

Connecting the Dots: Anatomical Network Analysis in Morphological EvoDevo

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Abstract Morphological EvoDevo is a field of biological inquiry in which explicit relations between evolutionary patterns and growth or morphogenetic processes are made. Historically, morphological EvoDevo results from the coming together of several traditions, notably *Naturphilosophie*, embryology, the study of heterochrony, and developmental constraints. A special feature binding different approaches to morphological EvoDevo is the use of formalisms and mathematical models. Here we will introduce *anatomical network analysis*, a new approach centered on connectivity patterns formed by anatomical parts, with its own concepts and tools specifically designed for the study of morphological EvoDevo questions. Riedl's concept of *burden* is tightly related to the use of anatomical networks, providing a nexus between the evolutionary patterns and the structural constraints that shape them.

Keywords Morphology · EvoDevo · Anatomical network analysis (AnNA)

Organismal form is a visible witness to the evolutionary process; a process that has generated, via multiple speciation events, the amazing diversity of living beings past and present on our planet. Organismal form is also the result of many processes acting at different levels of organization during morphogenesis, from the genes that codify and regulate the expression of different proteins involved in cell migration, division, and differentiation, to the geometric and developmental constraints involved in the

epigenetics that carry the embryo toward successive stable forms until the adult (Müller and Newman 2003; Hallgrímsson and Hall 2011).

Form is a rich concept that includes not only the shape of anatomical parts, but also their size, arrangement, relative orientation, and connections of these parts (Rasskin-Gutman and Buscalioni 2001; Rasskin-Gutman 2003). The field of geometric morphometrics (GM) treats the concept of form as a set of landmarks, endowing it with a precise formula: $Form = Shape + Size$; that is, in GM, form encompasses two comparative components, one that denotes the relative position of landmarks that describe the form and one that captures its scale, the centroid-size component (for a recent review see Adams et al. 2013). However, morphology goes beyond the precise geometric and statistical definition of shape and size provided by the successful field of GM; it encompasses different levels of morphological information that, together, account for the actual form of organisms. A separate analysis of each level allows for the identification of its level-specific processes, which otherwise would be hidden from observation because of multiple layers of simultaneous information (Rasskin-Gutman and Buscalioni 2001). The level of connections specifically targets how different anatomical parts are related to each other physically (Rasskin-Gutman 2003); in addition, it provides a morphological assessment of part-to-part functional and developmental relationships, including growth (Esteve-Altava et al. 2011).

Morphology is the very object of evolutionary theory where self-organization finds stability and where natural selection finds its main target. This dual nature of morphology makes it a suitable goal for evolutionary developmental biology (EvoDevo), viz., finding the morphogenetic processes that allow for the stability of the growing embryo amid the selective pressure and

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evolutionarily inherited structural constraints from the phylogenetic history of each species. In the past four decades, EvoDevo has split into two very different disciplines: one that carries the tradition of morphological studies with it, and another that is concerned with the comparative analysis of developmental gene regulatory networks (Rasskin-Gutman 2009). *Morphological EvoDevo* has focused on the comparative analysis of embryological patterns and processes in the tradition of von Baer and Haeckel; criticism of Haeckel's biogenetic law has brought about a rich research program carried by authors like Gavin de Beer, Conrad Waddington, Ivan Schmalhausen, Stephen J. Gould, and Pere Alberch, among others. On the other hand, *molecular EvoDevo* started out as a discipline with the advent of molecular biology, in particular with the analysis of gene expression patterns and their regulation. In a seminal paper (Jacob 1977) the basis for today's molecular EvoDevo was laid out; there, evolution was seen as a *tinkering* process by which organisms would take old structures and use them in new ways at all levels of biological organization. In Jacob's own words (1977, p. 1165):

What makes one vertebrate different from another is a change in the time of expression and in the relative amounts of gene products rather than the small differences observed in the structure of these products. It is a matter of regulation rather than of structure.

The discovery of shared early developmental patterning genes, what came to be known as the *developmental genetic toolkit* (Carroll et al. 2005), broadly extended this idea and, with it, the field of molecular EvoDevo until today.

Morphological EvoDevo started its strong research field with studies of heterochrony and developmental constraints, but it has switched its focus towards other issues such as modularity, innovation, emergence of morphological traits, and phenotypic plasticity (Müller 2007). Modeling approaches to undertake the analysis of these EvoDevo problems have been around for decades, in particular morphometrics and the study of allometry (both founded by the classic work of D'Arcy Thompson). Complexity sciences have also provided concepts and methodological tools to help in the study of these tasks, in particular modularity (Callebaut and Rasskin-Gutman 2005). In this context, network theory has been instrumental in bringing into biology the study of complex systems, i.e., describing parts of organisms at the molecular level as sets of interacting elements within a complex system (Hasty et al. 2001; Barabasi and Oltvai 2004; Sorkin and Von Zastrow 2009). However, networks were not used for the analysis of morphological problems until recently (Rasskin-Gutman 2003), and only in the past

five years has a full methodology started to take shape. In the following sections we will lay out the concepts and the methodological tools for undertaking efficient analyses of morphological problems using anatomical network models. We will start with an account of the conceptual use of connectivity as a biological feature from a historical perspective, which will provide the foundation to understand its current use in anatomical networks. Then, we will introduce the concepts and tools borrowed from network science, which we have adapted to study the skull in tetrapods. Our work has led to the foundation of *anatomical network analysis* (AnNA), a methodology that aims at having a broader scope for the understanding of morphological EvoDevo.

Historical Background

Classic morphologists such as Johann Wolfgang von Goethe, George Cuvier, Étienne Geoffroy Saint-Hilaire, or Richard Owen laid down the fundamental principles of comparative anatomy in the nineteenth century. All these authors employed, in one way or another, the concept of *connections among anatomical parts* as a main aid to comparing the anatomy of different species. From then on, some key authors in theoretical biology and mathematical biology have attempted to formalize the intuitive notion of similarity and morphological organization derived from connectivity relations by means of axiomatic and mathematical models. In the next sections we will outline the historical development of connectivity from its beginnings in the 19th century up to the present day.

19th Century: “*Le Principe des Connexions*”

In the early 19th century, Étienne Geoffroy Saint-Hilaire (1818) proposed the use of the “principle of connections” as a method to study animal form (1818, p. 38): “*Le principe des connexions, comme une autre boussole, rapproche donc les différents points du théâtre de nos explorations*” (The principle of connections, like any other compass, thus reconciles the different points of the theater of our explorations). This principle set out a new research program in pure morphology at the structural level (reviewed in Nuño de la Rosa 2012), formalizing the intuitive notion of similarity of those days: a body part in an animal is recognized as the same part in other animals neither by its function nor by its shape, but by its situation and contact with other parts. Other notable naturalists before Geoffroy, such as Pierre Belon and Johann Wolfgang Goethe, also made use of this principle as a way to recognize similarities, a tradition that goes back to Aristotle. For instance, Goethe used this principle implicitly to

point out the presence of an intermaxillary bone in the human mandible. However, Geoffroy was the first to establish connections as an operational criterion to identify morphological similarity among different parts and organisms, using structural relations among parts, rather than their shape and function. In doing so, he placed the criterion of structure before function and shape in the recognition of homologies.

