). As outlined above in Section 2 for studies of heterochrony and heterokairy, species that are relatively easy to obtain, house and care for in large numbers will also lend themselves to critical window studies. In terms of the system studied and whether interest is predominately in physiology, morphology, biochemistry or a combination of all three, the possibilities for critical window studies are numerous. Critical window experiments can be designed to target any regulatory or organ system using a vast array of relevant stressors, and this places critical window research as a vital contributor to the integrative nature of comparative developmental physiology.

Just as endpoints under examination in critical window studies may be at any level of organization, they may also be measured immediately following an exposure during a critical window or at later stage endpoints, including those post hatching/birth. While developmental plasticity may allow the survival of developmental stages under an altered environment, it also has possible long-term consequences for an animal (Burggren and Reyna, 2011; Kelly et al., 2012). Thus, endpoints may only be assessed in adults, as a means of addressing how the developmental environment influences the resultant adult phenotype. Furthermore, plasticity may only occur during certain critical windows of development due to the costs associated with phenotypic reversal or due to the negative effects on the adult (Bateson et al., 2004). Phenotypic changes during critical windows may be reversed, with animals capable of self repair (Hill and Janz, 2003). Alternatively, as occurs in the fetal origins of human diseases, changes may only appear at later stages and persist long term (Barker, 2004; Gluckman and Hanson, 2004), as discussed in more detail in Section 4.

Future studies examining critical windows will be most successful when they utilize well-replicated experimental designs consisting of multiple exposure windows and stressor doses, as this will ensure the most accurate determination of periods of phenotypic plasticity or susceptibility. Using experimental approaches that recognize critical windows are the result of the interaction between time in development, stressor dose, and the magnitude of phenotypic change will advance our knowledge of developmental critical windows and their importance in comparative integrative physiology.

4. Variation in intrauterine and neonatal development

4.1. Variation in perinatal human growth and adult disease

Evolution is more likely to occur in species that are able to adapt to a changing environment, and natural variation or plasticity in perinatal development helps to ensure population survival. With advanced technology and improved nutrition, human lifespan has been extended, and the long-term implications of early growth variance, as well as the timing and critical developmental window during which the variance occurs, have become apparent. The inverse relationship between body weight through two years of age and adult blood pressure in humans has become the prototypic example of the association between growth restriction during a critical window of development and subsequent adult disease (Barker et al., 1989; Bergvall et al., 2007; Eriksson et al., 2007). Environmental insults are known to influence the predisposition of growth-restricted individuals towards hypertension, psychological stress and increasing adult weight (Eriksson et al., 2007; Jones et al., 2007; Pyhälä et al., 2009). Neonatal growth restriction and adult obesity both exert detrimental effects on blood pressure, and growth restricted infants are at risk of central adiposity (Tian et al., 2006). Early life growth restriction is likely to be followed by lower adult body weight, and this is associated with hypertension and neural sympathetic overactivation (Hack et al., 2003, 2014; Boguszewski et al., 2004; Eriksson et al., 2007).

Although a relative paucity of human studies have enrolled both male and female subjects, available data are consistent with significant sex differences in developmental origins of hypertension (Intapad et al., 2014). Men with former intrauterine growth restriction (IUGR) have increased blood pressures and vascular resistance, and these cardiovascular risk factors are disproportionally exacerbated by psychological stress compared to normal birthweight men or IUGR women (Jones et al., 2008; Miles et al., 2011). Beyond hypertension, early placental insufficiency and growth restriction alter the morphology of vulnerable organs, including the pancreas and brain, and these morphologic alterations have been associated with impaired insulin secretion and adverse psychosocial outcomes (Darlow et al., 2013; Rozance et al., 2014). Ultimately, perinatal growth restriction increases not just morbidity, but also cardiovascular mortality (Barker et al., 1989).

In an analogous fashion, larger perinatal weight gain increases the likelihood of adult obesity and obesity-related morbidities (Tian et al., 2006). Maternal adiposity and excessive gestational weight gain increase the risk of a large newborn (neonatal macrosomia), resulting in the potential for trans-generational environmental or epigenetic inheritance of metabolic syndrome, a major risk factor for coronary heart disease (Pinney and Simmons, 2012; Yu et al., 2013). The increasing prevalence of childhood obesity has led to calls to

mitigate key prenatal and neonatal risk factors (Gillman and Ludwig, 2013). The characterization of mechanistic animal models is a critical step towards identification of interventions that preserve the balance between insufficient perinatal growth and the risk of obesity-related disease.

