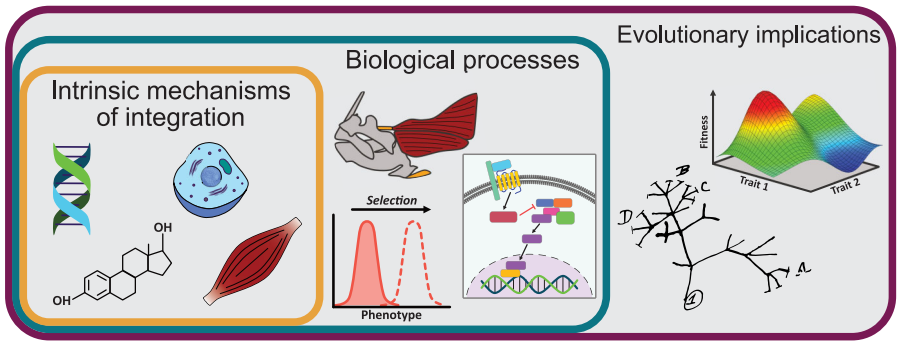

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SYMPOSIUM ARTICLE

Integrating Integration: The Intrinsic Mechanisms and Biological Processes that Shape Patterns of Trait Covariation Over Evolutionary Time

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Synopsis One ubiquitous pattern of organismal form that has long fascinated biologists is the covariation of seemingly unrelated traits across the body. This has led many to study phenotypic integration—the tendency of a biological or developmental system to give rise to such correlations. While integration has been observed across broad phylogenetic and broad temporal scales, our understanding of the underlying mechanisms is limited to broad categories of causation, such as development and selection. However, we believe that developing a more granular understanding of these mechanisms will be critical to more fully elucidate the evolutionary consequences of integration and to resolve past discrepancies in empirical data. To this end, we offer a list of intrinsic and molecular mechanisms that we hypothesize could drive integration of organismal form. We also present a list of biological processes, the set of intra- and inter-individual interactions affecting an organism, which may shape the deployment of these intrinsic mechanisms. Finally, we discuss how understanding these mechanisms could lead to different predictions about the temporal patterns of integration and even the evolvability of a system. Neither our list of mechanisms, nor our proposed consequences, are comprehensive; rather we hope that this discussion will encourage evolutionary and molecular biologists alike to build a deeper mechanistic understanding of organismal covariation, from cell-cell communications to macroevolutionary trends.

Introduction

Phenotypic integration and modularity

One hallmark of modern biology is the idea that organisms are the product of a complex interplay of processes occurring across many levels of biological organization. A realization of this perspective is the emergence of phenotypic integration as an important consideration in the study of anatomy, development, and evolution (Klingenberg 2008, 2014; Kane and Higham 2015; Farina et al. 2019). In this context, integration is the process through which covariation of two or more traits arises within an organism. And while integration and covariation are often used interchangeably, covariation simply refers to the statistical pattern, whereas integration is the predisposition to covary, and therefore implies an underlying biological mechanism (Klingenberg 2008; Hallgrímsson et al. 2009; Armbruster et al. 2014).

The degree of integration tends to be highly variable when examined across an entire organism. This leads to the parcellation of the body into quasi-independent sets of traits that covary more strongly with one another than with other parts of the body, a pattern known as modularity (Mitteroecker and Bookstein 2007; Klingenberg 2008, 2014; Zelditch and Goswami 2021). However, since modularity is emergent from integration, its mechanistic basis is necessarily complicated and multifaceted. We therefore focus our

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discussion of mechanisms on the more reductive and tractable trait that is integration.

Evolutionary patterns of phenotypic integration

One clear result of the ever-increasing number of studies on phenotypic integration is that it is ubiquitous across levels of biological organization in animals, plants, and likely all eukaryotes (Murren 2002, 2012; Armbruster et al. 2014; Conner et al. 2014; Diggle 2014). Furthermore, there is tremendous variability in the temporal scale on which integration arises and affects evolution. Even among species and populations within the same clade, for instance actinopterygians, we find that integrated suites of phenotypes shape divergence events across all evolutionary time scales; from order ten thousand years (Foster et al. 1992; Kim and Velando 2015; Dunker et al. 2024; St. John et al. 2024; Avery et al. 2026), to order one hundred thousand years (Cooper et al. 2011; Hu et al. 2014; Le Pabic et al. 2016 ; Conith and Albertson 2021), to order ten million years or more (Tsuboi et al. 2014; Evans et al. 2021; Larouche et al. 2023). Yet, while many of these studies show robust evolutionary trends, they rarely uncover the underlying mechanism driving these patterns.

Given the diversity of manifestations of phenotypic integration, we might predict that the nature of integration and its evolutionary implications would depend on both the clade in which it is examined as well as the scale (cladistic and temporal) of the analysis. It is therefore unsurprising that when researchers try to ask general questions about phenotypic integration, they often get conflicting results. For instance, one major question that gets asked repeatedly in studies of integration is whether it promotes or inhibits phenotypic evolution. When examined at a macroevolutionary level some studies find that integration increases evolutionary rates and/or morphological disparity (Marroig et al. 2009; Watanabe et al. 2019; Burns et al. 2023), while others find that it decreases rates and/or disparity (Goswami and Polly 2010; Goswami et al. 2016; Felice et al. 2018). Similarly, at the microevolutionary level (intraspecific and congeneric), there is support for increases (Hu et al. 2014; Penna et al. 2017) and decreases (Goswami et al. 2015) in both evolutionary metrics. Further, some find that integration constrains the axes of disparity while having no effect on rate or disparity (Goswami et al. 2014; Felice et al. 2018; Stayton 2024). While some of this discrepancy may be attributable to differences in the specific evolutionary pressures that shaped a given clade, for instance, whether the axis of selection aligns with the axis of trait covariation, it may also be explained by differing proximate mechanisms of trait integration (Evans et al. 2023). However, this can only be the case if there are multiple mechanisms that cause integration, and if they are sufficiently (and operationally) different from one another to change expected evolutionary outcomes.

