Understanding 'Non-genetic' Inheritance: Insights from Molecular-Evolutionary Crosstalk

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Abstract

Understanding the evolutionary and ecological roles of 'non-genetic' inheritance is daunting due to the complexity and diversity of epigenetic mechanisms. We draw on precise insights from molecular structures and events to identify three general features of 'non-genetic' inheritance systems that are central to broader investigations: (i) they are functionally interdependent with, rather than separate from, DNA sequence; (ii) each of these mechanisms is not uniform but instead varies phylogenetically and operationally; and (iii) epigenetic elements are probabilistic, interactive regulatory factors and not deterministic 'epi-alleles' with defined genomic locations and effects.

We explain each feature and offer research recommendations. Finally, we consider existing evolutionary models for 'non-genetic' inheritance and present a new model that implements a unifying *inherited gene regulation* approach.

Inheritance beyond DNA poses key questions for evolution and ecology

Biologists are currently engaged in a lively conversation about whether it is necessary to expand our view of biological inheritance to include 'non-genetic' factors [1–3]. In particular, mechanisms such as DNA methylation, histone modifications, and small non-coding RNAs have been interpreted as additional 'streams' of phenotypic information distinct from DNA sequence transmission [4–8]. In comparison with DNA sequence variation, which is transmitted with great fidelity across numerous generations, these factors have complex and potentially unpredictable dynamics: they may arise stochastically or be induced by specific environmental conditions, and they may persist from one to several (or possibly many) generations (reviewed by [9–11] in plants and by [12,13] in animals; further references in [14,15]). Biologists in many fields are now confronting an unexpected question: Must we fundamentally revise our understanding of inheritance to incorporate these new insights? Here, a group of molecular and evolutionary biologists join forces to collectively clarify the empirical foundation for bringing these findings into evolutionary and ecological research.

For evolutionary biologists, the phenotypic impact of inherited 'non-genetic' factors and their potential contribution to evolutionary adaptation and diversification are pressing issues (note that below the term *non-genetic* is subjected to critique, hence the quotes). Although many empirical questions remain [16,17], mounting evidence indicates that transgenerational mechanisms may indeed substantially influence phenotypic outcomes in a wide range of organisms. In some cases, the inherited effects are negative. For instance, mammalian or insect parents with a nutrient-poor, high-fat, or high-sugar diet may transmit to offspring altered DNA methylation states or tRNA fragments that result in metabolic or developmental disorders [18–20]. Alternatively, stressful maternal or paternal conditions may induce specific, gametically transmitted changes that promote adaptive phenotypes in offspring encountering similar stresses. Such inherited but non-sequence based adaptations have been documented in a vast array of taxa (reviewed by [21–24]), although precise molecular mechanisms for these transgenerational effects have been ascertained in relatively few cases. In one study of mammalian behavior, male mice that were experimentally conditioned to have a fear response to a specific odor produced sperm in which the relevant odor receptor was hypomethylated, such that their progeny showed altered neuroanatomy and expressed the appropriate fear response to the threat stimulus [25]. Targeted defensive responses are also initiated by juvenile plants in many taxa, as a result of inherited modifications induced in parents attacked by herbivores or pathogens [26]. In *Mimulus*, for instance, simulated insect damage to the leaves of parent plants resulted in substantial down-regulation of a transcription factor in their progeny, leading to altered expression of over 900 genes [27] and increased production of defensive leaf trichomes [28]. Although the paternal transmission mechanism of this inherited effect is yet undetermined, the maternal component is transmitted by changes in DNA methylation [29]. Environmentally-induced methylation changes are also implicated in the production of drought-tolerant and shade-adaptive phenotypes by offspring of correspondingly stressed parental *Polygonum* plants [30,31]; such induced methylation changes appear to be widespread in plants [3,11].

Because parentally-induced effects could cause specific adaptive adjustments in many individuals (and their descendants) in a population after only one generation, they are of particular interest to ecologists and evolutionary biologists as a potential source of tolerance to climate change and other novel, rapidly unfolding challenges [32,33]. A common reef fish provides an intriguing recent example: Ryu and colleagues [34] showed that juvenile *Acanthochromis* were able to acclimate successfully to elevated water temperatures simulating future marine conditions (+3°C), as a result of inherited methylation changes to numerous genes involved in oxygen consumption and metabolism that were induced in parents exposed to elevated temperatures. A similar transgenerational effect was found in *Acanthochromis* in response to a second dimension of global change – ocean acidification. Expression of numerous genes was altered in individual offspring exposed to elevated CO_2, but only if parent fish had not previously been exposed to high CO_2, and some epigenetic regulator genes exhibited divergence due to parental phenotype [35].

In addition to phenotypic effects on individual organisms, 'non-genetic' factors may play important roles in adaptive evolution. Either induced or spontaneous epigenetic variants may contribute to heritable variation in natural populations [36–38], potentially providing additional evolutionary potential [6]. If sufficiently plentiful and stable, such heterogeneous epigenetic states could provide an alternative substrate of standing variation to fuel

natural selection for local population divergence [39–41]. Although previously rare, studies of epigenetic variation in wild populations have recently become more common (e.g. [42]; further examples listed in [43–47]). Such variation has been shown to play a role in several aspects of population dynamics relevant to local differentiation (e.g., invasion potential [48,49], migration propensity [50], developmental morph determination [51,52], and host-parasite interactions [53]). In some cases, population comparisons across environmental or climatic gradients have demonstrated a role for epigenetic modifications in local adaptation [38,54,55]. However, it is challenging for field population surveys to determine whether epigenetic variation has been directly induced by environmental conditions, inherited from an induced ancestor (transgenerational epigenetic inheritance), or derived from locally selected and stable epigenetic variants (often termed 'epialleles' or 'epimutations') (e.g. [39]).

Importantly, even if epigenetic factors are transient relative to DNA sequence variants (i.e. inherited for only one to several generations), they may substantially change selection gradients and hence have far-reaching evolutionary impact [5,56,57]. Theoretical models show that the inheritance of 'non-genetic' factors affects the speed of evolution and the precision of adaptation as well as the genetic parameters (such as heritability) which are used for making evolutionary inferences (discussion and further references in [15,58]).

Toward a more precise view of 'non-genetic' inheritance

Although much progress has been made in understanding the ecological and evolutionary role of 'non-genetic' inheritance, a sound understanding is hampered by three commonly made simplifications regarding the nature and function of these molecular systems. First, the very term 'non-genetic' is inaccurate: inherited epigenetic factors and DNA sequence are not distinct but functionally interdependent, which is why epigenetic effects on offspring are often found to vary by genotype [31,37,59,60]. Second, processes such as methylation are not singular, uniform mechanisms, but rather, operate in a multiplicity of ways depending on both organism and mode of induction (e.g. [61]). Third, epigenetic variants are often conceptualized as (short-lived) 'epialleles' with well-defined genomic locations and effects; in contrast, these molecular factors are highly non-deterministic, and their impact reflects complex regulatory interactions across parallel pathways.