4.2. Modeling the developmental origins of adult hypertension

While international cohort studies of humans have defined the scope of the problem, animal models have been indispensable in the isolation of the developmental windows and environmental factors that lead to fetal or perinatal programmed hypertension. The sexual dimorphism highlighted has been a consistent theme in additional preclinical growth restriction models, including studies in sheep, pigs, and rats (Roghair et al., 2009; Intapad et al., 2014). Consistent with human epidemiological data, studies in isogenic mice have demonstrated that male mice have a heightened sensitivity to even mild naturally-occurring neonatal growth restriction, and early catch-up growth prevents stress-exacerbated hypertension, but increases adiposity and diabetes susceptibility (Roghair and Aldape, 2007; Hermann et al., 2009). The mouse is a non-precocial species that requires at least 2 weeks of postnatal growth to reach a neuro-developmental stage analogous to term infants (Romijn et al., 1991). Therefore, studies using 1 day–14 day old postnatal mice can best model third trimester IUGR and the neonatal growth restriction seen almost uniformly in premature infants (Hack et al., 2003).

Among the potential etiologies for programmed hypertension, there is experimental support for primary vascular dysfunction, as well as secondarily increased vascular resistance as a consequence of increased sympathetic tone (Intapad et al., 2013; Mizuno et al., 2013). Regardless of the etiology, reactive oxygen species and the renin–angiotensin system have often been implicated in the propagation of growth restriction-associated vascular dysfunction and hypertension (Yzydorczyk et al., 2006; Roghair et al., 2009; Mizuno et al., 2014). Both antioxidants and renin–angiotensin system antagonists have thus been shown to mitigate programmed hypertension in a number of species and model systems (Sherman and Langley-Evans, 1998; Roghair et al., 2011). Further studies are needed to define the independent roles of nutrient deficiency, hypoxia, renal insufficiency and other potential triggers, along with the critical developmental windows, in the inception of these long-term alterations.

While investigations into the origins of hypertension proceed, equal attention will need to be placed on female subjects. Investigations are more challenging in females given the variation in blood pressure seen throughout estrus, as highlighted by the work of Alexander and colleagues showing growth restricted female rats are hypertensive before puberty and following menopause, but are protected by endogenous ovarian function and exogenous estrogen therapy (Ojeda et al., 2007, 2011). In addition to sex-specific hormonal levels, male and female neurodevelopment appears to follow different trajectories, with studies in both humans and mice supporting a relative delay in neurologic susceptibility of male offspring (Hintz et al., 2006; Hermann et al., 2009). Further studies are needed to define and potentially exploit the pathways that underlie these critical differences in sex-specific brain and cardiovascular development.

4.3. Identification of pathway-specific interventions: best practices using leptin as a target

As mentioned above in studies that expose mammals to altered oxygen levels, the perinatal period can represent a critical period that permanently alters the adult phenotype. Searching for a targeted intervention that could prevent hypertension without sacrificing metabolic health, focus has turned to the critical windows for administration of the adipocytokine, leptin. In adults, leptin is secreted by mature adipocytes to signal the extent of fat stores to the hypothalamic regions responsible for appetite regulation. During the critical window of late human gestation and neonatal murine development, leptin exerts vital neurotrophic effects that help establish adult leptin sensitivity (Erkonen et al., 2011). Because growth restricted infants lack adipose reserves and are leptin deficient (Pighetti et al., 2003), perinatal supplementation may maintain neurodevelopment and metabolic regulation in the absence of the need for obesity-inducing hypercaloric nutrition.