Current understanding of mechanisms underlying integration

Past reviews of phenotypic integration have categorized the overarching processes that could drive covariation of traits, but only in a general way (Cheverud 1996; Arnold 2005; Klingenberg 2008, 2014; Armbruster et al. 2014; Farina et al. 2019). Namely, it is proposed that genetic, developmental, functional, evolutionary, and ontogenetic processes could all cause trait integration. The commonality across each category is that the same biological process or force is acting on multiple tissues simultaneously, causing variation in one tissue to be accompanied by a predictable change in another. Within some of these categories there have been abstract explanations of possible mechanisms, for instance, developmental pathways could cause integration either through bifurcation of a single pathway or through inductive signaling from one pathway to another (Klingenberg 2008). There are also cases where basic mechanisms such as pleiotropy have been associated with integration (Cheverud 1996; Pigliucci 2003; Murren 2012). In other studies, developmental variation has been described across multiple, putatively related, structures in response to a known developmental alteration (e.g., Xu et al. 2015; Hu and Albertson 2017). However, these discussions rarely go as far as to link these ideas to specific molecular processes (but see Archambeault et al. 2020; Rodríguez-Ramírez et al. 2023), nor show how such processes link concepts of integration across scales. As a result, our understanding of integration falls short of providing the framework necessary to ascertain why past studies have given diverging results and to make predictions about specific evolutionary outcomes going forward.

Given the implied molecular nature of many of the mechanisms that are likely to drive integration, we call for a greater collaboration between molecular biologists and evolutionary biologists to elucidate these mechanisms. This call to action echoes that of previous work illustrating how complex, layered, and hierarchical sets of developmental interactions could be studied as a means of ascertaining the proximate effectors of integration (Wagner 1984; Hallgrímsson et al. 2009). We believe that such cross-field collaborations will benefit both groups of researchers. Evolutionary biologists will gain access to cutting-edge tools and a deeper understanding of the complex, cell-level processes that underlie development. Simultaneously, molecular biologists will gain comparative insights on development as an evolutionary phenotype. Specifically, they would be able to take known examples of trait covariation

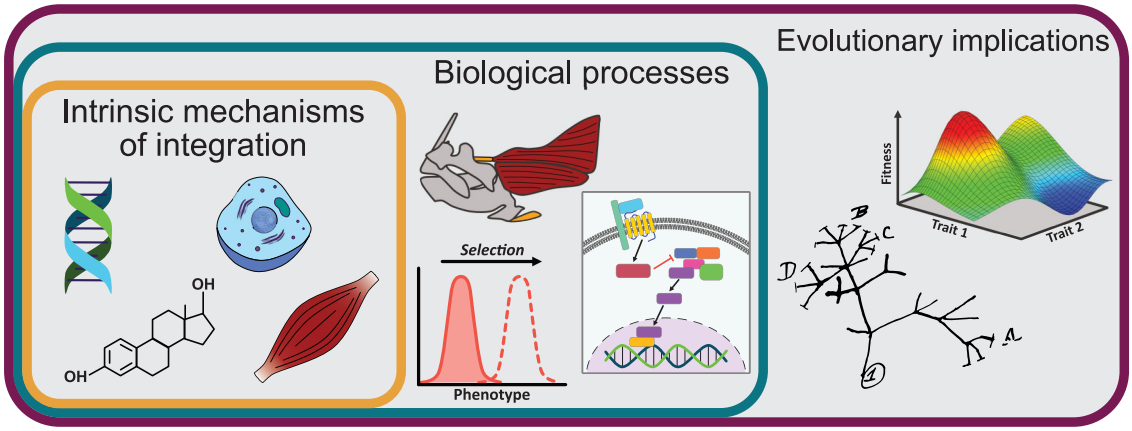

Fig. 1 Phenotypic integration can be established and shaped through many different channels. Here, we divide them into intrinsic mechanisms that can create integration (section 2) and the broader set of biological processes that shape the deployment of these intrinsic mechanisms or, in some cases, create *de novo* patterns of integration (section 3). In turn, these mechanisms and processes are fundamental components of the evolutionary process and thus shape the evolutionary patterns defining the history of life (section 4).

from evolutionary biology to uncover overlaps in developmental processes, which may reveal unexpected connections between tissues across the body.

To aid in this effort, we present a comprehensive, but not exhaustive, list of possible intrinsic mechanisms that could drive phenotypic integration. We then discuss how higher level biological processes can shape the deployment of these mechanisms. Finally, we show how an understanding of these mechanisms and processes can affect evolutionary outcomes (Fig. 1). We focus our discussion on the integration of anatomical traits in vertebrates, but these same mechanisms can also drive integration in other phenotypes and across clades, and we cite this broader literature where possible. Additionally, we note that although we offer a discrete list of mechanisms and processes, the reality is that they exist as a complex interconnected network. We therefore invite the reader to draw their own connections between mechanisms and processes as they explore integration in their own area of study.

Ultimately we hope that, in addition to spurring increased consideration of the importance of mechanisms in evolutionary understanding, researchers will use this as a way to generate alternate hypotheses during experimental design. Especially as many of the cellular processes that we present have not yet been implicated in trait covariation and are instead plausible mechanisms that remain to be tested. Accordingly, many of the sections below lack specific examples; instead, giving an overview of how integration could stem from the given mechanism or process.

Intrinsic mechanisms of integration

Integration can arise across many different levels of biological organization, from genes up to organism-wide processes. Yet at all levels of organization, integration arises due to the development of multiple tissues becoming coordinated through some type of signal, be it chemical or physical. Therefore, in this section we examine the intrinsic mechanisms through which signals can be generated and received as well as how the spatial domain of these signals can be altered. And while many of the higher level mechanisms can ultimately be traced back to a more proximate cellular basis, we highlight the emergent properties at those higher levels that can be altered to affect integration.

DNA/genes

The genome is a form of information storage that is deployed over ontogeny to create and maintain an organism. Therefore, the transcription of a gene can be understood as the creation of a signal, and integration can arise if this signal is created or transceived in different tissues in a way that coordinates their development.