Armed with this powerful tool, Geoffroy and his followers attempted to discover the same structures even when the form and function of body parts were different or intermediary forms were unknown; that is, they tried to find what we would today call *homologies* across the body plans of the four *embranchements* proposed by Georges Cuvier (Appel 1987). For instance, Geoffroy found homologies between the ear bones in mammals, reptiles, and birds and those of the operculum in fishes. In Geoffroy's own words, "an organ is sooner altered, atrophied, or annihilated than transposed" (Appel 1987, p. 99). Laurencet and Meyranx used the principle of connections in an essay on the organization of mollusks, suggesting unification between the vertebrate and cephalopod embranchements; their presentation in the *Académie des Sciences* was the trigger that started the famous debate between Cuvier and Geoffroy (Appel 1987; Le Guyader 2003). Following the debate, and notwithstanding interpretations of its results (Russell 1916; Gould 2002; Le Guyader 2003), the principle of connections became a tool to identify homology for prominent zoologists, such as Richard Owen, Milne Edwards, and, more recently, Adolf Remane (Appel 1987; Ochoa and Barahona 2009). Despite its promising beginnings, the aim of building a formal framework for morphology based on connectivity was timidly approached during the 20th century. Many authors carried out a series of independent, disconnected attempts to make ad hoc uses of connectivity analysis in disparate biological fields. However, as we will describe in the following section, no methodological advances were effectively made until the 21st century.

The 20th Century: Disconnected Ideas about the Use of Connectivity

In 1945, Joseph H. Woodger proposed a formal framework to deal with phenotypic transformations during development based on correspondences between parts of an organism (Woodger 1945). Woodger's aims were to identify similarities between parts of an organism and to study phenotypic transformations during development and evolution, using group theory to codify topological information. Anatomical parts were classified by three rules: (1) being distal to, (2) being postaxial to, and (3) being articulated to other parts (Fig. 1a). In this framework, two parts

are *the same* in different organisms or developmental stages if they establish the same set of relations, that is, if they have a structural correspondence. Just like the principle of connections, structural correspondence captures homology in different organisms, identifying morphological types (Rieppel 2006). Furthermore, Woodger introduced the concept of *Bauplan* to define the "homologous structural plan underlying evolutionary transformations within a taxonomic group" (Raff 1996, p. 196). Although the concepts of *Bauplan* and type, as well as the very work of Woodger, received much criticism from advocates of *population thinking* in the context of the Modern Synthesis (Simpson 1961; Mayr 1982; Hull 1988), current evolutionary theory can accommodate completely his ideas (Rieppel 2006; Nicholson and Gawne 2013). Whatever position we adopt about the ideas proposed by Woodger, his structural formalization of correspondence was an important step in the search for rational criteria to capture the intuitive observation of similarities (i.e., homology identification) between organisms.

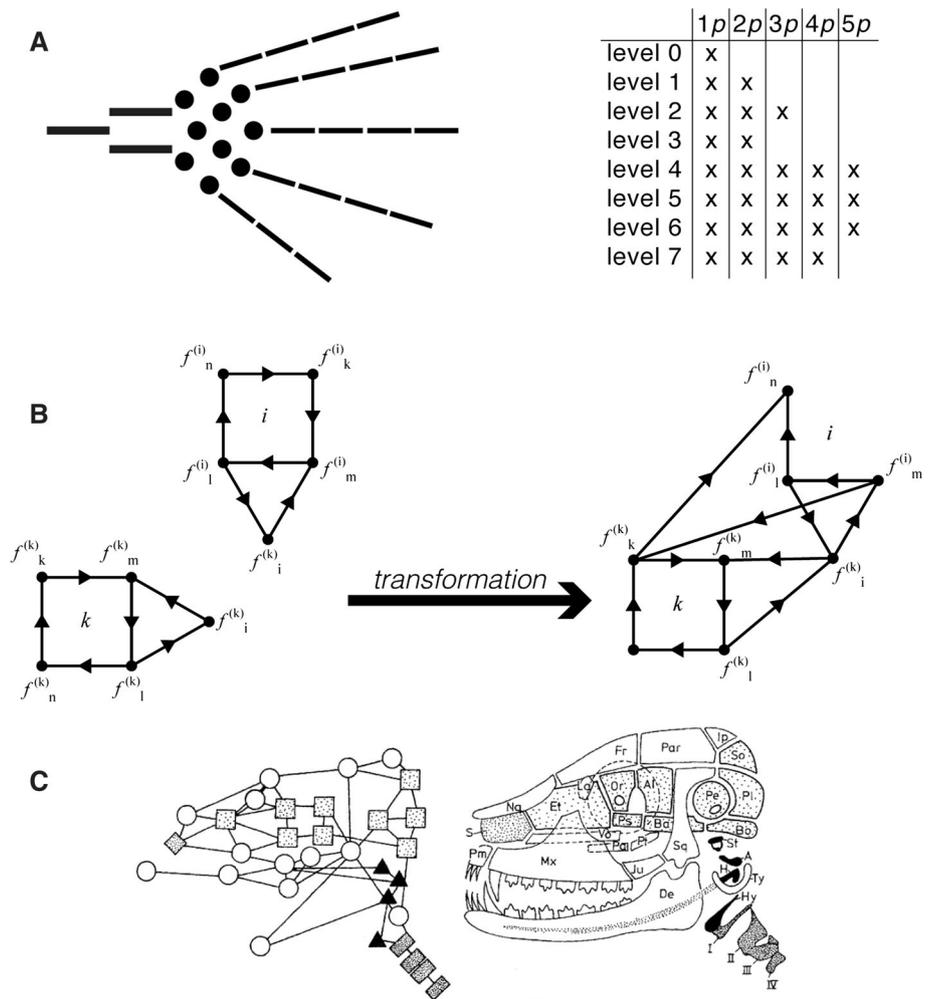
Nicolas Rashevsky is known as one of the founders of mathematical biology. His work on relational biology revolved around the integration of organismal functions into a systemic framework based on relations, which he formalized as networks (Rashevsky 1954). To analyze the changes in functional organization, Rashevsky used the transformations of the networks; a notion inspired by, or at least related to, the topological transformation introduced by D'Arcy Thompson (1942). Rashevsky called this the *principle of bio-topological mapping* (Fig. 1b):

the topological spaces or complexes by which different organisms are represented are all obtained from one or at most from a few primordial spaces or complexes by the same transformation, which contains one or more parameters, to different values of which correspond different organisms (Rashevsky 1954, p. 325).

He was also a pioneer in representing the complexity of biological organization by means of graph theory, a step beyond the use of group theory that we find in Woodger. However, the application of this framework of analysis to morphological systems was only discussed briefly (Rashevsky 1960). In such a framework, nodes would represent anatomical structures and links functional relations. However, neither Rashevsky nor his prominent disciple Rosen (1991, 2000), or any theoretical morphologists afterwards, developed further this intriguing research program (but see morpho-functional network diagrams used by Dullemeijer 1974).