Considering the potential permanent effects of neonatal leptin supplementation on adult metabolic and cardiovascular outcomes, the therapeutic indication, dose, and timing of the intervention were important considerations (Fig. 4). Both preterm and IUGR term infants have significant reductions in circulating leptin levels that may benefit from supplementation (Su et al., 2002; Pighetti et al., 2003) but macrosomic infants are already hyperleptinemic and thus unlikely to benefit (Vickers et al., 2008) further demonstrating the perinatal period is a critical window in human development. This paradigm has been well supported by studies in rats and mice showing that neonatal leptin supplementation improves the cardiometabolic health of undernourished pups, but increases the risk of diet-induced obesity and hypertension in non-leptin deficient controls (Trevenzoli et al., 2007; Vickers et al., 2008; Erkonen et al., 2011). Likewise, when neonatal leptin is administered to appropriately grown mice and rats at supraphysiologic doses (2.5 to 6 mg/kg/d), a metabolic state reminiscent of early-onset obesity is promoted, and long-term impairment in leptin-triggered anorexia is observed (Samuelsson et al., 2013). Finally, the timing of the leptin replacement must match the phase of leptin-sensitive neurodevelopment. In humans, this includes the third trimester of gestation, but in mice, the relevant critical window of susceptibility extends from postnatal days 4–14 (Bouret et al., 2004; Grove et al., 2005). Physiologic leptin administration to lactating rats during this neonatal window confers protection to the offspring from diet-induced obesity in association with favorable alterations in hypothalamic gene expression (Picó et al., 2007; Stocker et al., 2007). Likewise, placing pups in large litters to elicit neonatal undernutrition leads to a lean, leptin-responsive adult phenotype; while placement in small litters to elicit neonatal overnutrition predisposes the pups to obesity in association with an impairment in leptin-triggered hypothalamic signaling (Remmers et al., 2008; Velkoska et al., 2008; Rodrigues et al., 2009).

During the appropriate critical period, it has been demonstrated that precise correction of growth restriction-induced neonatal leptin deficiency with neonatal leptin supplementation (0.08 mg/kg/d) blocks the programming of stress-related hypertension, elicits neurotrophic effects on the hypothalamus, and improves both learning and social interaction (Erkonen et al., 2011; Meyer et al., 2014). To further clarify the effect of isolated leptin deficiency during development, a leptin antagonist was administered to well grown neonatal mice, and

adult phenotypes were compared four months after the treatment concluded. Compared to saline-treated controls, leptin antagonist-treated mice had reduced brain volumes, locomotor hyperactivity and increased cerebral cortex leptin receptor expression (Dexter et al., 2014). These results confirmed that neonatal leptin modulation could exert effects in normally grown mice. The inverse correlation between leptin sensitivity and neonatal leptin levels (increased sensitivity with neonatal deficiency, decreased sensitivity with neonatal excess) is consistent with investigations showing obesity-related (supraphysiologic) hyperleptinemia is associated with selective resistance to leptin's metabolic effects (Rahmouni et al., 2005). The relative insensitivity of the blood pressure *versus* the metabolic pathway highlights the need to understand better the site-specific regulatory pathways downstream of the leptin receptor so that targeted interventions can be developed that might selective attenuate leptin's hypertensive responses, without interfering with the hormone's appetite suppressant effects. Such investigations would ideally incorporate the roles of central angiotensin and estrogen receptors in the modulation of programmed adult hypertension (Clegg et al., 2006).

When the fetus or infant is challenged with inadequate or excessive nutrient delivery at a critical phase of growth and maturation, trade-offs that sustain metabolic balance may elicit long-term cardiovascular manifestations. Perinatal growth restriction significantly increases the risk of life-threatening cardiovascular disease (Barker et al., 1989). While there is current rationale for overnutrition to minimize the risk of irreversibly stunted brain development, more targeted investigations must be developed that incorporate optimized nutrient content (Cooke et al., 2006). As a homeostatic regulator, leptin administration at physiologic doses during a critical window, the third trimester through the neonatal period, may help mitigate the detrimental effects of an adverse perinatal environment.

5. Epigenetic influences in comparative developmental physiology

5.1. Introduction

Interest in epigenetics has expanded enormously in the last decade –in fact, a near exponential growth in studies focusing on epigenetic aspects of biology has been evident over the several decades since the introduction of the term "epigenetics" by Waddington in the 1940s –see Burggren (2014). Of late, epigenetics has been of considerable interest to the medical field because of its influence in human health. As just one of many examples, the "*epigenetics of cancer*" has been extensively investigated because of the obvious search for causative factors of this disease that are not related to actual genetic mutations, but rather the aberrant expression of the normal suite of genes (Barrow and Michels, 2014; Shukla and Meeran, 2014). Far less frequent – but equally interesting – are epigenetic studies that examine the transfer of phenotype *across* generations, rather than the modification of phenotype during development, maturation and senescence within a generation. In fact, a recent meta-analysis suggests that over the last 3 years intragenerational epigenetic studies outnumber transgenerational epigenetic studies by nearly 30 to 1 (Burggren and Crews, 2014). In this section, we focus primarily on *transgenerational* epigenetic phenomena and their implications for comparative physiology.