Pleiotropy

The most direct way that genes can integrate traits is through pleiotropy. This has been one of the most widely discussed mechanisms of integration (Wagner 1984; Cheverud 1996; Leamy et al. 1999, 2002; Ehrich et al. 2003; Wolf et al. 2005; Kay and Surget-Groba 2022), in part because widespread pleiotropy is a defining characteristic of metazoan development (Carroll et al. 2013). Pleiotropy occurs when a single gene is functionally tied to multiple traits. Variation in such genes can then directly cause concurrent changes in each of these phenotypes, thereby integrating them. In addition to being widely discussed, it is also one of the few mechanisms that has been directly demonstrated in a natural population (Porto et al. 2016; Conith and Albertson 2021; Rodríguez-Ramírez et al. 2023).

Linkage

Although it can present identically to genetic pleiotropy in association studies, linkage disequilibrium (LD) is a distinct mechanism from multi-effect genes with unique evolutionary implications. Two alleles at different loci are said to be in LD when they are inherited together more often than would be expected under a fully independent segregation model. If each allele is independently associated with a trait, then LD would cause these two traits to appear integrated.

Generally, LD occurs because the two loci are either physically proximate in the genome or because of reduced recombination rates. While some causes of decreased recombination will maintain LD over time, such as chromosomal inversions or hard selective sweeps, LD will generally break down on an evolutionary time scale. Unfortunately, experimental recombinant populations in the lab are created over much shorter time spans (e.g., 2 or few generations) and therefore have artificially high levels of LD, making it difficult to discern pleiotropy from linkage. Therefore, mapping studies are likely to see increased rates of type I errors in terms of finding pleiotropic loci, and require complementary studies (e.g., expression, knockdown) to increase confidence in a gene's ability to regulate the development of two or more traits.

Gene regulation

Another genetic mechanism of integration involves gene regulation, which is essentially a sub-category of pleiotropy. One instantiation of this would be integration resulting from shared, upstream, *cis*-acting regulators driving gene expression in multiple tissues. Alternatively, a gene product (including non-coding RNAs) may function as a *trans*-acting factor whose role is to affect downstream genes. For instance, a gene encoding a transcription factor which is involved in the development of multiple tissues would be considered pleiotropic and could lead to integration. We highlight this mechanism because regulatory evolution is considered an important means through which organismal diversity arises (Stern 2000; Carroll 2005), and because as a potential source of pleiotropy, it holds significant potential to underlie the evolution of integration. Indeed, while the evolution of genetic enhancers has long been recognized as a mechanism through which pleiotropy can be avoided (Carroll 2005), we emphasize its potential to drive new pleiotropic patterns. If, for example, a mutation introduces a new transcription factor binding site in the regulatory sequence of a gene, and if that transcription factor is present in two or more tissues, then we would expect to see novel pleiotropy.

Proteins and molecules

One of the fundamental functions of a gene is to encode the instructions for making a protein. Subsequently, these chains of amino acids carry much more functional, structural, and biochemical complexity than DNA, while also being able to move throughout the body. Because of this they are able to convey spatial information, engage in signaling both locally and globally, and catalyze reactions among other molecules, all of which carry implications for integration.

The effects of these mechanisms are largely based around the ability for cells to send signals to one another. While autocrine and juxtacrine signals are less likely to cause integration due to their localized effects (but see below), paracrine and endocrine signals are both well suited to the multi-tissue effects necessary for integration.

Paracrine signals

Paracrine signals are cell-to-cell messages that are spread through diffusion. As such, the scope of their effect depends on ontogenetic timing, the amount of ligand produced, and the biophysics of diffusion. They are likely most potent for integration during early stages of development when their diffusion range in the embryo will ultimately span large regions of the adult.

Cytokines/growth factors: Although cytokines are often associated with body-wide immune responses, they also carry more local cell differentiation, cell proliferation, and cell cycle suppression signals. Similarly, growth factors are a large family of signaling molecules that are associated with cell differentiation and proliferation. The specificity of these signals depends on the localization and/or range of travel, as well as the competency of cells to receive and/or interpret them. When disparate tissues receive the same signal, and respond in a coordinated manner, phenotypic integration is a likely outcome.

Morphogens: One particular type of signaling molecule that specifically defines developmental patterning is the morphogen. As these molecules diffuse through the embryo, they create a gradient whose strength is a function of distance from the source. Importantly, cells are able to discern differences in morphogen concentrations, and thus local development proceeds in a concentration-dependent manner. By using the concentration of several morphogens that diffuse along different anatomical axes, cells gain positional identity within a developmental field and can alter developmental trajectories accordingly. In theory, altering the concentration of a morphogen should result in a coordinated response across the entire developmental field, thereby creating integrated downstream effects that span all descendent cells.

Morphogens can also be potent integrative forces when they are shared between developmental fields. For instance, the vertebrate heart develops adjacent to the precursors of the face, and each field requires many of the same or related signals. Thus, these distinct organs are molecularly linked in early development. This linkage is evident in mutants with coordinated heart and craniofacial defects (Albertson and Yelick 2005; Zbasnik et al. 2022), but whether it has any effect on the evolution of these structures remains unknown. Another interesting example of cross-talk between developmental fields involves the gut and hindlimbs, both of which require related signals early in development. Initiation of hindlimb development requires expression of *Pitx1* in the limb bud, while gut development requires asymmetric expression of the paralogous gene, *Pitx2,* which patterns the gut along the left-right axis. Reduction of the hindlimb/fin in three- and nine-spine stickleback is due to loss-of-function mutations in *Pitx1;* however, evolved fin reduction is often asymmetric owing to a partial rescue of limb development on the side where *Pitx2* is expressed (Marcil et al. 2003; Shapiro et al. 2004).

Reaction–diffusion mechanisms: One special use case of spatial signaling that could carry unique implications for integration is found in Turing-like reaction-diffusion mechanisms. This developmental process is characterized by cells that produce both inhibitory and excitatory signals, which then interact to create spatially complex developmental patterns. Ultimately the number or shape of elements that are formed is determined by the interaction between these molecules, their biophysical properties (e.g., rate of diffusion), and their environment (e.g., shape of the tissue in which they diffuse). For instance, pigmentation patterning, turtle shell morphology, and digit number are all phenotypes that arise from Turing mechanisms (Moustakas-Verho et al. 2014; Raspopovic et al. 2014; Hiscock et al. 2017; Kondo et al. 2021). That each element in a repeated series (e.g., digits) develops using the same signals implies a high degree of integration across the series; however, the outcome of a Turing mechanism is dependent on the size and shape of the tissue in which it is deployed. Therefore, the magnitude of covariation may change across the series if the tissue is irregularly shaped. For instance, the reason why digit size is not uniform in most vertebrates is likely due, at least in part, to the fact that the developing limb bud is paddle-shaped and not square (Hiscock et al. 2017).