In the following sections, we elaborate these general features in detail, explaining how each feature emerges from inherent properties of the underlying molecular structures and biochemical events. By translating a fine-grained literature on molecular mechanisms into broader and more generally applicable properties, we aim to promote the effective integration of these inheritance mechanisms into evolutionary and ecological studies; for each of the three features, we also indicate how it points to targeted experimental approaches and new avenues of investigation. We provide a companion table summarising existing terms and their implications; a theory box briefly examines existing evolutionary models for 'non-genetic' inheritance and presents a broad-based model that avoids problematic assumptions. Readers will require only a broad familiarity with molecular mechanisms such as DNA methylation, histone modifications, and regulatory RNAs (for further detail see excellent recent reviews by [11,62–64].

To clarify the key shared features of the diverse molecular systems that underlie epigenetic or 'non-genetic' inheritance, we introduce the unifying concept of *inherited gene regulation*. Instead of reiterating mechanistic idiosyncracies of epigenetic or 'non-genetic' modes of inheritance, the collective functional term *IGR* focuses on the common effect of these transgenerational systems: they alter genome activity and hence gene expression in progeny. *IGR* denotes *regulatory aspects of inheritance that are not mediated by primary DNA sequence* **(Figure 1)**. While this term includes genome-associated mechanisms such as DNA methylation, histone modification and regulatory RNAs, it also encompasses both nuclear and cytoplasmic cellular components and is not limited to DNA-bound factors (**Box 1**). The term *IGR* shifts the focus away from heritable factors that are functionally neutral (for example, DNA methylation marks that do not affect gene expression), and instead emphasizes variants that alter gene expression and hence may be of ecological and evolutionary relevance.

Figure 1

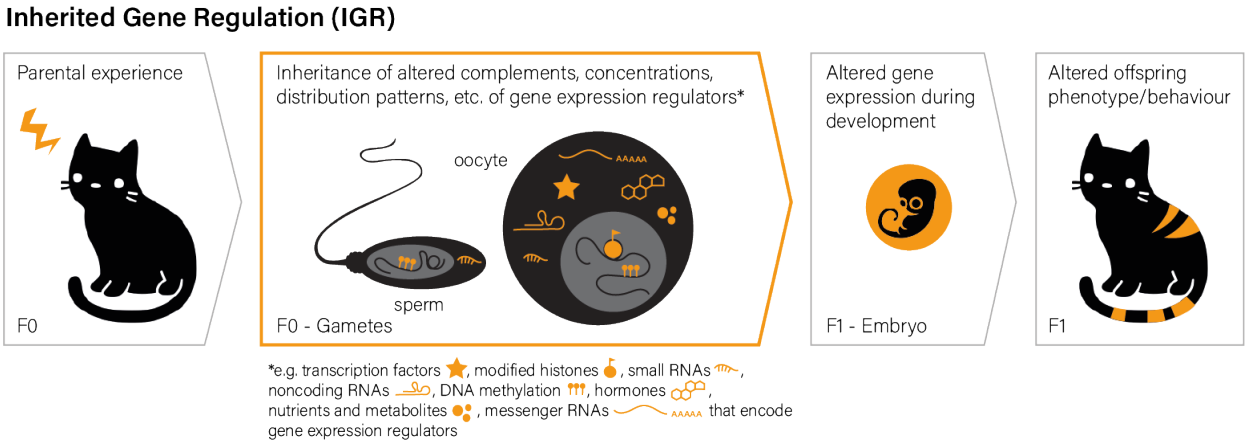

Figure 1. Inherited Gene Regulation.
The concept of Inherited Gene Regulation (IGR) encompasses all inherited factors that modify gene expression in offspring. This includes a wide range of molecular pathways, molecule types, and cellular compartments.

Box 1. Inherited Gene Regulation (IGR): a unifying concept

The diverse molecular mechanisms commonly referred to as 'epigenetic' or, more broadly, 'non-genetic' or extra-genetic inheritance (including DNA methylation, histone modification and regulatory RNAs) all share a common effect: they alter specific aspects of genome activity and thereby regulate progeny gene expression. For example, the addition of methyl groups to DNA typically suppresses transcriptional activity in plants and mammals, while histone modifications can be linked to gene activation as well as repression. Both mechanisms can act by regulating accessibility of DNA to transcriptional activators (reviewed by [65] and [66]) or by altering other aspects of gene expression such as transcript stability, nuclear export or translation efficiency. Small RNAs, for example, may induce the degradation or silencing of transcripts [67], and/or cause gene silencing by recruiting modifying enzymes [68] (reviewed by [69]).

Regulatory aspects of inheritance that are not mediated by primary DNA sequence can be collectively understood as **inherited gene regulation (*IGR*; Figure 1).** Adopting this unifying term offers several advantages. While 'epigenetic,' 'non-genetic,' and 'extra-genetic' bear a multiplicity of meanings in the current literature (**Table 1**), *IGR* is an inclusive term that focuses attention on the outcome of a diverse array of underlying molecular mechanisms. This conceptual framework encompasses the genome-associated molecular mechanisms often denoted as 'epigenetic' along with other germ-cell mediated modes of inheritance. Also, an *IGR* perspective does not discriminate between nuclear and cytoplasmic cellular compartments, and is not limited to DNA-bound factors. Accordingly, *IGR* also includes the maternal RNA contribution that controls a major portion of early development in many non-mammalian animals [70], since many maternal RNAs encode master gene expression regulators. *IGR* can also include maternal provisioning to eggs and seeds, as hormones and nutrients are known to be potent gene expression regulators [71]. *IGR* may even include other aspects of 'non-genetic' or 'inclusive' inheritance, such as parental care behaviors or ecological conditions created by parental habitat modification, if these influence gene expression in offspring ([72,73]; **Figure 1**). *IGR* also accommodates the closely related issue of 'transgenerational plasticity' - inherited effects of parental environment on phenotypic expression in progeny [23,32,74,74] – and connects to the elegant concept of "inheritance of the gene interpretation machinery" [5], which likewise unifies a variety of inheritance modalities in terms of their regulatory impact. Depending on the research context, specific modes of *IGR* can be distinguished using prefixes (e.g. sperm-mediated *IGR*, gamete-mediated *IGR*, DNAme-mediated *IGR*, hormone-mediated *IGR*, metabolite-mediated *IGR*, or RNA-mediated *IGR*). This allows for descriptive precision while maintaining a conceptual unity which points to broader implications.