5.2. Transgenerational epigenetics and variation in physiological phenotypes

Biologists studying epigenetics have not generally considered the epigenetic inheritance of modified physiological processes. Yet, epigenetic effects, through the now well documented mechanisms of DNA methylation, histone modification, non-coding RNAs, *etc.* (Gluckman et al., 2007; Ho and Burggren, 2010; Martin-Subero, 2011; Cantone and Fisher, 2013; Moore et al., 2013), have many avenues by which to affect physiological processes, especially because of underlying molecular, cellular and gross morphological phenotypic modifications. Indeed, one could ask "How could physiological phenotype NOT be modified if profound phenotypic alterations at lower organizational levels are being inherited by epigenetic mechanisms?" However, as Denis Noble has recently observed, molecular and cellular modifications do not necessarily translate into physiological changes, because there are many layers of potential buffering between a molecular or cellular phenotype and an actual modification of physiological performance (Noble, 2015). Consider, for example, a modification of the K_{max} of a rate-limiting enzyme that negatively affects the normal function of the Krebs cycle. Such a phenotype could potentially reduce the scope for whole body metabolic rate. Yet, this molecular phenotypic modification could be mitigated in a whole host of ways, including increased capillarization of muscle tissue, increased blood oxygen carrying capacity, reduced oxygen distance for oxygen diffusion in the gas exchange organs, *etc.* Individually or collectively, these intermediate level phenotypic modifications could result in a "normal" whole animal metabolic rate even with compromised enzymes. Consequently, epigenetic inheritance of molecular/cellular phenotypic modifications should not be assumed to immediately translate into the epigenetic inheritance of modified physiological phenotype. However, the transgenerational epigenetic induction or transfer of modified physiological phenotypes has been reported in numerous instances. Epigenetically inherited physiological phenotypic modifications include hypoxia tolerance, stress responses, heavy metal resistance, cardiovascular performance, and growth rates, to name but a few. It is beyond the scope of this section to review these changes — consequently the reader is referred to recent reviews (Ho and Burggren, 2010, 2012; Crews et al., 2012; Jablonka, 2013; Burggren, 2014; Burggren and Crews, 2014). Nonetheless, epigenetic inheritance of physiological phenotype is still considerably under-documented compared to phenotype modifications at lower organizational levels.

5.3. Forms and dynamics of epigenetic inheritance

In assessing transgenerational epigenetic inheritance of a modified physiological phenotype (indeed, of any modified phenotype), it is important to realize that such inheritance can take two general forms (Fig. 5). So-called "context-dependent" epigenetic inheritance arises as a result of direct exposure of the F_0 animal to a stressor (*e.g.* hypoxia, hypothermia). Modified phenotype will persist across multiple generations as long as the stressor persists. This contrasts with "germline dependent" inheritance, which occurs as a result of the experiences of the germline (*i.e.*, the eggs and sperm and even the stem cells that produce them) potentially persisting across generations even in the absence of the original causative stressor (Burggren and Crews, 2014). Transgenerational phenomena such as maternal and paternal effects are examples of germline dependent inheritance. Physiological examples of maternal/paternal phenomena effects have also been documented.

Irrespective of the form of epigenetic modification, epigenetically inherited traits that cross multiple generations (*e.g.* F_1 through F_n) or even across multiple broods within a generation (*e.g.* $F_{1.1}$, $F_{1.2}$, $F_{1,3}$) may show interesting "epigenetic dynamics". The loss of the modified phenotype is not necessarily digital, but may more accurately be portrayed as analog, where the modified phenotype might wash out (or even wash in) across multiple generations or broods (Burggren, 2015). Subtle effects such as these can complicate the measurement, and even detection of, physiological or other phenotypes that are epigenetically inherited across multiple generations.