It was Riedl (1978) who first used network diagrams in a purely morphological context. He represented the mammalian skull anatomy using positional relationships (i.e.,

Fig. 1 Representations of connectivity patterns during the 20th century. **a** Structural correspondence in the tetrapod limb by Woodger. **b** Biotopological mapping between cells by Rashevsky. **c** The diagrammatic morphotype of the mammalian skull by Riedl. Modified from Woodger (1945), Rashevsky (1954), and Riedl (1978)



connectivity) to identify homologies. In his book *Order in Living Organisms*, Riedl offers a deep reasoning on the origin and maintenance of body plans and evolutionary trends by means of the concept of *burden* in structures and events. This concept explains the integration of organismal form and its patterns of variation as a consequence of an increase in hierarchical nested developmental constraints on traits during evolution, linking evolution and development (Wagner and Laubichler 2004; Schoch 2010; Esteve-Altava et al. 2013a). In his defense of the necessity of concepts like morphotype and ground plan, Riedl introduced the idea of a *diagrammatic morphotype* (Fig. 1c), in which only minimal descriptors of form are used “as in a structural formula” (Riedl 1978, p. 249). By doing this, Riedl was able to capture the topological information that defines the morphotype of the tetrapod skull in terms of homology and developmental dependencies. To our knowledge, Riedl’s diagram is the first use of a network for the abstract representation of morphological relationships in a skull. Other uses of network diagrams in skulls have focused on the functional properties of skeletal parts, such

as the analysis of force lines of dermal skull bones in basal tetrapods (Thomson 1995) and biomechanical analysis in dinosaurs (Weishampel 1993).

The 21st Century: Anatomical Network Modeling

In the last decade, Rasskin-Gutman renewed the interest in studying morphology at a connectivity level within the frame of evolutionary developmental biology (Rasskin-Gutman and Buscalioni 2001; Rasskin-Gutman 2003). To formalize the topology or structural relation of parts in the pelvic girdle of archosaurs, Rasskin-Gutman introduced models based on network theory, in which each node represented a pelvic bone—ilium, ischium, and pubis—and each connection represented their physical junctions (Rasskin-Gutman and Buscalioni 2001). The use of networks was extended to study archosaur skulls (Rasskin-Gutman 2003), in which nodes represented skull bones and connections represented sutures. A network analysis was carried out to quantify the degree distribution (frequency of bones with a given number of connections) and to

identify building blocks (small, regular motifs such as triangular loops). This analysis, along with computational simulations, revealed the structural relation among the bones that are involved in the formation of skull openings. Furthermore, it demonstrated that the analysis of connectivity patterns is suitable for studying macroevolutionary structural changes. Rasskin-Gutman (2003) also introduced the use of computational models to explore changes in connectivity patterns in the skull of archosaurs and to explore the morphospace of connections. This 2-D computational model, based on cellular automata, was programmed to generate skull-like networks according to empirical connectivity patterns found in the skull of archosaurs; using different initial growth rules, bones were added or deleted until the maximum number of bones was reached. The results of this model were used to infer macroevolutionary dynamics in skull connectivity patterns, such as emergence of novelties, convergence, and stasis, as well as to offer a null hypothesis of skull evolution at a connectivity level.

In the past five years, we have developed a framework for the analysis of 3-D connectivity patterns in anatomical structures using concepts and tools borrowed from network theory. Our work has focused on different aspects of the evolution and development of the vertebrate skull: morphological complexity, integration, and modularity (Esteve-Altava et al. 2011, 2013a, b, 2014); the modular organization of the human skull in adults, newborns, and congenital malformations such as craniosynostosis (Esteve-Altava et al. 2013a; Esteve-Altava and Rasskin-Gutman 2014a); and the exploration of theoretical morphospaces (Esteve-Altava and Rasskin-Gutman 2014b). Although some concepts and methods are very intuitive, many others need further description to grasp the meaning and application of each network tool for other anatomical studies. In the next section, we will describe our analytical framework, which we will refer to as AnNA.

Anatomical Network Analysis (AnNA)

A network is a combination of two sets of elements: the set of nodes (N) and the set of connections (K), in which each connection has two endpoints that symbolize a relation between two nodes. Connections can be directed, denoting a causal relationship between the nodes, or undirected, denoting simply that there exists a relation. The most common representation of a network is a diagram of dots joined by lines (Fig. 2); the position of each dot and the length of each connection are irrelevant as long as the same relations between nodes are kept invariable. The nodes and connections are the raw descriptors of a network model; in anatomical networks, they are undirected and represent the

anatomical parts and their structural relations, respectively. This morphological information on part-to-part relationships is coded in an adjacency matrix: a symmetric binary matrix of size $N \times N$, where 1 indicates presence and 0 indicates absence of connection between two parts. The adjacency matrix directly defines the connectivity pattern of the anatomical network: the number and the particular distribution of structural relations among parts. In the following sections we will outline the main parameters and properties borrowed from network theory and explicitly used in AnNA.

Network Parameters and Properties

AnNA is carried out at different levels: from the anatomical parts that compose the network model, to the anatomical system as a whole, and from this, to the properties that define its morphological organization. In this section, we describe formally each parameter and property in AnNA. For clarity, we have divided its description into three levels: parts, system, and organization (Table 1). After this description, we will provide, in the next section, a broad morphological interpretation of all these parameters and properties.

Network Parameters for Anatomical Parts

At the level of anatomical parts, network parameters are measured for individual nodes, or pairs of nodes, defining the role of each part in the system.

The node connectivity (Eq. 1) is the sum of connections a specific node has to other nodes in the network:

$$k_i = \sum A_{ij} \quad (1)$$

The clustering coefficient (Eq. 2) measures the presence of connections between the neighbors of a node, specifically the number of interconnections among neighbors. The clustering coefficient is defined as the ratio between the total number of links connecting a node's nearest neighbors and the total number of all possible edges between all these nearest neighbors, which is $k_i(k_i-1)/2$:

$$c_i = \sum \tau_i / \sum k_i(k_i - 1) \quad (2)$$

where τ_i is the number of connections among the neighbors of node i .

The shortest path length between two nodes (Eq. 3) is a pairwise measure of their shortest distance:

$$l_{i,j} = \min_d(i,j) \quad (3)$$

where $\min(i, j)$ is the minimum distance in number of links to connect nodes i and j . By default, links have length one; thus, the shortest path between two connected nodes is 1.