Term	Epigenetics		Parental Effects	Epigenetic Inheritance		Lamarckian Inheritance	Transgenerational Plasticity	Epiallele	Non-genetic Inheritance	Extra-genetic Inheritance	Soft Inheritance	Inclusive Inheritance	Expanded Inheritance
# of hits in WoS[1]	97190		4884	2072		651	245	313	176	7	28	9	1
Dominating fields (>10% of hits); field associations are not mutually exclusive.	Biochem. & Mol. Biol (16800) Oncology (15748) Genet. & Hered. (13813) Cell Biol.(12977)		Ecology (1359) Evol. Biology (805) Genet. & Hered. (781) Agriculture (768) Zoology (512)	Genet. & Hered. (534) Biochem & Mol. Biol. (521) Cell Biol. (381) Multidisc.Sciences (221)		Computer Sci.& Artificial Intell. (116) Computer Sci. Theory Methods (65)	Ecology (112) Evol. Biol. (67) Plant Sciences (50) Genet. & Hered. (30) Biology (25)	Genet. & Hered. (139) Bioch. & Mol. Biol. (79) Plant Sciences (54) Multidisc. Sciences (37)	Ecology (50) Evol. Biol. (42) Genet. & Hered. (36) Biology (21) Bioch. & Mol. Biol. (19)	Evol. Biol. (4) Ecology (2) Genet. & Hered. (2) Behav. Sci. (1)	Biology (8) Evol. Biol. (8) Ecology (7) Genet. & Hered. (6) History & Phil. of Sci. (4)	Biology (2) Ecology (2) Genet. & Hered. (2) Evol. Biol. (1)	Ecology (1) Evol. Biol. (1)
Information carrier	Modified histones, proteins, RNAs, covalent DNA modifications (e.g. methylation)	Any physiological mechanism affecting phenotype except changes in DNA sequence	Molecules and behaviours	Modified histones, proteins, RNAs, covalent DNA modifications (e.g. methylation)	Any physiological mechanism affecting phenotype except changes in DNA sequence	Not defined	Not defined	DNA methylation	Not defined	Not defined	Not defined	Not defined	Not defined
Physical location of information	Mature gamete	Not defined	Not defined	Mature gamete	Not defined	Not defined	Not defined	Nucleus of the mature gamete	Not defined	Not defined	Not defined	Not defined	Not defined
Sensitive period	Gametogenesis	Throughout life	Throughout life	Gametogenesis	Throughout life	Throughout life	Throughout life	Gametogenesis	Throughout life	Throughout life	Throughout life	Throughout life	Throughout life
Immediate consequence in offspring	Altered gene expression	Inheritance of phenotype	Phenotypic variation	Altered gene expression	Inheritance of phenotype	Inheritance of phenotype	Phenotypic variation	Phenotypic variation	Inheritance of phenotype	Inheritance of phenotype	Inheritance of phenotype	Inheritance of phenotype	Inheritance of phenotype
Challenges and current questions associated with the term	**Mechanism:** Lack of mechanistic clarity, particularly as to which information carriers qualify (e.g. [75] vs.[76]). **Scope:** Information transfer after meiosis or fertilization (episomes, placenta) may or may not qualify. **Disparity:** Dichotomous use between molecular and evolutionary fields, roughly characterized by a mechanism-focused vs outcome-focused approach, see also "epigenetic inheritance" **Historical shifts:** Waddington's original conception [77] is different to more modern interpretations [78]		**Mechanism:** Outcome-focused term that is non-specific with regards to mechanism (e.g. [79–81]. **Prevalence:** Relatively uncommon in molecular fields.	**Mechanism:** Lack of mechanistic clarity (see "epigenetic") means that it is difficult to categorize potential examples [82]. **Disparity:** Dichotomous use in mechanism-focused fields (biochemistry, genetics and molecular biology; e.g. [83]) versus outcome-focused fields (whole-organism, ecology, and evolutionary biology; e.g. [84]).		**Mechanism:** Lack of mechanistic clarity may cause mis-categorization [85]. **Connotations:** May carry associations with anti-Darwinian evolution in some contexts [86]. **Historical shifts:** Used in its historical meaning and context in some fields alongside more modern usage [87]. **Prevalence:** Relatively uncommon in biological fields.	**Mechanism:** Non-specific with regard to mechanism [88]. **Scope:** What qualifies as "trans"-generational? e.g. F1 vs F2/3 generations ([89] vs. [90]), trans- vs. intergenerational plasticity ([91] vs. [92])	**Connotations:** May suggest a genetic-like nature and therefore be misleading: while epialleles do exist, they appear to be rare [11] and their contributions to phenotypic variance are likely to be complex [93].	**Mechanism:** Non-specific about mechanisms [90,94] **Connotations:** 'Non-genetic' may imply a lack of role of genes, which is not reflective of the potential interplay between inheritance systems [95] **Prevalence:** Not (currently) in common usage.	**Mechanism:** Non-specific about mechanisms [90,94] **Connotations:** 'Non-genetic' may imply a lack of role of genes, which is not reflective of the potential interplay between inheritance systems [95] **Prevalence:** Not (currently) in common usage.	**Mechanism:** Non-specific about mechanisms [95] **Scope:** "Diagnosis by exclusion": catch-all term [96] for anything but 'hard' (DNA-mediated) inheritance. **Connotations:** Creates a dichotomy between DNA-based and other inheritance mechanisms which is not reflective of the potential interplay between inheritance systems [95]. **Prevalence:** Not (currently) in common usage.	**Mechanism:** Non-specific about mechanisms [97] **Prevalence:** Not (currently) in common usage.	**Mechanism:** Non-specific about mechanisms [97] **Prevalence:** Not (currently) in common usage.
[1]Search terms in WoS core collection, search field: topic, linked with "or", on 5th January 2020	*"epigenetic", "epigenetics", "epigenetically"*		*"parental effects", "maternal effects", "paternal effects"*	*"epigenetic inheritance", "epigenetically inherited"*		*"Lamarckian inheritance", "Lamarckian"*	*"transgenerational plasticity", "trans-generational plasticity", "intergenerational plasticity", "inter-generational plasticity"*	*"epiallele", "epialleles", "epiallelic"*	*"non-genetic inheritance", "nongenetic inheritance", "non-genetically inherited", "nongenetically inherited"*	*"extra-genetic inheritance", "extragenetic inheritance", "extra-genetically inherited", "extragenetically inherited"*	*"soft inheritance"*	*"inclusive inheritance", "inclusively inherited"*	*"expanded inheritance"*

Table 1. Terminology. *The currently used terminology differs between fields, has differing implications, and is associated with specific challenges related to scope and specificity.*

Feature One: 'Non-genetic' and genetic aspects of inheritance are inseparably intertwined

'Non-genetic' and genetic aspects of inheritance are often viewed as separate streams of information. Accordingly, current statistical and modelling approaches often rely on the (linear) decomposition into 'genetic' and 'non-genetic' effects and on the relative quantification of these effects (see **Box 2**). Yet gene-sequence variation and heritable variation in the regulation of gene sequences are intertwined in an intricate manner, making such a decomposition highly problematic.