5.4. Epigenetics in comparative and evolutionary physiology

Intragenerational epigenetic studies tracking phenotypic modifications within an organism's lifetime, though squarely within the domain of medical epigenetics, are of interest to comparative and evolutionary physiologists. Indeed, these investigations tend to focus on the underlying mechanisms to explain phenotypic plasticity and especially developmental phenotypic plasticity (West-Eberhard, 2003; de Jong and Leyser, 2012; Kelly et al., 2012; Snell-Rood, 2012; Forsman, 2014; Woods, 2014). Such studies almost invariably spill over into considerations of fetal programming (Symonds et al., 2007; Nijland et al., 2008; Vickers, 2011) and heterokairy (Spicer and Burggren, 2003; Spicer and Rundle, 2007; Spicer et al., 2011; Mendez-Sanchez and Burggren, 2014). Transgenerational epigenetic studies *per se* are more inclined to provide insights into the influence of rapid phenotypic adjustments — so-called "emergency phenotypes" (G. Claireaux, unpubl.). These phenotypes may allow organisms to survive an abrupt, adverse change in environment (*e.g.* temperature, nutrition) that develops over the course of multiple generations. Yet, these modified phenotypes (which could have significant energetic and/or physiological cost) can be "sunsetted" should the environment return to more favorable conditions. In rapidly changing, unpredictable environments, epigenetically inherited physiological traits may actually better serve the organism than traits arising by mutation and natural selection (Burggren, 2014). In this respect, transgenerational epigenetic mechanisms will increase a population's fitness by allowing reproduction of individuals that survive transient environmental stressors over an evolutionary time frame. Finally, just as is evident from medical epigenetics, epigenetically inherited physiological phenotypes may in fact be maladaptive, in which case the loss of the phenotype in subsequent generations (assuming the animal survives to reproduce) would restore previous levels of fitness to the population.

5.5. Best practices for experimental designs in physiological epigenetics

Experimental designs for studies probing epigenetic inheritance of modified physiological phenotypes start with a sound choice of animal models. If one is interested in a physiological effect in a specific animal, *e.g.* the marmot, then the experiments are done on marmots. However, given that our understanding of physiological epigenetic effects is still in its relative infancy and that we are still just beginning to understand the epigenetic modification of physiological phenotype, the case is compelling for animal models that provide tractable solutions and quick, abundant data that allow exploration of "universal truths" in physiological epigenetics. Hence, the same features that make certain species good models for other physiological experiments equally apply to epigenetic studies. Models that have short generation times, that are inexpensive to maintain and rear, that are highly fecund

and, of course, lend themselves to physiological measurements, are highly useful in epigenetic studies. Some familiar species now appear in the animal epigenetic literature — *e.g.* the zebrafish (*D. rerio*), nematode (*Caenorhabditis elegans*), and fruit fly (*Drosophila melanogaster*). However, some species can be excellent models for epigenetic studies because of special or unusual characteristics. Animals that can reproduce clonally by asexual reproduction (*e.g.* the water flea *Daphnia*) can help eliminate complexities introduced across generations by genetic variation in offspring (Andrewartha and Burggren, 2012; Verhoeven and Preite, 2013). Animals that can regenerate major portions of their body when bi- or dissected (*e.g.* starfish, flatworms) may also prove to be interesting animal models for studying the epigenetic dynamics of transgenerational physiological inheritance. If a mature animal consists in part of the original F_0 parent *and* newly regenerated limbs or other body parts, what does F_1 actually mean in this developmental mosaic?

Experiments in physiological epigenetics may also be enhanced by considering signal-to-noise ratios (*i.e.* the size of the epigenetic effect against background variation in physiological performance). In this regard, physiological experiments in epigenetics are not different from any other physiological studies. However, when looking for subtle transgenerational effects, being able to measure a strong signal with both accuracy and precision may be especially important. Consider, for example, that an epigenetically inherited physiological phenotype that might wax or wane over a couple of generations. Under these circumstances, one might erroneously assume that either there is no epigenetic effect (when in fact a phenomenon slowly grows over the F_1 and F_2 generations) or that the effect disappears in the F_1 generation (when in fact it persists at low levels in subsequent generations) (Burggren, 2015).

To conclude, epigenetic influences on physiological processes can range from subtle to profound, and within or across generations. While not all physiological studies need to specifically address epigenetic issues, investigators are best served by recognizing that epigenetic effects can contribute significantly to what might otherwise be attributed to variation of indeterminate origin that cannot be controlled for (Burggren, 2014).

6. Conclusion

The influence of the environment on shaping the timing of physiological, morphological and/or biochemical phenotypes within a species, both within a generation during development as well as across generations, is an area that requires the interdisciplinary experimental approaches characteristic of comparative physiology. Variation in phenotypes may be obvious for some features, such as an organism’s body size. However, variation in variables such as metabolism, enzyme activities, organ function, and timing of morphological or physiological development may require variation to be examined within and across generations, including the size of critical windows and the variation in timing of developmental events.