Endocrine signals

Hormones: Hormones are a broad class of molecules whose shared feature is their ability to send signals that span the entire body. As such, the signal itself has essentially no spatial specificity. Instead, local responses are mediated by tissue-specific hormone metabolism, activity of carrier proteins, as well as presence, absence, and binding affinity of receptors in different tissues (Ketterson et al. 2009). Additionally, once a cell receives the hormone signal, there can be variation in the induced signal cascade. When multiple tissues are capable of receiving a hormonal signal, often referred to as hormonal pleiotropy, then their responses will be integrated (Ketterson et al. 2009; Evans et al. 2021). Importantly, these hormonally integrated phenotypes will only be present if the relevant hormone is expressed at a time when the tissues are receptive, meaning that the presence of the hormonal signal can have temporal, if not spatial, specificity.

Enzymes: Not all hormones are themselves proteins, as steroid hormones are synthesized from cholesterol. However, the process of synthesizing these hormones depends on enzymatic proteins, those that catalyze chemical reactions. Therefore, this facet of hormonal signaling can be affected at the protein level through changes to the number and functionality of enzymes. From here, the ability to affect integration is similar to other hormones.

Protein complexes

The next step up in terms of biochemical complexity and functional scope comes from combining proteins into sub-cellular level complexes. Many of these structures vastly expand what cells are able to accomplish by sensitizing the cell to their physical and chemical environment as well as by granting them the ability to alter their internal and local external environments.

Intramembranous receptors

A common theme among mechanisms of integration is that they involve sending or transmitting some sort of signal. However, signals are only useful if they are received and transduced. Therefore, a crucial part of cellular signaling, and a potent way of integrating sets of tissues, is through variation in the presence/absence and in the molecular affinity of receptors within different tissues. By sensitizing multiple tissues to the same developmental signals, organisms can link their growth patterns and cause them to be integrated. This is particularly true in the case of endocrine signals, or in paracrine signals during early development, as both of these signals can span large portions of the body. While many receptors are single proteins, others are the result of protein complexes, including both homo- and heterodimers. This complexity in receptor structure/function increases the types of signals that may be received and transduced. Distinct cell lineages that are

enriched for the same types of receptors therefore have the potential to receive/respond to the same signals, increasing the likelihood of integration between the resulting tissues.

Transmembrane transporters

In addition to the receptors that transduce an external signal, the cell membrane contains several types of structures that allow molecules to be transported into or out of the cell. These include ion channels, ion pumps, membrane transport proteins, and even vesicles formed from the membrane itself. The integrative effects of some of these structures are likely to be similar to intramembranous receptors if they simply pass along a signal to be transcribed in the nucleus. However, other transmembrane structures allow the cell to change its internal and local environment to alter its own developmental field. One prominent example of this is in bioelectric patterning that is established by active ion pumps.

Bioelectric patterning

An emergent property of the active transport of ions by transmembrane transporters in populations of cells is the establishment of electrical gradients within the body. Much like morphogens, this spatially resolved signal is used in establishing anatomical axes, field identity, and in guiding cell motility (Levin 2014). However, unlike purely diffusion-based gradients, bioelectric gradients are established through active ion transport and can therefore take on more spatially complex and widespread distributions. This is particularly relevant to trait integration because bioelectric signals often override chemical ones when the two conflict (Levin 2014). Therefore, bioelectric fields represent a mechanism through which complex patterns of integration can be established. Evidence for the potential of bioelectric signals to integrate disparate tissues comes from human channelopathies, disorders that arise due to dysfunctional ion channels and affect different tissue types (Kim 2014), including the nervous system, heart, skeletal muscle, and even the facial and appendicular skeleton.

Chaperone molecules

This class of functional unit spans from individual proteins to larger oligomeric constructs, but uniting all chaperone molecules is the ability to alter functional protein levels. This is accomplished primarily through two functions: guiding protein folding and transferring proteins out of the endoplasmic reticulum. In both cases, the chaperone molecules are directly responsible for the concentration of correctly folded, and therefore functionally active, proteins that leave the endoplasmic reticulum and the cell. Therefore, changes to these molecules in different tissues could affect many of the above intrinsic processes. However, many chaperone molecules are classified as heat shock proteins and therefore are primarily altered in response to physiological stress. Therefore, their ability to act as an integrative force over evolutionary time may be limited by an organism's ability to canalize their effects.

Organelles

Organelles are a set of functionally distinct subunits within the cell. While many organelles function in maintaining internal cellular function, others allow for the transduction of external signals. One of the more complex organelles of the eukaryotic cell is the primary cilium. Each of these non-motile projections from the cellular membrane sensitizes a cell to a particular signal, acting as a mechanoreceptor, chemoreceptor, or photoreceptor (Pazour and Witman 2003). Of these, mechanoreception has been the most directly implicated in phenotypic integration of skeletal tissues, particularly in the ability of bone to remodel itself in response to mechanical load (Goetz et al. 2009; Navon et al. 2020). For instance, zebrafish mutants with defective cilia exhibit bone dysmorphologies, primarily in bones with complex patterns of soft tissue attachments (Gilbert et al. 2021). Chemoreception is also an excellent candidate for integration since transmembrane receptors from several signaling pathways localize to, and are transduced through, the primary cilium (Pala et al. 2017; Mill et al. 2023; Li et al. 2025). Additionally, chemoreceptive primary cilia can help sensitize cells to the general chemical and bioelectric environment of the body, as ion channels also localize to primary cilia (Delling et al. 2013; Pablo et al. 2017). Exactly how these signals are transduced through the cilium into the cell remains an active area of research, but given that nearly every cell type possesses primary cilia, this organelle holds tremendous potential to promote integration through the sensitization of cells to one or more signals. This is evidenced by the stereotyped multi-tissue effects that are associated with ciliopathies, a category of birth defects caused by primary cilia dysfunction.