DNA sequence invariably plays a role in *IGR* for a number of reasons (summarized in **Figure 2A**). For instance, DNA methylation marks are set, recognized, maintained, and erased by methyltransferases and other proteins that are encoded in the genome. Accordingly, allelic variants of genes encoding these proteins can potentially affect epigenetic induction and reversal dynamics [59]. Biochemical comparisons of DNA methyltransferase isoforms and of mutants in epigenetic modifiers suggest that minor sequence differences can have significant effects on where, when, and how an epigenetic modifier acts [98,99]. The fact that experimental strains or inbred lines differ with regard to effects of parental conditions on offspring phenotypes (e.g. 100–103) is also consistent with the view that genetic variation may influence *IGR*.

In addition, DNA sequence features of the loci targeted by *IGR* mechanisms may influence regulatory dynamics. Whether or not a DNA methylation mark can be set depends, for example, on the presence of CpG dinucleotides. In mice and humans, the majority of CpG dinucleotides in intergenic regions are methylated by default, independently of environmental conditions or transcriptional status; methylation of these CpGs is thus largely genetically determined. Phenotypically relevant conditional and regulatory methylation mostly occurs in specific small areas of the genome, for example in promoters. DNA sequence at target sites also plays a role for other mechanisms implicated in *IGR*. For example, histone modifying enzymes are frequently recruited to genes by transcription factors that are in turn recruited by specific DNA sequence motifs in promoters or enhancers [104].

Finally, *IGR* dependency on DNA sequence arises because *IGR* mechanisms integrate information contained in the sequence context surrounding a targeted locus [105]. In mice and *Arabidopsis*, DNA methylation marks contribute to gene silencing when embedded in a CpG-dense promoter, while DNA methylation marks embedded in coding sequences are associated with the timing of transcription initiation events, and DNA methylation marks in intergenic regions have little impact on genome activity.

In parallel to these three types of sequence dependencies, molecular mechanisms underlying *IGR* feature partial sequence independence through regulatory and 'read-write' mechanisms. The activity of the proteins setting, maintaining, reading and erasing epigenetic marks depends on post-transcriptional regulation rather than on sequence. For example, DNA methylation erasure in mammalian germ cells is specifically prevented in certain nuclear compartments and genomic regions [106], and histone methylation marks are removed in *C. elegans* germ cells only upon a specific cell-cell signal and only at specific genes [107]. In addition, many IGR mechanisms encompass read-write modules that recognize a modification and reiterate or amplify it. Such copy-write mechanisms allow epigenetic marks to spread in cis on chromosomes [108], to be copied to the new DNA strand during cell division [109], and to self-perpetuate over time. Examples include small RNA production in plants [110], C. elegans, and fission yeast [111,112], the transfer of DNA methylation marks to the newly synthesized DNA strand [113], or the maintenance of local histone methylation patterns [114–116]. Read-write modules mediate the prolonged inheritance of environmental signals [111,117] and account for pathway-specific fidelity properties [118] of *IGR* mechanisms.

In summary, modifications such as DNA methylation do not arise deterministically from genome sequence, nor are they sequence-independent. Rather, they operate on a continuum from completely sequence-independent, to partially regulated, to entirely sequence-determined. The location on this continuum is not fixed for any one mechanism: genetic versus regulatory aspects may gain or lose relative importance depending on the genomic location, on the developmental timepoint, on the tissue, or on environmental conditions.

Empirically disentangling the contribution of individual genetic or regulatory aspects to a particular (inherited) phenotype is challenging, and often fails even for single loci in controlled conditions in isogenic strains or cell culture within single generations. One complicating factor is that *IGR* mechanisms act pleiotropically (e.g. on many loci simultaneously) and in a cascading fashion. In other words, a point mutation in one gene can