This review has provided examples of best practices and important areas for future developmental physiology research. Video footage of high-throughput (*e.g.*, 96 well plates) development for individual embryos of model (*D. rerio*, *X. laevis*) and emerging-model

organisms (*R. balthica*) provides an excellent avenue to replicate and examine changes in the timing of developmental events in response to physiological stress (*e.g.*, temperature or oxygen level changes) (Tills et al., 2013a). These types of data allow for more explicit links to be generated between heterochrony and heterokairy that to date are, unfortunately, largely treated as separate phenomena in the literature (Spicer and Rundle, 2007).

Most developmental physiology experiments utilize a single stressor dose at a single developmental time point, but 3-dimensional experimental designs utilizing multiple stressors and developmental time points will allow for the importance of the interaction between critical window size, stressor dose, and phenotypic modification to be demonstrated (Burggren and Mueller, 2015). These 3-dimensional experimental designs should adhere to the “Krogh Principle”; (Krogh, 1929), as appropriate model organisms are those that can be housed and cared for in large numbers — such as fish and amphibian embryos, as well as aquatic invertebrates.

Mitigating increased susceptibility to human disease during fetal and neonatal development, such as cardiovascular disease and hypertension, includes targeted intervention that may prevent hypertension without sacrificing metabolic health. Administration or alteration of pharmacological agents or hormones, such as leptin, should focus on determining the appropriate dosage *and* critical window for administration (Bouret et al., 2004; Grove et al., 2005; Erkonen et al., 2011).

Epigenetic literature to date is largely skewed towards intragenerational phenomena, and future research would benefit from added emphasis on transgenerational experiments and integration between these two phenomena. In addition, molecular or cellular changes following environmental changes that arise through developmental phenotypic plasticity or epigenetic alterations may not be reflected in whole organismal phenotypic change, so care must be taken when arriving at definitive conclusions regarding effects of environmental perturbations. Lastly, clonal animals may reduce variation across treatments within an experiment (Andrewartha and Burggren, 2012; Verhoeven and Preite, 2013), and animals capable of regenerating from portions of their whole bodies (*e.g.* starfish, flatworms) may be animal models that can provide unique insights into transgenerational physiological inheritance.

Comparative developmental physiology will continue to provide insight into fundamental questions regarding the timing, duration and heritability of developmental periods that are critical to normal phenotypic expression.

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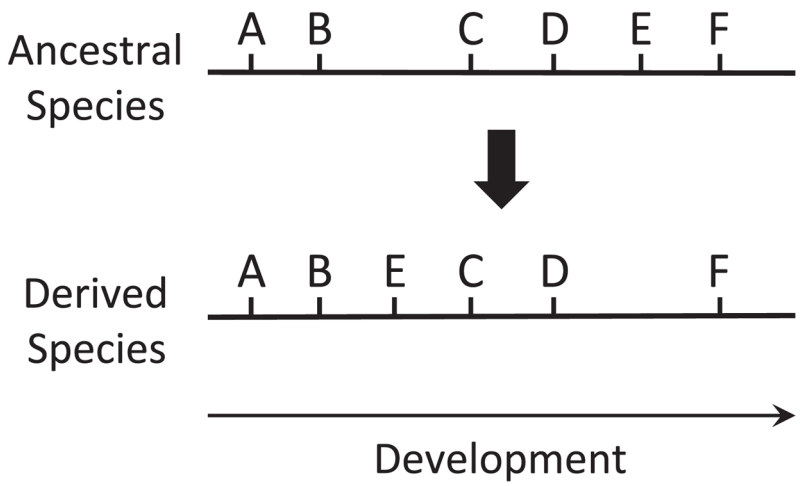

Fig. 1.

Sequence heterochrony. The timing of six developmental events in an ancestral taxon and a derived ancestor. Note that event E shows an evolutionary change in its timing, occurring earlier in the developmental sequence in the descendant compared with the ancestral species. Comparisons of such sequence data can allow the identification of potentially adaptive heterochronies.