Cytoskeleton, extracellular matrix, and cell adhesion molecules

If mechanosensation can drive integration, then the structures that transmit these forces will also carry integrative potential by affecting the strength and pattern of force transmission through different tissues. One such component of the cell is the cytoskeleton, a highly dynamic network of structural proteins within the cell that is critical in cellular mechanotransduction. It has been shown to sensitize the cell to tension and shear in the extracellular matrix and can induce cell differentia-

tion and migration. The cytoskeleton can also transduce force to the nucleus leading to a genome-wide transcription response (Oses et al. 2023). Similar to other mechanisms at this level of biological organization, variation in the cytoskeleton could change the sensitivity of a given population of cells to their mechanical environment, which could either integrate or dis-integrate these cells with other tissues receiving the same mechanical signal.

External to individual cells, but also important in granting structure and transmitting forces, is the extracellular matrix (ECM). The ECM is composed of various different proteins, polymers, and minerals, the precise combination of which helps define the mechanical properties of a given tissue (Fomovsky et al. 2010). Therefore, by changing the relative makeup of the ECM within a given tissue, the patterning and strength of mechanical transduction can be substantially altered. At a finer scale, individual cells can be differentially sensitized to these forces by changing how tightly bound to the ECM and to each other they are. This is accomplished primarily through cell adhesion molecules on the surface of cells.

Cells

When we discuss anatomical integration, we often mean the coordinated behavior of cells within anatomical units. Therefore, the cell is a critical level at which integration can arise. Specifically, it is the cell's position at the interface of molecular and structural processes that makes it a unique actor in integration. Here we focus the discussion less on molecular mechanisms and more on emergent cell behaviors.

Cell division

Cell proliferation is a tightly regulated process that is inherent to organismal development. This process is both temporally and spatially complex, as there are often stark differences in the rate of cell division between tissues and over ontogeny. Interestingly, when adjacent tissues proliferate at different rates, they generate forces on one another, resulting in a complex landscape of stress and strain relationships across a developing body. Because cells are able to sense their local mechanical environment (see above), the distribution of forces is a signal that could drive covariation. Additionally, cells are able to sense and respond to their chemical environment. One example of this is the proliferation-migration tradeoff (e.g., grow-or-go) in tumors, an evolutionary strategy whereby "go" cells are considered colonizers that adopt a migratory cell fate (see "cell migration" below) as resources become limited in their growing local environment (e.g., Kotler and Brown 2020).

In addition to generating a mechanical environment that could guide integrated development, cell division can also directly integrate two adjacent endochondral bones if they share a growth plate. The dynamics of growth plates are complex and involve multiple cell behaviors, any of which could cause coordinated development of the structures around it (e.g., Fuente et al. 2018). Some of the major developmental modifications that could be achieved by modifying cell proliferation within the growth plate include the position and orientation of the growth plate with respect to the long axis of the developing bone and the symmetry of signaling across the growth plate (Le Pabic et al. 2016).

Cell migration

In addition to cell division, integration could arise due to patterns of cell migration. In its simplest form this could happen as cells migrate from one precursor population to another, causing the donor structure to be diminished and the receiving structure to be expanded. For example, changes in the relative size of embryonic pharyngeal arches 1 and 2 have been associated with different size neural crest streams, leading to interspecific variation in the relative size of the first and second arches (Powder et al. 2014). Another potential source of integration could arise through shared developmental origins. Specifically, if cells from one embryonic progenitor population are able to migrate through the body and form different structures, then early developmental changes in the precursor population (e.g., altered gene expression, size of the original population) could cause coordinated changes to all the resulting structures.

Cell death

Apoptosis, or programmed cell death, is another critical process that is utilized at all stages of development and is therefore able to induce covariation in several ways. The most well-known role of apoptosis in morphogenesis is found in its deployment as a "stone sculptor," where it is used to eliminate tissue, thereby shaping the resulting structure (Suzanne and Steller 2013). Because apoptosis is being used to create a negative space in the anatomy, variation in its deployment could directly shape two distinct but adjacent structures. This effect can either directly shape a structure or, perhaps more potent, it can shape early populations of cells that will ultimately give rise to other structures. For instance, the teleost pectoral fin originated from a solid cartilaginous fin pad, from which individual bony elements are sculpted, seemingly via apoptosis (Grandel and Schulte-Merker 1998; Woltering et al. 2018). This process is reminiscent of the way digits form in tetrapods—e.g., apoptosis of the interdigital membrane.

Additionally, cell death is often accompanied by the creation of actomyosin rings that extrude the dying cells and pull in other cells to take their place. This has been shown to generate forces that play a necessary role in shaping nearby tissues (Murrell et al. 2015; Weißenbruch and Mayor 2024). So in addition to shaping the negative space between two structures, apoptosis might also be involved in coordinating the shapes of adjacent structures. Although these are just a few examples of the roles that apoptosis takes in development, they highlight the importance of considering destructive developmental processes as well as constructive ones when considering how covariation might arise.

Tissue

While there is a great deal of functional and developmental complexity present at the level of tissues, most of the intrinsic integrative potential of a tissue can be traced to one or more of the processes described above. However, there is one set of attributes that arise at the level of the tissue that is a strong candidate for potentiating covariation: material properties.

Material properties

Biological tissues are composed of an amalgam of cells, extracellular matrix, and fluids. The relative proportions of each, as well as the specific types of cells and matrix, are variable across different types of tissues. Even within the same class of tissues, there can be significant compositional variation across the body and across clades. Emergent from the composition of a given tissue are its material properties, traits such as tensile strength, toughness, and ductility. Therefore, there is variation in the mechanical properties of the body, both within and between tissue types.

This is particularly relevant to integration when there is variation in the material properties of connective tissues such as muscles, tendons, ligaments, and bones. Since these tissues generate and transmit forces throughout the body, changing their material properties will fundamentally alter the mechanical environment of the structures to which they connect. If these structures are mechanosensitive, or even sometimes in the absence of mechanosensation (e.g., Lieberman et al. 2008), then this will serve to integrate or dis-integrate their development, with the effect depending on the precise mechanical changes in the connective tissues.