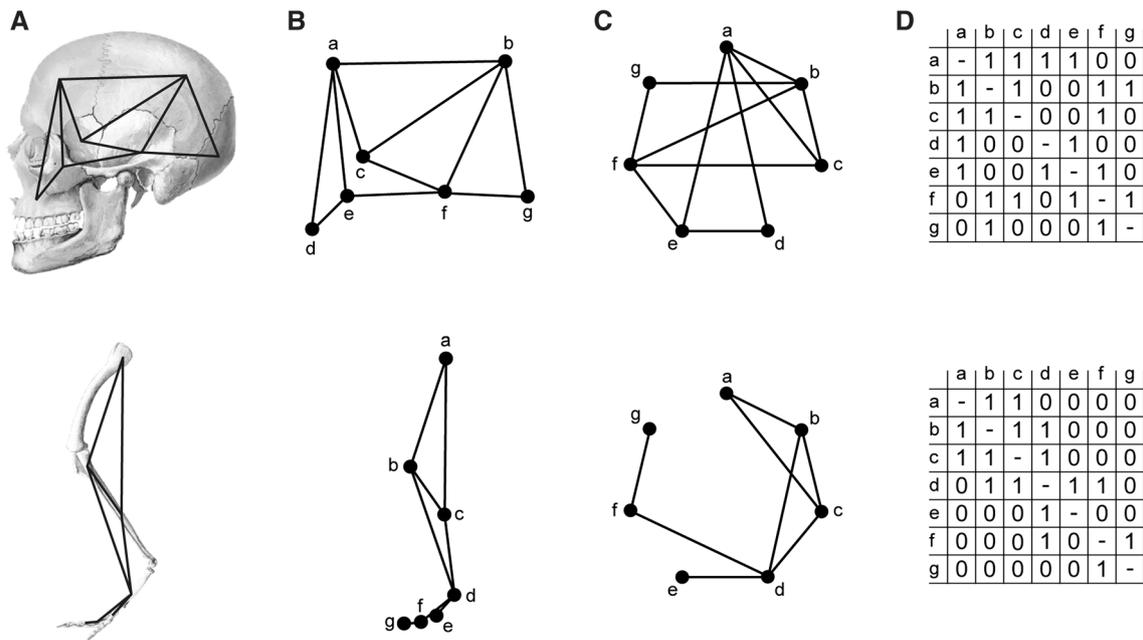


Fig. 2 Abstraction process to build anatomical network models in the human skull and a bird lower limb. **a** Identification of anatomical parts (bones) and relations (articulations, *perpendicular lines*). **b** Network representation using nodes to represent bones and connections between nodes to represent sutures. **c** Circular

Non-connected nodes have higher shortest path length depending on how well connected they are through the connection to others; many paths exist between any two nodes, which may or may not be the shortest ones.

The topological overlap between two nodes (Eq. 4) is a normalized measure of similarity that estimates the extent to which two nodes connect to the same other nodes.

$$TO_{i,j} = TO_{j,i} = J(n_i, n_j) / \min_k(i,j) \tag{4}$$

where $J(n_i, n_j)$ is the total amount of neighbors in common between two nodes and $\min_k(i,j)$ is the lowest connectivity of both nodes. Two nodes that share all their connections with the same other nodes have a TO of 1, whereas two nodes without any neighbor in common have a TO of 0. This similarity index has been extensively used to analyze modularity in different types of networks (Ravasz et al. 2002; Solé et al. 2006).

The within-module connectivity coefficient of a node (Eq. 5) is the normalized number of connections this node has to other nodes in the same module:

$$z_i = (k_{si} - \hat{k}_s) / \sigma_{k_s} \tag{5}$$

where k_{si} is the number of connections of node i within its module s and \hat{k}_s is the average of connections of all nodes within the module.

The node participation index (Eq. 6) is a measure of how uniform is the distribution of connections to nodes that do not belong to the same module:

representation of the network model to show that other morphological features such as position, orientation, shape, and size are not registered in the anatomical network. **d** connectivity pattern coded in a binary adjacency matrix: 1s for presence, 0s for absence of connections. From Esteve-Altava et al. (2011)

$$p_i = 1 - \sum (k_{si}/k_i)^2 \tag{6}$$

For sufficiently large numbers of modules, if a node has all its connections within its module $p_i = 0$; however, if the distribution of all node connections is uniform to all other modules $p_i = 1$.

Network Parameters for the Anatomical System

The parameters measured for the entire network are derived from the combination of node parameters. These parameters are useful for comparing different systems, for example, biological and non-biological systems, biological systems at different scales, and different systems of the same kind.

The density of connections (Eq. 7) is the number of existing connections with respect to the maximum possible. It measures completeness: how many connections are realized of the total possible; a complete network has a density equal to 1.

$$D = 2K/N(N - 1) \tag{7}$$

The mean clustering coefficient (Eq. 8) is the arithmetic mean of the clustering coefficient of all nodes in the network. It measures the number of triangular motifs in the networks.

$$C = \sum c_i/N \tag{8}$$

Table 1 Parameters used in anatomical network analysis and their morphological interpretation

Abstraction level	Network theory	Morphology
Model	Node (n)	Anatomical part
	Link (k)	Anatomical relation
	Adjacency matrix (A_{ij})	Connectivity pattern
Anatomical parts	Connectivity (Eq. 1)	Burden rank
	Clustering coefficient (Eq. 2)	Correlation
	Shortest path length (Eq. 3)	Effective proximity
	Topological overlap (Eq. 4)	Structural equivalence
	Within-module connectivity (Eq. 5)	Intra-module integration
	Participation index (Eq. 6)	Inter-modules integration
	Anatomical system	Density (Eq. 7)
Mean clustering coefficient (Eq. 8)		Complexity
Mean shortest path length (Eq. 9)		Complexity
Heterogeneity (Eq. 10)		Anisomerism
Connectivity distribution (Eq. 11)		Bauplan
Clustering coefficient distribution (Eq. 12)		Bauplan
Q-value (Eq. 13)		Strength of modularity
Morphological organization		Small world (Eq. 14)
	Community structure	Modularity
	Hierarchy	Near-decomposability

The mean shortest path length (Eq. 9) is the arithmetic mean of the shortest path length between all pairs of nodes in the network. Together, C and L are used to identify the presence of the small-world effect when compared with randomly generated networks.

$$L = \sum l_{ij}/1 - N \quad (9)$$

The heterogeneity (Eq. 10) is the variance of connectivity of the nodes in the network.

$$H = \sigma_K/\mu_K \quad (10)$$

A finer analysis of the network's heterogeneity is possible by looking at the specific frequency of nodes with a given number of connections in the network: the connectivity distribution (Eq. 11).

$$P(k) = n_k/N \quad (11)$$

The $P(k)$ is the probability of finding a node with a given number of connections in the network; in networks with a small number of nodes, it is commonly quantified as the cumulative $P(k)$ in order to avoid statistical fluctuations. The functional form of the $P(k)$ characterizes the architecture of the network.

Similarly, the clustering coefficient distribution (Eq. 12) measures the clustering coefficient mean of all nodes with k connections. The $C(k)$ function is used to characterize the structure of the network; for example, a right-skewed distribution indicates the presence of highly clustered groups

of nodes, forming sub-modules or blocks (Mason and Verwoerd 2007).

$$C(k) = c_{i,k}/N \quad (12)$$

The modularity Q-value (Eq. 13) measures the strength of a modular organization by quantifying the number of connections between nodes within the same module, in comparison with the number of connections that would be expected if the empirical network were a random network (Newman and Girvan 2004).

$$Q = \sum_{m=1}^M \left[\frac{k_{sm}}{K} - \frac{k_m}{2K} \right] \quad (13)$$

where M is the number of modules, k_{sm} is the sum of connections from nodes to the same module m , and k_m is the expected sum of all connections within module m in a random network.

Network Properties of Morphological Organization

The structural properties of a network are to be inferred by comparing the values of network parameters, or a combination of parameters, with the values of those parameters in baseline null models (e.g., random, scale-free, or Gabriel).