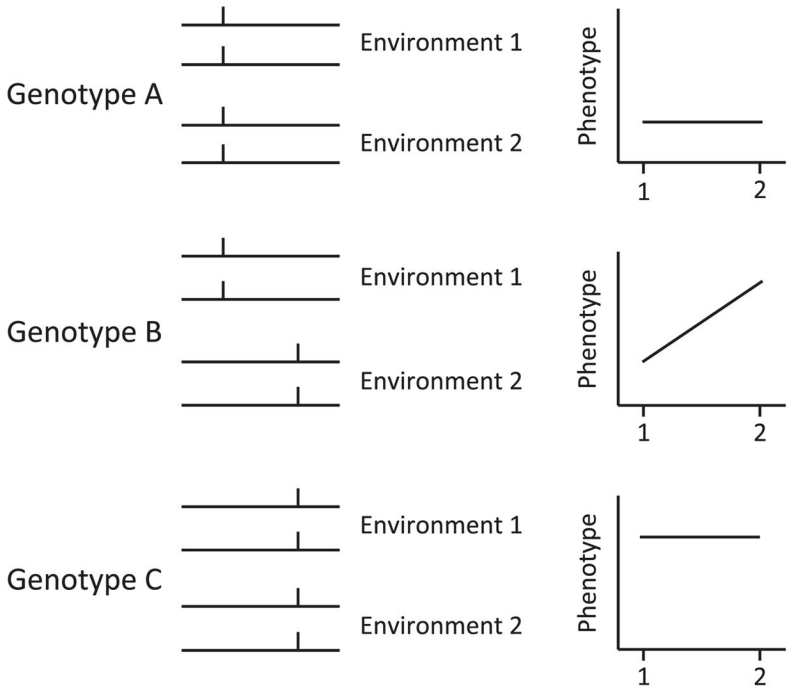

Fig. 2.

The potential link between heterochrony and heterokairy. The timing during development of the same event for two individuals from the same genotype in two environments. Genotype A shows an early timing but no plasticity of this event. Genotype B shows plasticity in the timing of this event. If this plasticity allows Genotype B to colonizes a new habitat (Environment 2), the new timing of this event could become fixed through genetic assimilation, leading to a loss of plasticity and potentially giving rise to a new genotype — Genotype C.

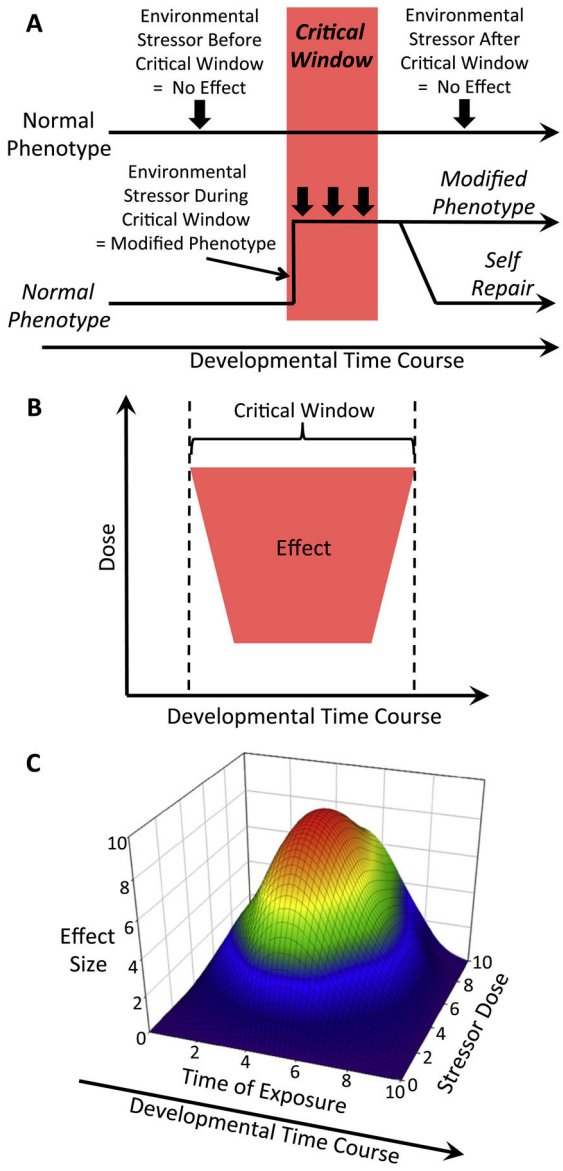

Fig. 3.