Figure 2

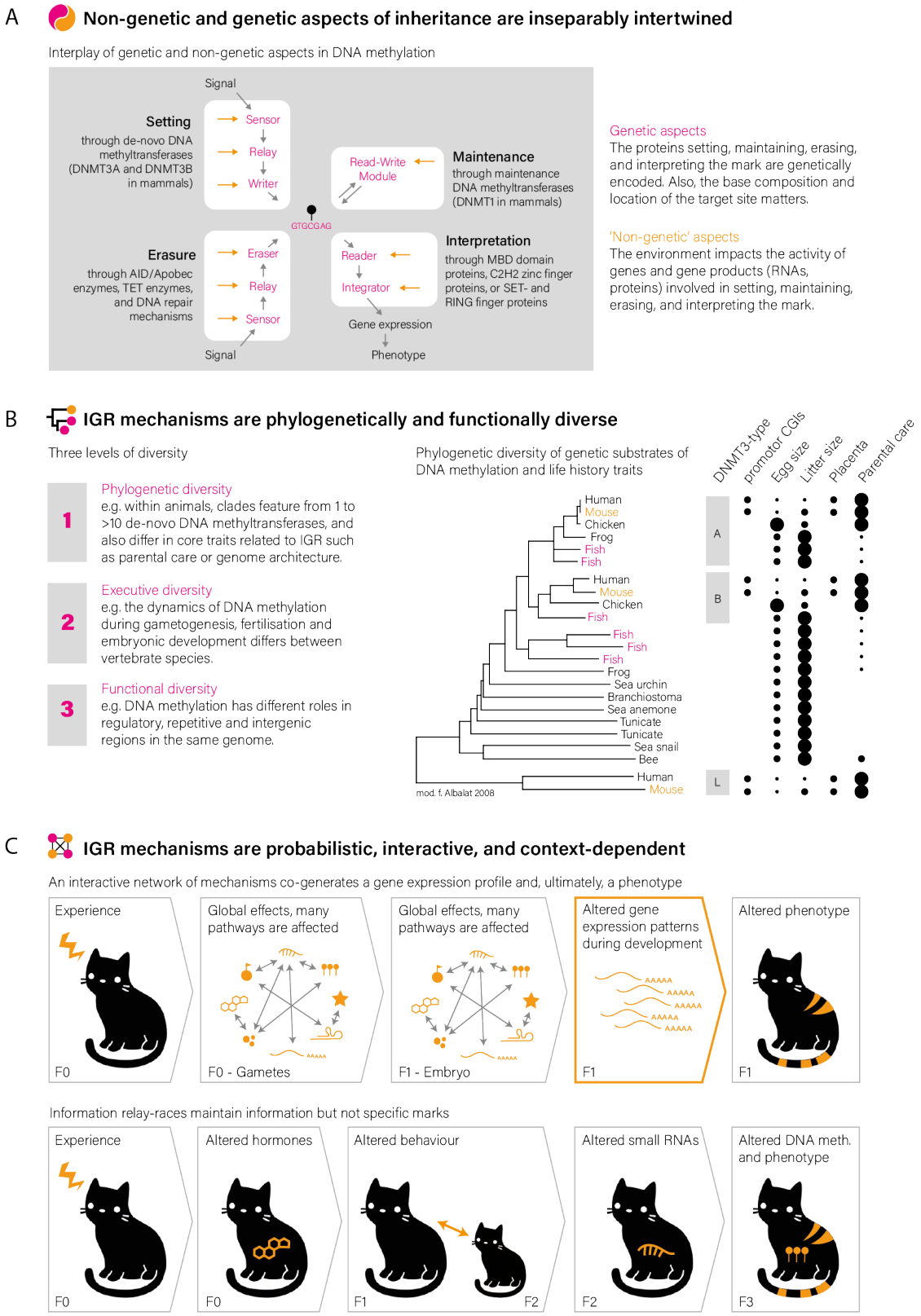

Figure 2. Fundamental features of IGR.
A. In IGR, non-genetic and genetic aspects are inseparably intertwined. *The pathways setting, maintaining, erasing, and interpreting a particular mark receive input from genetic and from regulatory sources. This is*

exemplified in the figure on the example of DNA methylation, the concept however applies equally to other mechanisms. ***B****.* ***IGR mechanisms are phylogenetically and functionally diverse.*** *The diversity stems from the phylogenetic level (different species feature different gene numbers and types, and different life history traits related to reproduction and inheritance), from the level of dynamics (the same pathway may display different dynamics across fertilisation in two species), and from the level of function (the same mark may have different functions in the same species, depending on the genomic context). The aspect of phylogenetic diversity is further exemplified using a phylogenetic tree of de-novo DNA methyltransferase genes, modified from [194]. Two of the best-studied animal models, mouse and human, feature DNMT3L genes which are absent from non-mammals. Similarly, de-novo methyltransferases from many animals cannot be assigned to any of the two 'canonical' types A and B. Core traits related to reproduction are equally variable across phylogenetic scales* ***C. IGR mechanisms are probabilistic, interactive, and context dependent.*** *In IGR, molecular mechanisms collaborate in creating a gene-regulatory landscape that culminates in a gene expression pattern and a phenotype. Also, regulatory information can be preserved across time and across generations in the absence of stable mark inheritance through re-coding. For a legend of symbols, see Figure 1.*

directly or indirectly entail genome-wide changes in, for example, the DNA methylation landscape [119]. In addition, *IGR* factors may persist for multiple generations [111] and thus produce complex, multi-generation genotype x environment interaction effects [120]. Non-penetrant, difficult-to-attribute and variable phenotypes with evident co-regulation by genetic and gene regulatory mechanisms are therefore rarely followed up by investigators, even in isogenic strains or cell culture.

The intricate interplay of genetic and regulatory aspects in *IGR* poses considerable conceptual and experimental challenges. To face these challenges, we give three recommendations. First, assume from the start that genetic and non-genetic factors both contribute to any inherited phenomenon, and only drop this assumption in case of unequivocal and complete evidence to the contrary. Genetic variation may impact a phenotype through its influence on the genes involved in mediating *IGR*, and these genes act pleiotropically. Therefore, a role for genetic variation in any inherited phenotype can only be excluded if the whole genome sequences of the investigated generation as well as the previous generation(s) are known to the last base, and/or if candidate sequence differences are followed up functionally (for example by crosses or transgenics). Similarly, a quantification of non-genetic aspects of an inherited phenotype requires controlled transplantation experiments. Whenever genome sequence cannot be completely determined or controlled in multiple generations, when functional tests to explore the impact of sequence differences are lacking, or when transplantation experiments are not feasible, caution is warranted regarding statements on relative contributions of genetic inheritance versus *IGR.* In some contexts, attempts to disentangle genetic and regulatory factors across genomes and generations may somewhat be missing the point. Investigations of functional and fitness outcomes may not need to try to distinguish these two elements of gene expression regulation. Instead, efforts could be made to develop methodology that integrates both levels and treats them as one, in a systems biology approach (see also **Box 2**).

Second, treat the genome as a non-uniform, multifaceted, multifunctional and differentiated landscape, and focus attempts to disentangle genetic from regulatory impacts on functionally meaningful genome regions. Analyses could focus on regions that have previously been shown to influence gene expression in the investigated species (for example, CpG-rich promotors in mammals), or could separately analyze marks in exons, introns, promotors, enhancers, repetitive elements, and intergenic regions. An excellent example is a recent effort in stickleback [121]. Such approaches require a well-annotated genome; accordingly, empirical approaches should always, as a first step, aim to generate a reasonable genome annotation. If genome annotation and functional data are unavailable and cannot be generated, an alternate first step could be to monitor the response of the modification of interest across a panel of different tissues and developmental stages to single out regions with inherently dynamic responses (for example using gene expression analyses by e.g. RNA sequencing or assays probing for DNA accessibility, e.g. ATAC sequencing [122]), since these regions are more likely to be functionally relevant in generating phenotypes.

Third, consider exploring the role of genetic variation in *IGR* in your experimental model. Strain-specific capacities for *IGR* and the effects of natural (rather than experimentally generated) genetic variation in key molecular pathways of *IGR* are poorly understood. The field would vastly benefit from an in-depth exploration of the interplay between genetic variation and the strength and form of *IGR*.

Feature Two: *IGR* mechanisms are phylogenetically and functionally diverse

The mechanisms underlying IGR are evolutionarily ancient [123], and it is tempting to treat them as if they were as universal as the principles underlying DNA-based inheritance. However, this may be misleading, as non-genetic inheritance mechanisms are highly diverse. For instance, both models and experiments often assume that DNA methylation is a singular mechanism with a uniform mode of action across the genome and in different taxa. Yet although DNA methylation plays a major role in gene repression in mammals and in plants, in the fruit fly genome this mark is absent and instead other repressive mechanisms are involved in IGR [124].