The small-world effect is a pervasive feature of many real networks, which are characterized by highly clustered nodes, as in a regular network, and a small mean shortest path length, as in a random network. To identify the

presence of the small-world effect in the network, the values of C and L are compared with those of random equivalent networks with the same number of nodes and connections: small-world networks have higher values of C than regular and random networks, and similar or lower values of L (Watts and Strogatz 1998). However, a common problem in the analysis of the small-world effect in networks with few nodes (e.g., $n < 100$) is that the value of C cannot be sufficiently large to be discriminated from that of a random network; hence, the detection of the small world is unreliable. To circumvent this problem, the analysis can be corrected so that for any number of nodes, a network is small world if it fulfills the following condition (Eq. 14; Humphries and Gurney 2008):

$$[(C/C_r)/(L/L_r)] \geq 0.012n^{1.11} \quad (14)$$

The presence of community structure or modules is also a common feature in many different biological and non-biological networks; in a modular network, nodes tend to form groups of densely interconnected nodes. Thus, a connectivity module is simply defined as a group of nodes with more connections among them than to other nodes outside the module. However, module identification in a network is an NP-complete problem (non-deterministic polynomial time problem), which means that the evaluation of all possible partitions to find the best solution takes too much computational time (the number of possible partitions of a set of n nodes is called the n th Bell number; for instance, the Bell number for a small network of 21 nodes, like the human skull, is $\sim 4.75e^{14}$). For this reason, the identification of connectivity modules often relies on heuristic assumptions that overcome this problem: traditional clustering techniques based on similarity/dissimilarity matrices, oriented algorithms based on prior information, or spin-glass models (Porter et al. 2009).

We have used one of the above-mentioned heuristic approaches to identify connectivity modules in skull anatomical networks: an agglomerative hierarchical clustering analysis on a similarity matrix, for which we used the TO similarity matrix (Esteve-Altava et al. 2013a). This grouping method brings together nodes with a higher TO in single branches until all nodes form one single group. After each match, the TO matrix is recalculated and grouped nodes act as a new element in the grouping process. The final outcome is a dendrogram of nested groups of nodes. The best of all possible modular partitions can then be identified using Q as an optimization index (i.e., the stronger the modularity, the higher the Q).

In addition to modularity, some networks exhibit a hierarchical organization of connections, in which modules are, simultaneously, composed of smaller sub-modules (blocks or motifs). The number of nested layers of modules varies from one network to another, which can be different even within modules in the same network. Two network

parameters are used to identify a hierarchical organization: $P(k)$ and $C(k)$. Theoretically, both the $P(k)$ and $C(k)$ in hierarchical networks fit a power-law distribution, an instance of the Pareto distribution (Newman 2005); in random networks, the $P(k)$ fits a Poisson distribution and the $C(k)$ is independent of the number of connections; whereas, in scale-free networks, the $P(k)$ fits a power-law and the $C(k)$ is also independent of the node connectivity (Ravasz and Barabási 2003; Wuchty et al. 2006). Right-skewed distributions, which are close to the power-law distribution, indicate that the neighbors of less-connected, most-abundant nodes are highly clustered, while neighbors of highly connected, less-abundant nodes are sparsely interconnected. Thus, $P(k)$ and $C(k)$ fitting right-skewed distributions suggest that hub nodes (see next section) are acting as interconnectors between blocks.

Morphological Interpretation

The use of network models in biology has also introduced many new concepts from more specialized literature on network theory in order to describe and explain biological systems, such as small world, scale-free, and hub (reviewed in Proulx et al. 2005; Knight and Pinney 2009). Building a network framework to study anatomical systems requires endowing network parameters with a morphological meaning, which is required to fully interpret the outcomes of the network analysis. To this end, we will refer to concepts deeply rooted in the morphological EvoDevo tradition (Table 1). Since we have developed AnNA for the study of the tetrapod skull, all examples refer explicitly to developmental or evolutionary aspects of the skull bones.

The elements of an anatomical network are anatomical parts (nodes) and the anatomical relation among them (connections). As such, they are discrete anatomical units with their own developmental features, internal (e.g., histological) structure, and biological functions. For instance, skull bones are hard parts with characteristic shapes that protect soft parts of the head and provide insertion surfaces for the muscles, whereas skull suture joints are fibrous attachments between bones that act as primary sites of bone growth and distribute stress forces (Esteve-Altava et al. 2013a). Knowing the roles of anatomical parts and their structural relation in development and function is paramount to correctly interpret network parameters and is the first necessary step in any study using AnNA.

The Role of Anatomical Parts

The role of each anatomical part in an anatomical network derives from the number and targets of its relation to other parts. Defining roles of anatomical parts is important to understanding more derived morphological properties.

Connections Measure the Burden Rank Boundaries between anatomical parts define the regions where parts interact functionally as well as during development, establishing codependences that will constrain variation. For instance, the role of craniofacial sutures as primary sites of bone growth determines the overall general morphology of the skull (Opperman 2000; Rice 2008). Moreover, suture joints also participate in other functions, such as intracranial movements of bones (Jaslow 1990) and strain sinks (Rafferty et al. 2003). Thus, it is expected that bones with more suture connections have central developmental and functional roles affecting the morphology of the entire skull. As a consequence, the more connections a part has, the more the number of codependences that it establishes with other bones during development. This can also be interpreted as the presence of more developmental constraints or shape correlations, because these connected anatomical parts are growing, acquiring shape, and functioning in coordination. Riedl's concept of burden (1978) captures this association between the number of connections (relations or dependences) between parts and the intensity of constraints, due to acquired developmental and evolutionary compromises (Schoch 2010). Thus, anatomical parts with higher connectivity would have a higher burden rank than those with fewer connections, which would carry a relatively minor role in morphological stability (Esteve-Altava et al. 2013a). These roles, played out during both development and evolution (Wagner and Laubichler 2004), are important to understanding the evolutionary patterns of skull morphology in vertebrates concerning bone losses and fusions (Esteve-Altava et al. 2013b, 2014). The relative amount of connections in an entire anatomical system (i.e., density) is the first of three features that characterize morphological complexity.

Clustering Coefficient Measures Network Correlation Among Parts The clustering coefficient of an anatomical part measures the presence of a relationship between its neighbors; that is, the establishment of triangular loops, a specific form of correlation in networks. Following the morphological interpretation of connectivity as developmental and functional codependence between parts (i.e., burden rank), this parameter captures some kind of second-order codependence (Dorogovtsev and Mendes 2003). Thus, the clustering coefficient is interpreted morphologically as contributing to a higher burden due to this kind of second-order correlation of an anatomical part with its neighbors; for the entire anatomical system, this is the second feature of morphological complexity.

Shortest Path Length Measures Effective Proximity Between Parts The shortest path length between two anatomical parts indicates their proximity in a network.

Since distances in networks are measured as the number of connections that separate two parts, proximity has to be understood as the range of interdependence between them. For instance, in the skull two bones in direct contact ($l_{i,j} = 1$) have more functional and developmental codependences than two bones that connect to the same third bone, but are not in direct contact ($l_{i,j} = 2$). As a consequence, correlation between bones would be higher if they are *near* and lower if they are *far away* in the network, for example, in stress-forces flow (Rafferty et al. 2003; Moazen et al. 2009) or in shape covariations (Woo 1931; Pearson and Woo 1935; for a different perspective see Magwene 2001, 2008). Thus, the path length is interpreted morphologically as the effective (in opposition to geometric) proximity between parts; for the entire anatomical system, this is the third feature of morphological complexity.