Biological processes affecting integration

To understand how the above mechanisms actually lead to integration in organisms and not just in discrete populations of cells, we must understand them in the context of the biological processes that shape the organism—the complex set of intra- and inter-individual interactions affecting an organism. Some of these processes are capable of driving covariation without an underlying shared intrinsic mechanism, while others simply explain the deployment and potency of the above mechanisms. In either case, we contend that it is necessary to consider these processes when studying any tangible examples of phenotypic integration.

Biophysics and biochemistry

Many of the above mechanisms depend on the transmission and reception of a molecular signal. While some of these processes are actively controlled by the organism, much of it is also a result of the biophysics of the cellular environment and the biochemistry of ligand-receptor interactions (Newman 2019). For instance, the distance and rate of diffusion of a paracrine signal can only be understood as a function of cellular level physics. Similarly, transcription factors are often able to bind to multiple target sequences, each with a different binding affinity. The exact strength of any given sequence is then best understood through biochemistry. These are just a few examples of the myriad ways in which biophysics and biochemistry intimately shape the outcome of integrative processes.

Pathways

Development is an emergent property of the interactions and regulation of many genes. When we build a mechanistic understanding of the hierarchical relationship between different genes and their products, we call this a pathway. So while the above mechanisms represent the causal elements of integration, it is impossible to fully understand their effects without understanding the genetic and developmental pathways through which they act.

Klingenberg (2008) established the idea that there are two ways that a pathway can lead to integration. The first is through bifurcation, where a pathway splits into multiple inductive paths that each then influence another trait. The second is through inductive signaling, where one pathway affects the strength of another pathway that is acting in parallel. This highlights the importance of understanding pathways as context because it shows the multiple ways in which the same mechanistic elements can act to integrate tissues, each of which has the potential to impact evolution in distinct ways. This is further complicated by the fact that multiple pathways can simultaneously affect the integration of the same two traits. However, the palimpsest model put forward by Hallgrimsson et al. (2009) provides a model

for disentangling these complex sets of developmental interactions.

Ontogeny

The nature of development changes continuously over the course of an organism's lifetime, from the early developmental processes of embryogenesis to the homeostatic mechanisms that maintain and reshape adults. This history of development is referred to as ontogeny, and an understanding of the history and the connections between stages has enormous implications for integration. These implications have been most famously explored alongside the analogy of a palimpsest, a manuscript that has been erased and written over with traces of the original writing remaining (Hallgrímsson et al. 2009). Since development is a hierarchical process, early patterns can leave traces that are detectable in all subsequent stages. Additionally, heterochrony provides an opportunity for these patterns to change between clades (Goswami et al. 2014). Therefore, when trying to understand the proximate mechanisms for the covariation of two traits, we need to understand their entire developmental history and how covariation could arise at any stage. Additionally, we must account for the life stage of an organism, as allometry can have potent integrating effects (Klingenberg 2013).

The actual effects of ontogeny are best understood by mapping the hierarchical relationships of tissues across development. As early development unfolds, cells undergo a series of differentiations and over time lose potency, or the breadth of the tissues that they are capable of becoming. This can be illustrated with a cell fate map in which each differentiation represents a branch in the map wherein each individual path has fewer potential outcomes downstream than before. If developmental modifications are made to the cells, or to their developmental environment, at any given point, then the effects could echo through all subsequent tissues that these cells give rise to. For instance, the patterning of orbital bones in blind morphs of the Mexican cavefish (*Astyanax mexicanus*) is highly dependent on the timing of eye loss, with earlier eye loss being associated with greater morphological changes to the number and positioning of the bones (Hamm et al. 2026). Therefore, cell fate maps are an important tool for understanding when covariation can arise in any given set of tissues.

Metamorphosis

Although not present in all clades, metamorphosis represents a potential way to circumvent the palimpsest, and to dis-integrate structures across life stages. This is especially true in holometabolous organisms—those that undergo complete metamorphosis. By separating larval and adult forms with a wholesale reorganization of the body during a pupating stage, these organisms can largely erase the holdover effects of early development on adult morphogenesis (Moran 1994).

Interestingly, animals that undergo partial metamorphosis, or transformation, may actually be more subject to integrative effects arising across ontogeny. This life history is characterized by drastic coordinated morphological changes that arise without a pupation stage separating juvenile and adult forms. Because of this, the adult morphology must be built more directly upon the framework of a morphologically dissimilar juvenile form (Moran 1994; Suzuki and Toh 2021). This often means that juvenile tissues are repurposed for adult structures, leading to constraints that might not have arisen under a direct developmental model (Evans et al. 2021).

Organismal processes

Plasticity

Although development is sometimes discussed as a deterministic process, it is actually highly sensitive to the internal and external environment of the organism. In many lineages, this sensitivity extends into adult life stages. In either case, the effect of the environment on development and ongoing remodeling of adults is referred to as plasticity. Therefore, we can think of the environment as a signal in development and plasticity as the ability to respond to this signal. As such, integration as a result of plasticity simply comes down to multiple tissues responding to the same environmental signal and concurrently altering their development in response (Schlichting and Pigliucci 1998). However, we note that both the environmental signal and the phenotypic response are high dimensional metrics from which causation can be difficult to parse (Lofeu and Kohlsdorf 2026). Yet many of the environmental factors that can influence development in this way are tractable at the level of the organism, as they are directly determined by the functional connections across the body and by an organism's behavior (see below).

While plasticity can be achieved simply by altering expression levels of already active genes, it can also alter development by changing the entire underlying genetic architecture of traits (Murren 2012). For instance, raising some fishes in different trophic environments is sufficient to alter the set of genes that are associated with many of their skull bones (Parsons et al. 2016), in part driven by mechanosensory processes (Navon et al. 2020; Gilbert et al. 2021). This change in genetic architecture would then create an entirely new set of interactions among the intrinsic variables controlling development, allowing for entirely new patterns of integration to arise in a novel environment. The most

extreme version of this is found in polyphenisms, an all-or-nothing response to the environment where changes to large suites of traits are all triggered simultaneously by the same stimulus.

Functional linkages

One of the major drivers of plasticity in adults is the force that is generated by muscles and by interactions with the physical environment. These forces are then transmitted through the muscles themselves and through other connective tissues. As discussed above, many of the intrinsic mechanisms of integration revolve around mechanotransduction; traits which sensitize tissues to these forces and allow them to alter their development in response. Therefore, one of the likely determinants of integration in many organisms will be the anatomical pattern of connections that are force-loaded during any given function. Indeed, these same linkages are often critical in the base development of an organism, independent of the external environment (Hu and Albertson 2017). By understanding the biomechanics and functional connections between different structures, we can understand what patterns of covariation might arise due to shared function.