Diversity in *IGR* mechanisms is relevant at three levels (**Figure 2B**). Firstly, although certain features may be broadly conserved, there is substantial phylogenetic diversification in the molecular machinery setting, maintaining, reading, and erasing epigenetic marks. The scope of this sequence-based diversity is emerging as non-model genomes become increasingly available, and the associated functional diversity is starting to

become apparent with the incorporation of species beyond mice and humans into functional assays. Examples for diversification include DNA methyltransferases (DNMTs) and histone-modifying mechanisms. The number and types of DNMTs are highly variable between species, even among vertebrates. Mammalian genomes feature 3 de-novo DNMT genes, while teleost fishes contain between 5 and 12 genes [125–127]. Regarding maintenance DNMTs, copepods feature three genes while placental mammalian genomes contain one gene [128], and marsupials have two genes [113]. Budding and fission yeast lack DNMTs altogether [129], whereas the pathogenic fungus *Cryptococcus neoformans* contains only a maintenance, but not a *de novo* methyltransferase [130]. In the context of histones, histone modifiers feature species-specific insertions which may affect how they are targeted to certain sites in the genome [131], and species differ in their complements of protamine genes [132], histone variant genes [133], and histone mark reader proteins [134]. Importantly, assumptions on differences between taxa to this day generalize from single species which are chosen to represent entire taxa, with potentially misleading conclusions. For example, the best-studied yeast is *S. cerevisiae,* which happens to not feature RNAi [135].

Secondly, even if two species groups feature roughly similar gene complements, individual gene function may differ between the groups. Taxa, species or strains also display diversity in the temporal and spatial dynamics of epigenetic modifications in germ cells and embryos. For instance, certain highly conserved histone modifiers (the catalytic units of the polycomb repressive complex PRC2) are essential for embryonic development in mammals but not in fish [136]. Modified histones are retained in human sperm to a much greater extent than in mouse sperm [137]. Data from mice initially suggested very thorough reprogramming of regulatory information in mammalian or even vertebrate germ cell development and fertilization, but this may be specific to mice and/or certain regions in the genome [138]. In zebrafish, paternal DNA methylation is not erased during germ cell development and is transferred to the embryo [139,140], but this may not be universal within fish [141,142]. Plants are known to retain DNA methylation patterns across several generations [118], while gene regulation in fruit flies is largely independent of DNA methylation [143].

Thirdly, the same mechanism may serve different purposes in different species, have high relevance in one species yet little significance in another, or may have different functions in different regions of the genome. For example, zebrafish accumulate DNA methylation within actively expressed genes rather than at silenced gene promotors as in humans and mice, and fish genomes do not contain CpG islands in promotors as in mammals [144,145]. DNA methylation upstream of a mammalian gene may inhibit gene expression, while DNA methylation within an actively transcribed gene may regulate the choice of transcription initiation site [146]. Moreover, taxa differ widely in their reliance on inherited maternally-transmitted molecules including RNA. While mice activate the zygotic genome almost immediately after fertilization, the initial post-zygotic development in zebrafish, *Drosophila*, and sea urchins occurs in the absence of zygotic transcription and is powerfully shaped by maternal RNAs [147–150], with effects of maternal RNAs potentially lasting up to late morphogenesis stages [151].

In summary – and in stark contrast to the universal code of DNA sequence inheritance – 'non-genetic' inheritance is a language with many dialects; results obtained from model systems may not be universally applicable due to the functional and phylogenetic diversity of IGR mechanisms. From this, we derive several recommendations.

First, generalizations from distantly-related species should ideally be replaced by known properties of the species (or at least the clade) of interest. This may involve finding specific anwers to the following questions: What is the species' complement of genes involved in the mechanism of interest - does the species feature RNA silencing pathways, how many DNA (de)methylases are there? Does the genome feature CpG islands in promotor regions? Is a particular molecular mechanism at all related to gene expression in the particular species? Do data suggest how relevant a particular mechanism is - are histones retained in sperm, are hormones deposited in the oocyte? What are relevant life history parameters linked to reproduction? For example, hormones and nutrients are a major component of bird, fish, and amphibian eggs, but are less prominent components of mammalian eggs (**Figure 2B**). Similar rationales apply to species-specific roles of maternally inherited RNA.

Second, bring a phylogenetic perspective to the choice of methods. For example, in a species featuring 50 Argonaute genes, such as *C. elegans*, the likelihood for a role of small RNAs in *IGR* is quite high, and targeting small RNAs experimentally may be promising, and RNA isolation protocols should be fine-tuned for small RNA species. In a species with major maternal RNA dependence,

RNA sequencing approaches of embryos need to acknowledge differences between maternal and zygotic RNAs with regard to polyadenylation status - the choice of random primers vs poly-T primers will affect the outcome of the experiment. In species that lack CpG islands, don't choose affinity-based techniques to measure DNA methylation, such as MeDIP or MBD capture, because they do not perform as well on loosely interspersed DNA methylation compared to CpG islands [152–154].

Consider generating the abovementioned data as a first step of the project if these specifics are not known for the species of interest. The limited knowledge on the differential evolution of *IGR* mechanisms and capacities across taxa may represent an obstacle for certin experimental approaches, but is also a wonderful research opportunity.

Third, utilize knowledge of a species' evolutionary and ecological history in experimental designs. As with any study of environmental effects, parental exposure should be ecologically relevant for the species of interest. In rodents, food scarcity, chronic predation stress, or overcrowding may trigger transgenerational responses, while parental obesity may not. The species should also have recently and/or historically evolved in an environment where cross-generational information matters [155,156]. A medium-lifespan species from a temperate climate with little brood care may display more germ-cell mediated *IGR* than a long- or very short-lived species, or an inbred laboratory species that has experienced invariant conditions for many generations. These considerations may seem obvious, and are much discussed in the evolutionary biology literature, but receive comparably little attention in research on molecular mechanisms.

Feature Three: *IGR* mechanisms are probabilistic, interactive, and context-dependent

The literature on 'non-genetic' inheritance often focuses on cases where a deterministic link exists between a bimodal pattern of epigenetic modification and a bimodal phenotype [157]. Such robustly heritable and deterministic scenarios, as *Kit* in mice [158] or flower phenotype in toadflax [159], certainly exist and are useful research models. In mammalian imprinting, the parent-of-origin predicts the expression state of some genes through DNA methylation [160]. However, these scenarios represent one far end of a spectrum, and may, in fact, be quite rare [161]. More often than not, the link between mechanism and outcome (such as between DNA methylation and a phenotype) is probabilistic, facultative and context-dependent rather than deterministic, universal, and linear.