Topological Overlap Measures Structural Equivalence Among Anatomical Parts Topological overlap measures the extent to which two anatomical parts share the same developmental and functional relations to other parts (codependences). Altogether, sharing codependences makes two parts structurally equivalent for the constraints affecting their variation; hence, it is expected that two structurally equivalent parts would tend to vary in a coordinate fashion.

Within-Module Connectivity Measures the Intra-Module Integration The within-module connectivity measures the contribution in number of connections of a part to its module. As the number of connections within modules increases, anatomical parts become more packed, increasing their network correlations, and reducing their effective proximity. This parameter captures the contribution of one part to the modularization of the system and the internal integration of its module.

Participation Index Measures the Inter-Modules Integration The participation index of an anatomical part to a modular organization captures the number of its connections to parts in other modules. Spreading connections between different modules contributes to increasing the integration among modules, and hence, to decreasing the system's compartmentation. Anatomical parts with higher participation are responsible for maintaining the integration among semi-independent modules.

Connectivity Patterns Measure the Role of Anatomical Parts The most straightforward way to define the role or importance of anatomical parts in an anatomical network uses the burden rank (i.e., the number of connections): a hub is a part with high burden rank (i.e., its connectivity is

one or two standard deviations above the mean), and a satellite is a part with low burden rank. This definition of roles, based solely on the burden rank in the entire system, is not completely useful when comparing the same anatomical system in species with many differences in the number of parts and relations among parts. A more precise definition of hub uses the intra-module contribution to integration of parts (i.e., z_i in Guimerà et al. 2007). This definition does not always agree with the definition of hubs as nodes with a higher burden rank because, although both are defined for individual parts, the latter only makes sense within a modular context; there, parts can act either as connectors, if they spread connections between different modules (connecting modules), or as local nodes, if they keep most connections within their modules. New roles emerge for anatomical parts when seen as how they contribute to the integration of the system. The role of each part within a given modular structure is characterized by its contribution to the integration of its module and its contribution to the integration among different modules: z_i and p_i . Specifically, the role of each part can be assigned to one of the following four categories: (1) local hub, when z_i is high and p_i is low; (2) connector hub, when z_i and p_i are high; (3) peripheral connector, when z_i is low and p_i is high; and (4) satellite, when z_i and p_i are high (Guimerà and Amaral 2005). In anatomical networks, a disparity of roles among parts reveals heterogeneity in the morphological organization, highlighting that not all parts have the same burden rank and their roles in the organization of modules vary. Moreover, the distinction between connectors and satellites suggests that different parts also contribute differentially to morphological integration.

Morphological Features of Anatomical Networks

Network Connectivity Estimates Morphological Complexity Three network parameters capture complementary aspects of complexity in an entire network: density of connections, mean clustering coefficient, and mean shortest path length. This definition of morphological complexity is based on the structural organization of parts (for alternative definitions of morphological complexity based on the number of part types see, e.g., McShea 1993; McShea and Brandon 2010; McShea and Hordijk 2013). Thus, the definition of morphological complexity that these parameters bring up resembles that of Herbert A. Simon (1962, p. 468): “a large number of parts that interact in a non-simple way.” The density of the anatomical network is the number of existing connections of the total possible given the number of parts. As it captures the overall burden rank of all anatomical parts, we expect that a system with higher density would show more constraints and, hence, less overall variation. Moreover, as a proxy of the number of functional responses,

density has been also interpreted as a measure of complexity in many other biological networks (Sporns 2002; Newman and Forgacs 2005; Dunne et al. 2002). Thus, density measures morphological complexity as it is related with the formation of more developmental and functional relations between parts. Furthermore, the mean clustering coefficient and the mean shortest path length of the network are both parameters related to developmental and functional correlation between parts, which can be seen also as related to information flow within the anatomical system. Thus, the clustering coefficient captures short-range information correlation, due to redundancy among neighbor bones; whereas the path length determines the speed of information transmission among effectively distant parts. Information flow occurs in morphological systems at different levels: developmental, functional, and evolutionary. For instance, in the skull, the developmental information flow occurs between different bones during their coordinated development and growth in the form of allometric growth patterns (Huxley 1932; Sardi et al. 2004; González et al. 2010). Thus, the premature closure of sutures leads to different types of deformities due to the compensatory and coordinate growth in other skull bones that are not directly involved in the suture closure (Hukki et al. 2008). The functional information flow is related to the daily activity of the morphological system in performing its functions, for example, the diffusion of stress forces acting on skull bones from injuries and feeding activity (Moazen et al. 2009), a flow that is determined by the presence and nature of suture connections. Finally, evolutionary information flow can be measured as correlated variation between traits because of genetic, developmental, and functional integration; this allows for a precise definition of morphological integration (in terms of shape covariation) and modularity in morphological systems (Klingenberg 2010). Therefore, the clustering coefficient and path length measure morphological complexity as it is related to the integration of developmental, functional, and evolutionary relations among anatomical parts.

Heterogeneity of Connections Measures Anisomerism It is an old claim in studies on evolutionary trends in skull morphology that a reduction in the number of bones (i.e., Williston’s Law) is compensated by a differentiation of the remaining bones (Esteve-Altava et al. 2013b). This pattern-process is known as anisomerism, in contrast to polysomerism that accounts for patterns of less specialized, similar anatomical elements. In this context, heterogeneity of connections has been interpreted as anisomerism: a break of regularity in the burden rank of anatomical parts (Esteve-Altava et al. 2013b, 2014).

Distributions of Connections Define the Bauplan The literature on complex networks refers to the connectivity

and clustering coefficient distributions of the network as the parameters that define the *architecture* of the network (Wuchty et al. 2006; Sales-Pardo et al. 2007; Horvath and Dong 2008; Barabási 2009; Knight and Pinney 2009). Moreover, these distributions provide information about the possible nature of the mechanisms that formed the network, for example, power-law distributions by preferential attachment, or binomial distribution by random processes (Albert and Barabási 2002). Indeed, using these two parameters, we can build anatomical networks with the same structure of connections (i.e., the family of all isomorphic networks). However, it is still controversial whether or not we can assume that sharing an architectural plan means also sharing analogous generative processes and properties (Fox-Keller 2005). In morphology, the concept of architectural plan, body plan, or *Bauplan* has more connotations than in network science. However, the interpretation of these two parameters as the morphological architectural plan makes sense at this structural, topological level. A step further in this interpretation would be to consider similarities in skull network architectures as an evidence of the unity of type or homology.

Modularity Q-value Measures the Strength of Modularity The morphological interpretation of this parameter comes directly from its formalization as strength or integration among modules; thus, the higher its value, the higher the integration within modules. In addition, when quantified at the same morphological level, modularity is inversely related to the strength of the integration among modules (Eble 2005); thus, the inverse of the Q-value is a potential measure of morphological integration among anatomical parts. An advantage of studying morphological integration using anatomical networks (i.e., at the connectivity level) is that we produce a priori hypotheses about morphological integration and modularity—directly from the patterns of organization of structures. Some of these hypotheses concern how different anatomical parts (e.g., groups of bones) are integrated between themselves, in other words, how morphological modules are organized (Eble 2005; Esteve-Altava et al. 2013a).