Behavior

There are many functional connections that exist across a body, but the degree to which each one is used, and therefore the degree to which it affects development, is determined by an organism's behavior. For instance, one of the major functional divergences in fish feeding comes down to whether they apply the highest forces during jaw adduction (maximizing bite performance), or during jaw abduction (maximizing suction feeding performance). Importantly, each of these behaviors utilizes a different series of bones, muscles, tendons, and ligaments to drive the motion (Gidmark et al. 2019). Additionally, many species are capable of switching between feeding modes depending on environmental conditions, meaning that it is a highly evolutionarily labile trait. Therefore, whether we see integration among jaw-abduction or jaw-adduction-linked traits is heavily influenced by trophic behavior. Although this is only one example, the degree of covariation among any functionally linked traits could similarly be affected by the extent to which their shared function is performed. Interestingly, in some cases the morphology and behavior are genetically integrated (Nicholson et al. 2026), making this behavioral effect on integration more evolutionarily stable.

Additionally, an organism's external environment is a crucial part of its developmental process, across embryonic stages and through to homeostatic upkeep in the adult. One classic example of this is temperature-dependent sex determination, where the sex of an individual depends entirely on the temperature that it was exposed to during a particular period of development. Beyond this, development can be shaped by an organism's chemical environment, photic environment, and by innumerable other environmental cues (Reppert et al. 1985; Moczek et al. 2011; De Coster and Van Larebeke 2012; Annamalai and Namasivayam 2015; Sultan 2017; Suzuki and Toh 2021). Therefore, any aspect of behavior that influences the environment of an animal or its offspring is liable to create or extinguish patterns of covariation by changing the course of development wholesale.

Evolutionary processes

Selection

Evolutionary fitness is a complex property that emerges from the interplay of the environment with many intrinsic processes, including genetic, biochemical, developmental, hormonal, anatomical, functional, behavioral, etc. So when an organism undergoes an adaptive process, we would expect selection to favor sets of traits within and across these levels that are able to effectively operate together. If the functionally effective trait space is narrow enough, then this will result in strong patterns of covariation among the relevant traits. Indeed, early examinations of phenotypic integration and modularity in animals viewed these processes solely as the result of selection for shared functionality (Terentjev 1931; Berg 1960; Olson and Miller 1999). Given the highly interconnected nature of many organisms, this functional approach could lead to coincident changes spanning the entire body.

Chromosomal/structural evolution

Units within an organism often get repeated over the course of evolution, both at the level of genes and in entire structures. For instance, some types of chromosomal evolution lead to two copies of the same gene, known as paralogs. There is also the repurposing of the same gene network to drive development of distinct tissues. A similar trend is described at higher organizational levels as serial homology, where entire anatomical units, such as a limb or body segment, are repeated within the same organism. In all cases, after the duplication there are at least two units that have shared developmental origins but are now free to diverge from one another, such as vertebrate gills giving rise to numerous other structures after the water-to-land transition (Crump et al. 2026). While this is not a mechanism itself, it does explain how different structures can be integrated due to shared origins and/or related signals over development.

Other confounding factors

While not processes through which integration (*sensu* Hallgrímsson et al. 2009) can be achieved, there are several other important ways in which covariation can occur in datasets. These must be considered when making decisions about methodology, data analysis, and data interpretation.

Evolutionary history

Evolution is often a slow process, meaning that the morphological divergence between any two species is governed in part by their coalescent time. When extended to an entire clade, this then implies that more closely related species will have greater morphological similarity than distantly related ones. Of course, given enough evolutionary time these associations can break down. However, for clades which are more recently evolved or which undergo very slow morphological evolution, we would expect trait covariation to arise simply from shared ancestry.

This phenomenon is often explicitly addressed in comparative work by utilizing phylogenetic independent contrasts or phylogenetic generalized least squares analyses (Garland et al. 1992; Revell 2010). So while phylogenetic relationships are often the cause of covariation across clades, these relationships are usually accounted for before answering other research questions.

Stochasticity

It is possible that two structures will appear to covary simply by random chance, particularly when there is a small sample size or insufficient cladistic breadth (Armbruster and Schwaegerle 1996; Goswami et al. 2014; Grabowski and Porto 2017). Given that evolutionary biologists often work in non-model organisms for which specimen collection can be limited by many external factors, this is an important factor to be mindful of.

Evolutionary implications of a mechanistic understanding of integration

In order for the mechanism driving integration to be evolutionarily relevant, they must be capable of altering evolutionary outcomes. We present two major ways in which this can happen, though this is far from a comprehensive list of the possible evolutionary implications. First, we discuss how different mechanisms of integration will have different degrees of temporal stability. Second, we consider the consequences of different mechanisms on the evolvability of the integrated elements.

To see how temporal stability might be affected by the mechanisms, we return to one of the major evolutionary questions posed in studies of phenotypic integration; does integration act to promote or constrain phenotypic evolution? Evolutionary responses are often considered at both the micro- and macro-scale, where microevolutionary changes need to be temporally stable in order to affect macroevolutionary outcomes. While some intrinsic mechanisms of integration are expected to persist over evolutionary time scales, others are more labile. Even just among the possible genetic causes of integration, we can see a stark difference between pleiotropy, linkage disequilibrium, and gene regulation. The least temporally stable mechanism is linkage, as this is quickly broken on evolutionary time scales unless a population undergoes a selective sweep and/or linkage arises from a structural change in the chromosome (e.g., inversion). More stable than linkage are cis-acting regulation mechanisms acting on multiple adjacent genes (e.g., co-regulated gene clusters) (Razin et al. 2021). Although this form of pleiotropy can be maintained for long periods of time, variation in such regulatory elements is a crucial element of rapid adaptive radiation (Wray 2007; Wittkopp and Kalay 2012; Marand et al. 2023). This suggests that these regulatory elements still maintain a fair amount of evolutionary lability. Finally, pleiotropy that occurs because of multiple downstream effects within the same pathway is likely to be the most temporally stable mechanism, especially when the gene is part of a conserved pathway. Because neutral or positive variation in these genes is rare, it is less likely that this type of integration will break down except over very long time scales.