In line with this concept, *IGR* mechanisms usually integrate information across larger genomic regions. The overall DNA methylation state of a CpG-rich mammalian promoter (for example, 'largely methylated') is important for gene activity, but not the state of individual cytosines: similar cells maintain similar DNA methylation profiles overall, but at slightly different nucleotide positions (See e.g.162, Fig. 3). It is tempting to treat individual occurrences of marks as independent bits of information, much like nucleotide polymorphisms (i.e. as 'epialleles'), but analyzing 'epimutations' rather than the overall state of a genome region might misleadingly detect statistically significant but functionally irrelevant differences between cells, tissues, or individuals.

The facultative role of IGR mechanisms is illustrated by some less widely known aspects of DNA methylation. For example, the widely accepted notion that DNA methylation in promotors silences genes in mammals applies only to a fraction of genes [163,164] – many genes with methylated promotors are expressed. Also, mammalian promotors are often depicted as either high or low in DNA methylation, but actually many promotors exhibit intermediate levels of methylation [162].

In addition, mechanisms and marks involved in *IGR* tend to co-occur, work in concert and influence each other to create a "chromatin landscape" and, ultimately, a gene expression profile that represents the integrated information of several mechanisms (**Figure 2C**; 165–167). Generally, DNA methylation patterns are highly correlated with histone modifications [168]; for example, DNA methylation at the *agouti* locus is accompanied by distinct histone modification patterns [169]. Transcription factor binding impacts the DNA methylation landscape [170,171], and major cross-lab research initiatives such as ENCODE [172] have been working since 2003 to understand which marks occur together, and what impact certain combinations of marks have on DNA accessibility and transcription [173]. These and other data reveal that the effect of a given mechanism is often highly context-dependent. For example, so-called "repressive" and "activating" histone marks co-occur on the very same nucleosome to create "bivalent domains" which are poised for expression but inactive [174].

Finally, *IGR* is often viewed as unstable because the associated mechanisms display erasure events – for example around fertilization – leading to broader questions with regard to heritability and long-term significance. However, the absence of a certain modification should not be equated to an absence of information. *IGR* mechanisms have cascading effects and recruit each other, resulting in 'relay races' of marks and information, where information is maintained but handed over from one mechanism to the next. Experimentally, such relay races are challenging to measure, since they require assessments of several different mechanisms over a time course, but data are accumulating even from germ cells. For example, during mouse meiosis, previously methylated regions are bound by a protein that preserves the "memory" of DNA methylation during DNA methylation erasure [175–177]. Mammalian promoters initially marked by H3K27 trimethylation in stem cells tend to accumulate DNA methylation during cellular differentiation [178]. Occasions where changes persist across generations, but not in identical format, have been observed after paternal toxicant exposure in mice [179] and in heat-exposed guinea pigs [180]. In *C. elegans*, small RNAs and proteins expressed in neurons interplay to mediate transgenerational inheritance [181], and osmotic stress of parents protects offspring by way of a developmental arrest and subsequent insulin signaling [182]. In yeast, feedback loops between small RNAs and repressive histone marks maintain signals over generations in a phenotypically neutral state, allowing later generations to potentially benefit from the information when required [183]. Such iterative re-coding of environmental information in a relay-race fashion can promote ecologically-meaningful impacts in multiple offspring generations even when individual marks are not robustly inherited and/or are repeatedly erased (**Figure 2C**).

In summary, *IGR* mechanisms act as a cluster of interdependent molecular nudges that together enhance the likelihood of a particular outcome on the gene expression level. This complexity creates challenges for empirical studies, which often aim to track down the effect of a single type of mechanism. Below we derive recommendations for experiments that investigate cross-generational effects of a particular parental exposure.

First, it may be most valuable to focus on the outcome level by prioritizing measures of gene expression at early life-cycle points. Examining gene expression early has several advantages. At this timepoint, the offspring's own developmental plasticity would have had limited time to overwrite any inherited alterations to gene regulation [184], thus avoiding false negative conclusion about non-heritability. A further benefit is that examining molecular phenotypes at an early stage could shorten intergenerational experiments and hence reduce resource demands. Importantly, tissue-specific approaches to gene expression analyses are always to be preferred – even if they necessitate delicate dissections – to avoid swamping signals that manifest only in particular tissues.

Second, design the experiment to investigate the 'cluster of nudges' in its entirety. If possible, attempt to detect altered DNA methylation, small RNAs, histone modifications, nutrients, metabolites, or hormone allocations in gametes of exposed parents before embarking on long term experiments studying adult phenotypes.

Third, exploit data generated as outlined above to identify the most promising experimental paradigm for adult life stages. *IGR* may manifest in adults only under very specific conditions of stress, resource deprivation etc. Data from gametes or embryos may help to determine promising experimental conditions. For example, embryos derived from metabolically-challenged parents may display a specific up-regulation of stress-related genes, which would warrant assessing adult offspring with a stress paradigm rather than a metabolism paradigm. Similarly, differential expression analyses of seeds of sun- versus shade-grown plants might reveal a down-regulation of small RNAs targeting root-related, which would warrant a focus on root morphology and nutrient uptake rather than leaf traits or transition to flowering in adults.

Box 2: Modelling the impact of 'non-genetic' inheritance

Mathematical and computational models play a crucial role in mapping the implications of epigenetic modifications, maternal effects and other aspects of 'non-genetic' inheritance for ecology and evolution (here, we use the collective term 'non-genetic' to reflect how these factors are modeled, with the caveat that they interact with or are influenced by genetic variants, as explained in Feature One). Even the relatively simple initial models (e.g. [56]) reveal that adding these additional modes of inheritance to genetic models can systematically and strongly affect the dynamics and outcome of adaptive evolution. At present, there are two dominant modelling approaches.