Properties of Morphological Organization

The Small-World Effect Defines a State of Order We assess the presence of the small-world effect in an anatomical network by comparing its mean clustering coefficient and mean shortest path length with those of random equivalent networks. Small-world networks are more clustered than random ones, sometimes more than regular networks too, and yet the effective proximity between nodes is as small as it is in random networks or even slightly smaller (Watts and Strogatz 1998). Thus,

identifying the presence of the small-world effect in an anatomical network indicates that it exhibits a particular order in the pattern of relations among parts, one between regularity and randomness. Riedl (1978, p. 31) intuitively glimpsed this property of morphological order as “a region of unspecified probability, a no-man’s-land between accident and necessity.” For example, as explained below, the skull of tetrapods shows this mixed pattern of order: some regions show an orderly arrangement of bones, while other regions are indistinguishable from randomness (Esteve-Altava et al. 2013a, b). The reason for this is the presence of short-cut connections between bones that otherwise would be *far away* (in a network sense). The bones that establish these special sutures are usually the skull hubs, or those bones identified as contributors to integration within modules (Esteve-Altava et al. 2013a). As a consequence, modularity emerges in these skull networks.

Community Structure Interpreted as Modularity The presence of community structure in an anatomical network model is interpreted directly as presence of morphological modules. Thus, a connectivity module is a group of anatomical parts with more connections (i.e., codependences) within the module than to other parts outside the module. Modularity, like integration, is a multilayered concept in morphology that arises at different levels of organization: developmental, genetic, functional, and evolutionary, and converges in morphological modules (Eble 2005). Traditionally, morphological modules are inferred from data of covariation of morphological traits, usually sets of landmarks that tend to change together; thus, they are also called variational modules (Wagner et al. 2007). However, connectivity modules differ from variational modules in that they are inferred from the topological arrangement of anatomical units, and not the shapes of these units. The morphological information for variational and connectivity modules comes from completely different sources (Raskin-Gutman 2003). Since anatomical relations among parts involve developmental and functional interactions, connectivity modules have also a developmental and functional foundation, in addition to being, in origin, structural or topological modules.

Hierarchy of Connections Defines Near-Decomposability A hierarchical structure exists in an anatomical network when parts form modules that are, in turn, subdivided into smaller modules or blocks. Since blocks are highly clustered, we can identify hierarchical organizations by looking at the architectural plan or *Bauplan* (as defined above, i.e., quantifying $P(k)$ and $C(k)$). The formation of small blocks occurs between less connected anatomical parts that tend to cluster, while more connected ones hierarchically integrate the blocks. As a consequence,

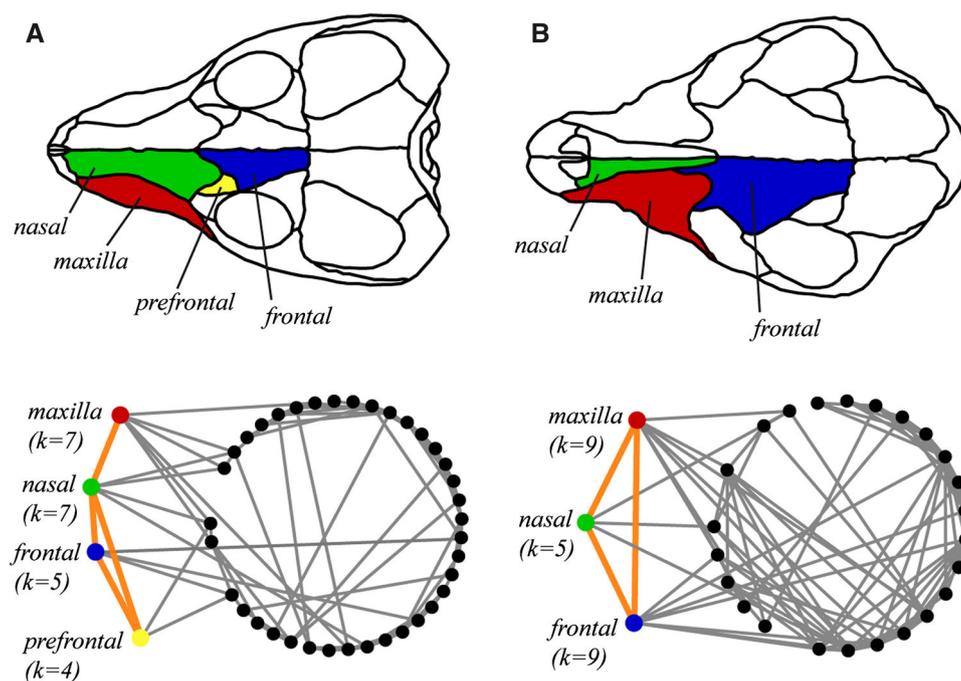


Fig. 3 The burden rank of bones has changed during mammal skull evolution due to loss and fusion of bones that have modified overall patterns of connectivity. **a** In the extinct therapsid *Thrinaxodon liorhinus*, the rostral roof comprises four bones: frontal, maxilla, nasal, and prefrontal. Each bone has its characteristic number of connections for the species, which defines its burden rank (maxilla, nasal = 7; frontal = 5; prefrontal = 4). According to our interpretation of the burden hypothesis for skull evolution, bones with a lower burden rank are more easily lost; thus, prefrontal would be a preferred target under selective pressures or developmental constraints toward

bone number reduction. **b** In modern mammals, such as *Canis lupus*, the prefrontal bone has been repeatedly lost. Various changes in the size and shape of the remaining bones of the roof have accompanied this loss; which, in the course of their development and growth, have configured a new set of connectivity relations, or burden ranks (maxilla, frontal = 9; nasal = 5). Now, according to the burden rank hypothesis, the nasal would be a preferred target for loss in future evolution, as has occurred before, for example, in modern turtles (Gaffney 1979). Modified from Esteve-Altava et al. (2013a)

morphological systems achieve a complex hierarchical organization composed of *semi-independent, quasi-decomposable modules* (Simon 1962). Morphologically complex structures evolve, changing in form, growing, and functioning in a coordinate way from one transitory stable form to another (Weiss 1971).

The Study of the Tetrapod Skull Using Anatomical Networks

Our recent work on the structural organization and complexity of the tetrapod skull using AnNA has focused on the following issues: the evolutionary trend in skull bone number reduction and its consequences (Esteve-Altava et al. 2013a, 2014); the architectural role of each particular bone in the structural organization of the skull (Esteve-Altava et al. 2013a); the morphological complexity, integration, and modularity of the skull (Esteve-Altava et al. 2013a; Esteve-Altava and Rasskin-Gutman 2014a); and the exploration of skull connectivity morphospace in a theoretical morphology context (Esteve-Altava and Rasskin-Gutman 2014b). These studies have demonstrated the adequacy of

AnNA to successfully address questions in morphological EvoDevo, providing new answers and suggesting new ideas. Specifically, AnNA has found a powerful ally in Riedl's concept of burden, an interpretation of how developmental constraints can cause evolutionary trends (Fig. 3).