Next, we examine how different mechanisms of integration affect the evolvability of a system. To understand this, we must first consider the type of selection and how the axis of selection relates to the axis of integration. Specifically, when directional selection aligns with integration, we expect evolutionary rates to increase because multiple adaptive phenotypes will be innately inherited together and therefore the population will explore less phenotypic space while moving towards the fitness peak (Fig. 2A) (Schluter 1996; Villmoare 2013; Goswami et al. 2014; Felice et al. 2018). And without a perpendicular force driving them off this axis of integration variation may decrease as the population approaches the adaptive peak, depending, of course, on the strength of stabilizing selection associated with the peak. Conversely, when the axis of selection is orthogonal to the axis of integration, we expect slower evolutionary rates because integration is constraining populations from moving along the axis of selection (Fig. 2B). Importantly, the degree to which evolutionary rates are de-

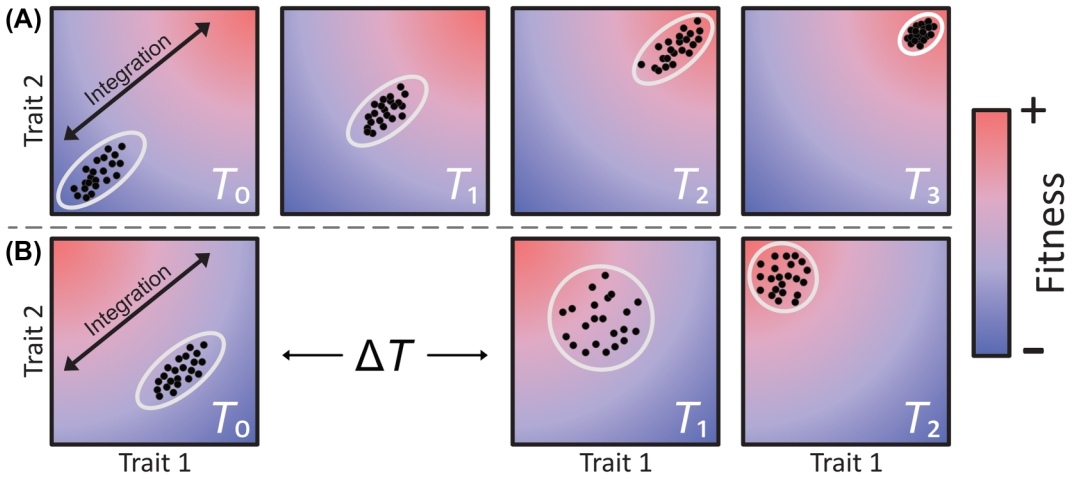

Fig. 2 The rate of evolution of a population in a hypothetical trait space is highly dependent on the relative orientation of the axis of integration versus the axis of selection (running from low to high fitness). When these axes are perpendicular, the rate also depends on the mechanism of integration. (A) When integration is parallel to the axis of selection, the population evolves rapidly while maintaining integration, regardless of the specific mechanism. (B) When integration is perpendicular to selection, there is a delay before the initiation of an adaptive response (ΔT). The duration of ΔT depends on the mechanism driving the observed pattern of integration, as this determines how difficult it is for evolution to break down integration. Time points (T_0, T_1, etc.) are relative within each panel and are not equal between the panels.

creased (the duration of ΔT in Fig. 2B) depends directly upon the mechanism driving integration and, therefore, how rapidly selection can dis-integrate the focal traits. Once clades escape the constraint imposed by integration, then we would expect to see increased morphological disparity as populations are able to more fully explore morphospace, even as they approach an adaptive peak.

The question then becomes, which mechanisms of integration will pose the least resistance to a perpendicular force of selection? One way to answer this is to return to the idea that many mechanisms of integration can be thought of as either integrating signals or integrating receivers, where the integrating factor needs to act on a specific set of tissues. This is accomplished in integrating signals by limiting the spatial range of the signal. For instance, many cells may be capable of transceiving morphogen signals, but only cells relatively near the source are exposed to the signal. Conversely, integrating sensors can drive covariation in response to a global signal as long as the receptors are only on some cells, as is often the case in endocrine responses. Given this dichotomy of integrating factors, we might predict that integrating signals would limit evolvability more than integrating receptors. In other words, because the specificity of signals comes from their limited spatial domain, the only mechanism through which their effects can be modified is to modulate their deployment. However, given the exquisite sensitivity of tissues to morphogens and their importance for proper development, altering their deployment might be difficult without precipitating negative developmental outcomes. On the other hand, integrative effects of shared receptors could be escaped relatively easily by disrupting the expression of receptors in one tissue without affecting the development of other tissues.

Conclusions

While the above examples illustrate how an understanding of integrative mechanisms can inform a deeper exploration of a narrow set of questions in morphological evolution, the merits of this sort of understanding can be extended much further, particularly when combined with the palimpsest model (Hallgrímsson et al. 2009). We suggest a set of outstanding evolutionary questions that we think are critical to advancing the fields of evo-devo and evolution more broadly, and whose results could depend heavily on the underlying mechanisms of integration:

- When is integration itself the target of adaptive evolution? How evolvable is integration?
- Are patterns of integration consistent across taxonomic scales, from intraspecific to clade-wide?
- Can integration help selection overcome drift by increasing the fitness effect of selective units by bundling multiple phenotypes?
- To what extent is integration affected by the evolution of phenotypic novelty?
- To what extent does developmental systems drift (DSD) impact integration? If developmental systems can link traits, then DSD ought to affect the strength of such linkages.

In short, we see great potential for the collaboration between molecular and cellular research with organismic and evolutionary biology to address these and other outstanding questions in the field and, in doing so, to advance an understanding of the causes and consequences of phenotypic integration.

Author contribution

David G. Matthews (Conceptualization, visualization, writing–original & draft, writing–review & editing), R. Craig Albertson (Conceptualization, Funding Acquisition, Supervision, Writing–original & draft, writing–review & editing)

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Conflict of interest

The authors declare no conflicts of interest.

Data availability

No new data was generated in the preparation of this manuscript.

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