1. Extensions of population genetic [PG] and quantitative genetic [QG] models: Here, classic PG and QG models are expanded by including 'non-genetic' inheritance to study possible effects on evolutionary dynamics. These models are further used for estimating genetic parameters such as heritabilities and the phenotypic resemblance of relatives (e.g. [185,186]). PG models tend to focus on the special case of epialleles at a single locus (e.g. [187,188]). Even for simple scenarios, such models are very complex, making them mathematically intractable. Accordingly, they are generally studied numerically or by means of computer simulations. Some PG models do not model the evolutionary dynamics directly, but instead assume that evolution corresponds to an adaptive walk on a fitness landscape [189]. Such models can be applied to a broad class of 'non-genetic' mechanisms [8], but have often entailed somewhat unrealistic assumptions regarding how fitness reflects the interplay of genetic and 'non-genetic' factors (e.g. that fitness can be split into separate genetic and epigenetic portions). QG models (e.g. [56,190,191]) are technically more tractable, but they are based on strong and empirically-untested assumptions (such as a normal distribution of genetic and 'non-genetic' effects, with stable variances and covariances). Perhaps most importantly, QG models tend to assume that genetic and 'non-genetic' effects are additive (or that selection is very weak, implying that non-additive effects are negligible). Such additivity assumptions are not supported by the available data (e.g. [192]) and do not align with the view that 'non-genetic' inheritance is best understood as inherited gene regulatory information. By means of a Price equation approach, PG and QG models can be viewed from a unified perspective [97]. This provides useful insights such as the result that 'non-genetic' inheritance can foster rapid adaptation when the population is far from a fitness peak, while it will often lead to a fitness reduction in an already well-adapted population [57]. To date, however, applications of the Price equation (e.g. [97]) have also relied on simplifying and potentially misleading assumptions such as the additivity of genetic and 'non-genetic' effects.

2. Conceptual models based on the interplay of 'information channels': Here, genetic and 'non-genetic' effects are viewed as cues providing potentially adaptive information about the state of the environment (e.g. [7,94]). These models seek to ask what kinds of cues (for instance, inherited parental effects versus an individual's current information) will evolve to be used in a given scenario (depending on such factors as temporal versus spatial environmental fluctuation and transgenerational correlation). In contrast to most PG and QG models, the information channel approach explicitly models the machinery integrating and interpreting different kinds of information. This allows an important additional question to be addressed: how do these information-integrating systems themselves evolve? At present, however, information channel models reflect highly simplifying assumptions regarding the nature of genetic and 'non-genetic' cues and the way these cues are processed: the phenotype results from the weighted summation of different cues, and the information-processing machinery is represented by the weighing factors.

To our knowledge, a mechanistic model for the evolutionary causes and consequences of 'non-genetic' inheritance that reflects the three key features of these systems (as explained in the main text) has not yet been proposed. Figure 3 illustrates a possible structure for such a model. The ‘interpretive machinery’ of a cell [97] is represented by a regulatory network whereby genetic and epigenetic factors interact in a variety of ways. The implementation of such a network in an evolutionary individual-based simulation model is straightforward (see [193]). Such a flexible network model could do justice to the various interactions among genetic, epigenetic and environmental factors, providing for an inclusive understanding of inherited gene regulatory information, and might serve as a useful check of the robustness of the predictions made by the approaches discussed above.

Figure 3

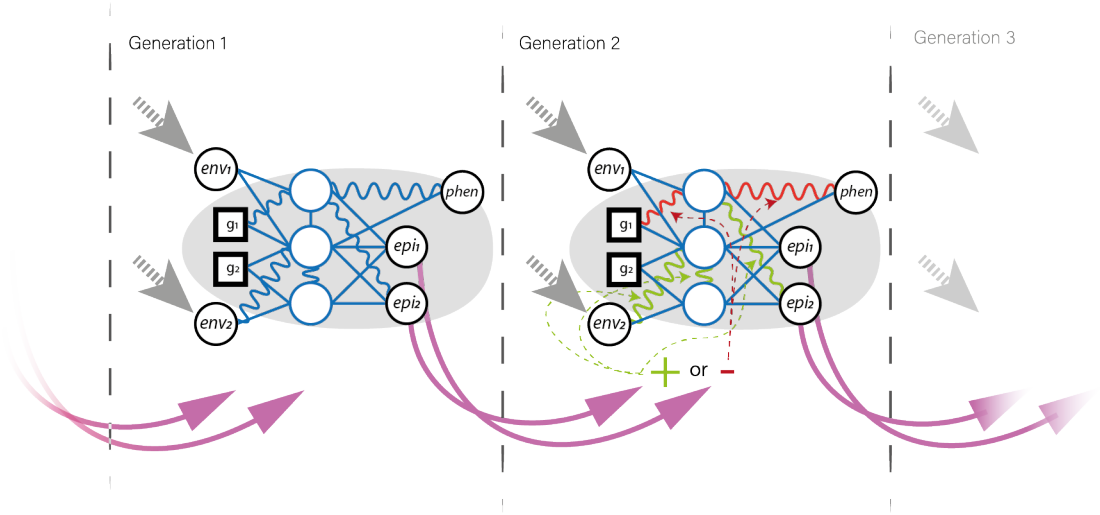

Figure 3. Gene regulatory network model incorporating IGR.
A gene regulatory network model allows mechanistic investigation of both the evolution of inherited gene regulation systems, and the implications of inherited gene regulation (including epigenetic inheritance) for the dynamics and outcome of evolution. The chart illustrates the transmission of epigenetic information from a parent in generation 1 to an offspring in generation 2. Both individuals harbor a gene regulatory network (GRN, blue lines) that determines the phenotype in response to genetic and environmental information. As in standard GRN models, the nodes (blue circles) represent regulatory genes, and the connections between nodes represent the influence of transcription factors (or other regulatory elements) on the expression of other genes. The GRN integrates environmental (env_i) and genetic (g_j) information to produce the phenotype (phen). To study the effects of inherited gene regulation, we propose to expand the standard GRN models in two ways. First, as illustrated in the parent, the GRN not only mediates the expression of the phenotype; it also induces the production of epigenetic factors (epi_k) that are transmitted to the offspring. Which epigenetic factors are produced depends on the genotype and the environment of the parent. Second, as illustrated in the offspring, the connections between the nodes of the GRN are not solely genetically determined. Some connections (indicated by waves) can be up (+) or down (-) regulated by the inherited epigenetic factors. The effects of these factors can be either unspecific (big + or – signs) or targeted to specific evolvable gene sequences (green and red arrows). Notice that in the proposed model the production of epigenetic factors in the offspring is partly determined by the epigenetic factors inherited from its parent, potentially resulting in a 'relay race' mode of regulation.

Conclusion

A key contemporary challenge is incorporating inheritance mechanisms beyond DNA sequence *per se* into evolutionary and ecological investigations. Some simplification of these dauntingly diverse and functionally complex mechanisms is reasonable and indeed necessary for this effort. By drawing on molecular insights to these mechanisms, this can be done in ways that maintain rather than distort key aspects of their functionality. 'Cross-talk' between evolutionary and molecular biologists provides a way to bridge this gap in understanding. Recognizing the common effect of highly diverse molecular mechanisms as *inherited gene regulation* is a first step toward identifying general features of 'non-genetic' inheritance systems. Characterizing such features can help replace some initial misconceptions with a more solid mechanistic foundation to inform ecology and evolution theory and research programs.

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