The skull is a complex structure that has evolved, taking up several functions such as brain protection, feeding, and communication; thus, it has been subject to selective pressure as well as constrained by structural necessities originating from the initial design exhibited in basal tetrapods. The general evolutionary trend of the skull is a net reduction in number of bones along with the specialization of the remaining ones; this trend is known as Williston's Law, and was recognized and fully enunciated by Gregory (1935). Using AnNA, we have shown that Williston's Law occurs as a mix of bone losses and fusions; however, there is a structural constraint that makes bones with higher burden rank less prone to be lost during the evolution of a lineage (Esteve-Altava et al. 2013b). Furthermore, we have found that the reduction in bone number during evolution is accompanied by a trend toward a more complex organization (Esteve-Altava et al. 2013b), rather than toward

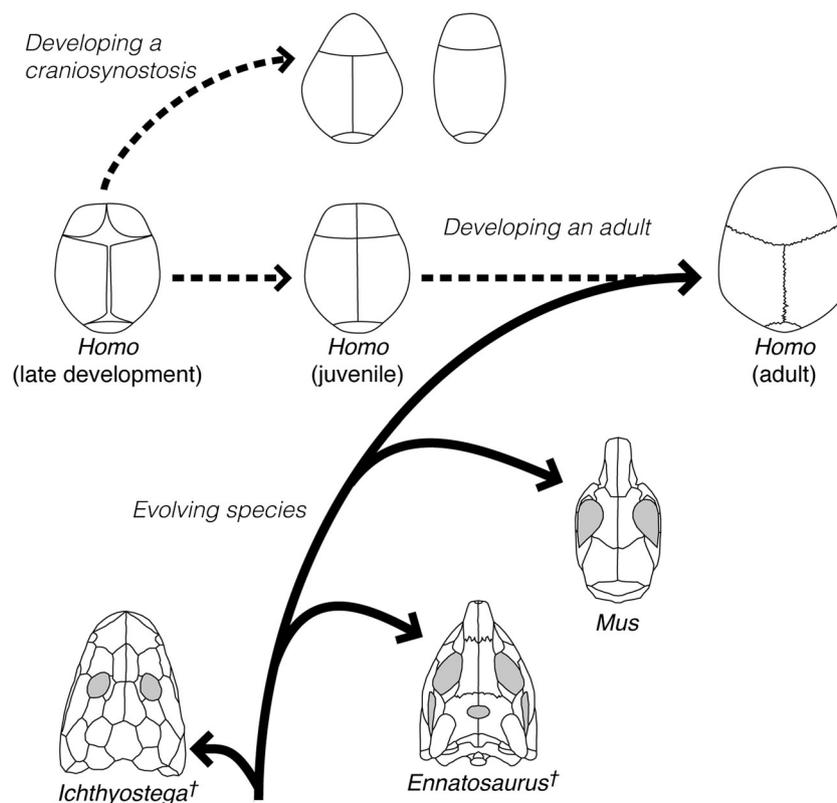


Fig. 4 Morphological changes associated with skull bone loss and fusion can be observed throughout evolution, and occur at an ontogenetic scale during normal and pathological development of the skull. In particular, bone fusions show many parallels between evolution and development; for example, the originally paired frontal bones of tetrapods and mammals fuse into a single unpaired bone during the evolution of primates, as happens during normal anthropoid primate late ontogeny. The timing of the metopic suture closure

is species-specific and is commonly modified by heterochrony. A premature closure of the metopic suture in ontogeny also leads to characteristic skull shape changes, such as frontal ridge and trigonocephaly. In the context of anatomical networks and burden rank hypothesis, the loss and fusion of bones, the reconfiguration of connectivity patterns, and the changes in shape and size of bones to accommodate them are tightly related in evolution and development. Modified from Esteve-Altava and Rasskin-Gutman (2014a)

simplification (Sidor 2001). The processes that take place during evolution to generate this trend in the skull involve the random loss of poorly connected bones and the selective fusion of the most-connected ones (Esteve-Altava et al. 2014). Further exploring this issue, we have built several generative morphospaces derived from null network models using random, preferential attachment, and geometric proximity growth rules. What we found was that geometric proximity between bones is the best explanation for the disparity of skull structures under two anatomical structural constraints found in all skulls: the presence of bilateral symmetry and the presence of unpaired bones (Esteve-Altava and Rasskin-Gutman 2014b). When we analyzed the temporal occupation of this morphospace we found that the tetrapod skull has followed an evolutionary path toward more constrained morphological organizations.

At the same time, our detailed work on the morphological integration and modularity of the human skull using AnNA has shown that the human skull is a small-world network; that is, the overall connectivity pattern of the

human skull is between randomness and regularity, and the connections that allow for the small-world network generate a heterogeneity that permits modularity (Esteve-Altava et al. 2013a). Thus, the human skull shows two connectivity modules: one facial organized around the ethmoid bone, and one cranial organized around the sphenoid bone. The facial module has a hierarchical sub-modular structure in four blocks: frontonasal, left maxillary, right maxillary, and ethmoidal; in contrast, the cranial module shows a regular structure. Further, each bone has a different structural role within the module it belongs to; thus, the ethmoid is a local hub with all its connections within the facial module; the sphenoid is a connector hub, spreading its connections between the facial and cranial modules; the frontal and zygomatics are peripheral connectors, that connect the facial and cranial modules rather than participating in their internal integration; and the vomer, the occipital, maxillas, temporals, parietals, lacrimals, nasals, nasal conchas, and palatines are satellites that contribute their few connections to integrate their own

module. Since network models of the skull are based on growth dynamics, they reflect fundamental differences in the ontogenetic patterns of growth and the structural constraints that each module generates (Esteve-Altava et al. 2013a). This modular organization found in the human skull seems to be conserved among primates and, to some extent, in mammals (unpublished results).

Recent work has also shown the similarities between craniosynostosis (premature and pathological fusion of skull bones) patterns and the evolution of tetrapod networks (Esteve-Altava and Rasskin-Gutman 2014a). We have compared skull network models of a normal newborn with different craniosynostosis conditions, the normal adult stage, and phylogenetically reconstructed forms of an ancestral tetrapod, a synapsid, and a placental mammal (Fig. 4). Our results have shown that the subtle changes in skull complexity at the developmental scale can change the modular substructure of the newborn skull to more integrated modules in the adult skull, but are not enough to generate the radical changes that occur at a macroevolutionary scale. Thus, the timing of closure of craniofacial sutures, together with the conserved patterns of morphological modularity, highlight a potential relation between the premature fusion of bones and the evolution of the shape of the skull in hominids (and probably in primates as well). This opens a new window of EvoDevo inquiry into the establishment of structural patterns in the tetrapod skull, centered on the properties of the networks derived from their articulation patterns.

Conclusion

The use of AnNA is very recent. The conceptual machinery accompanying AnNA has been developed in the past five years and has already shown compelling results for understanding the structural constraints that have shaped the tetrapod skull. Nevertheless, we are aware that a new methodological and conceptual framework can easily develop and evolve in ways that we cannot envision; we are ready to follow any path these constraints may take us on. We are presently working on extending the bone networks to include soft tissues, such as muscles and tendons, making the interpretation of the patterns more complex, but also more exciting. All in all, we believe that the possibilities for the use of AnNA in morphological EvoDevo problems are endless, and most beautiful.

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