A) A ‘one-dimensional’ critical window, in which phenotypic modification caused by a stressor is dependent upon when during development the stressor exposure occurs. Most studies use just one dose of a stressor and a critical window is defined by a detectable change in the phenotype following exposure. In some instances, the animal may show self-repair capabilities following removal of the stressor either immediately or later in the development or life history of the animal. B) A ‘two-dimensional’ critical window, in which a phenotypic modification is dependent upon time of exposure and stressor dose. This diagram demonstrates how the detection of a critical window and determination of its size is likely dependent upon the dose of a stressor used. A very low dose may detect no effect, and therefore no critical window. Subsequent increases in the stressor dose may detect an effect, with the presence of the effect occurring slightly earlier and later in development as higher doses are able to induce an effect at the outer bounds of the critical window. C) A ‘three-

dimensional' critical window, in which the magnitude of the phenotypic change is dependent upon the time of exposure and the stressor dose. This three-dimensional critical window design compares how effect size (*e.g.*, phenotypic change) varies based on when exposure occurs and the dose of the stressor applied. In this instance, it is not just if an effect is present or absent that defines a critical window, but the extent of phenotypic modification within the window, and how it may vary, is also considered.

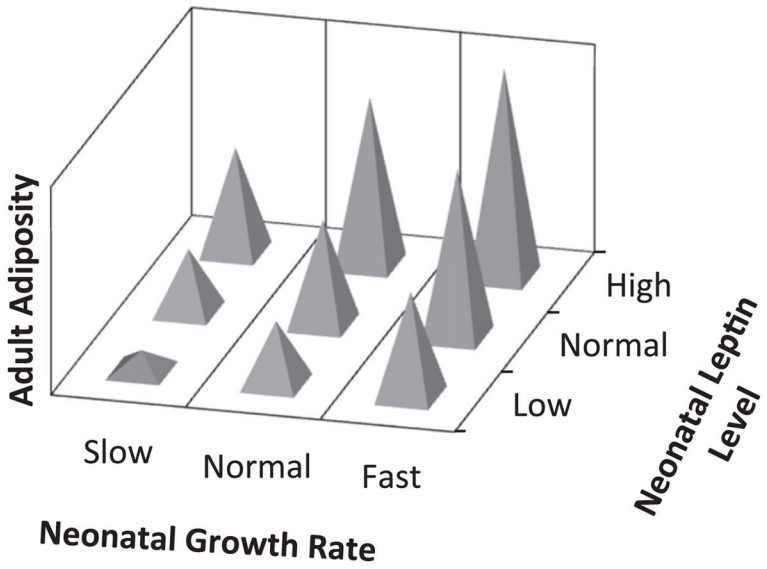

Fig. 4.

In rodents, the neonatal period is a critical window of hypothalamic development and adipose deposition. During this time, variations in growth rates and leptin levels interact and help establish the susceptibility to adult obesity.

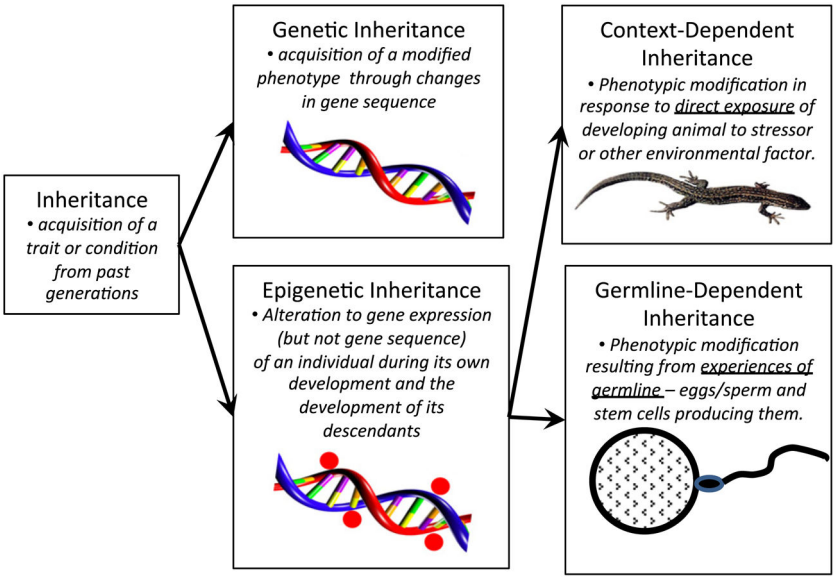

Fig. 5.

Phenotypes can be inherited through either genetic or epigenetic means. Epigenetic inheritance can be either context-dependent, where the developing animal is directly exposed to a stressor or germline-dependent, where the modification results from the experiences of the germline. (After Burggren and Crews, 2014).